Genetic and Epigenetic Role of IL-17A in Rheumatoid Arthritis

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Dedicated to My Parents…!

You have been there from the very start,
Took care of me, gave me your heart.
Thank you so much, for believing in me,
You gave me everything, the sky and the sea.

I have been blessed with parents so kind,
When I made mistakes, you didn't mind.
What I'm trying to really express,
Without you, I would have been less.

Thank you for teaching me how to live,
How to love others, and to forgive.
Thank you so much for being my light,
Because of you, my future is bright.

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Abdul Haseeb Khan

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List of Abbreviations

RA	Rheumatoid Arthritis
IL	Interleukin
DNA	Deoxyribonucleic Acid
RF	Rheumatoid Factor
ACPA	Anti-Citrullinated Protein Antibody
CRP	C-Reactive Protein
ESR	Erythrocyte Sedimentation Rate
SLE	Systemic Lupus Erythematosus
ACR	American College of Rheumatology
EULAR	European League Against Rheumatism
TNF	Tumor Necrosis Factor
CTLA	Cytotoxic T-Lymphocyte Antigen
DMARDs	Disease-modifying antirheumatic drugs
MHC	Major Histocompatibility Complex
TAE Buffer	Tris – Acetic Acid – EDTA Buffer
TE Buffer	Tris – EDTA Buffer
NaCl	Sodium Chloride
$MgCl_2$	Magnesium Chloride

EDTA	Ethylenediaminetetraacetic acid
WBCs	White Blood Cells
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
NCBI	The National Center for Biotechnology Information
CAPS	Cleaved Amplified Polymorphic Sequence assay
SNP	Single Nucleotide Polymorphism
SDS	Sodium Dodecyl Sulfate
MSP	Methylation Specific Primers

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Abstract

Background: Previous studies have revealed an association between interleukin 17A (IL-17A) polymorphisms and the prevalence of rheumatoid arthritis (RA) in Japanese and Caucasian patients. It was hypothesized that IL-17A polymorphisms might also affect RA susceptibility in the Pakistani population.

Methods: In this study, two polymorphisms including IL-17A SNPs rs8193036 and rs2275913 were examined in a case – control study of Pakistani population through Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR - RFLP). Methylation Specific PCR was followed by bisulfite conversion of genomic DNA to evaluate the methylation status of IL-17A promoter region.

Results: The study showed association value of *8.760* and the *p value 0.0125* for the genotype data for SNP rs2275913. While no association was found between rs8193036 and RA ($p = 0.5223$). IL-17A rs2275913 (A>G) was found associated with decreased risk of RA. Methylation status of IL17A promoter showed statistically significant association to RA ($p = 0.0001$).

Conclusion: The present study demonstrated that the IL17A SNP rs2275913 (A>G) significantly decreases the risk of RA. While no association was found between IL17A SNP rs8193036 (C>T) and risk of RA. Methylation status of the IL17A promoter region is significantly associated with the disease. However, these results were obtained from only a moderate-sized sample population. Replication of these findings in larger populations from diverse ethnicity are required to confirm these findings.

Keywords:

Rheumatoid Arthritis, RA, RFLP, PCR, rs8193036, rs2275913, Restriction Enzymes, Digestion, Epigenetics, Bisulfite conversion.

CHAPTER 1

INTRODUCTION

Rheumatoid arthritis (RA) is a disease manifested by inflammation of synovitis, articular cartilage damage and co-occurring of various other disorders, including problems in the bone, vasculature, brain and lungs (O'Shea *et al*., 2013). Hyperplasia, synovial inflammation, autoantibody production i.e. rheumatoid factor (RF) and anti–citrullinated protein antibody, bone and cartilage destruction and systemic features like pulmonary, psychological, skeletal and cardiovascular disorders are the characteristic features of rheumatoid arthritis (McInnes and Schett, 2011). The onset age of RA ranges between 35 and 55 years (Symmons *et al*., 2002).

The exact causes and triggers for RA are unknown, it is arguable that RA has multifactorial etiology including both genetic and environment factors. Immunological cascades involving autoantibodies cause in the release of inflammatory cytokines and synovial inflammation. Synovitis, if not controlled effectively, bone and cartilage destruction follows along dysfunction of nearby structures including muscles and tendons (Boissier *et al*., 2012). Progressive disability and handicap develops through damage to the joints. Approximately, 50% of the RA patients are unable to work or walk 10 years after the disease onset causing a significant health and cost burden on the patients and their families (Pugner *et al*., 2000).

0.5 - 1% of the adult population of developed regions are affected by RA (Carbonell *et al*., 2008). RA prevalence in developing countries is statistically variable. Lower incidence is reported in the studies from countries like Nigeria, Indonesia and Africaas compared to western countries, while India has the same prevalence of RA as reported in western world that is 0.75 – 0.8% (Choy, 2012; Carbonell *et al*., 2008; Symmons *et*

al., 2002). In Pakistan, 0.55 – 1.9 % of the population is affected by RA (Imran et al., 2015). Out of the 291 diseases and conditions studied, RA was ranked to be the 42nd highest cause for disability worldwide (Cross *et al*., 2014).

1.1. Clinical Diagnosis of RA

The RA diagnosis of can be made on the basis of presence of all the following clinical symptoms:

- Inflammation of three or more joints.
- Positive rheumatoid factor (RF) and/or anti-citrullinated peptide/protein antibody.
- Increased levels of C-reactive protein (CRP) or the erythrocyte sedimentation rate (ESR).
- After the exclusion of diseases with similar clinical features especially psoriatic arthritis, polyarticular gout or calcium pyrophosphate deposition disease, systemic lupus erythematosus (SLE) and acute viral polyarthritis.
- Symptoms are consistent for the duration of more the six weeks.

The graphical comparison of normal and RA affected joint is shown in Fig. 1.1 (Venables, 2014). The 2010 American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) classification criteria focus on features that would identify patients at an earlier stage of disease than would the previously used criteria that had been last revised in 1987 (Venables, 2014). The 1987 ACR criteria were formulated to distinguish patients with established rheumatoid arthritis (RA) from patients with other defined rheumatic diseases; the 2010 ACR/EULAR criteria for RA focused on identifying the factors, among patients newly presenting with

undifferentiated inflammatory synovitis, which could allow for the identification of patients for whom the risk of symptom persistence or structural damage is sufficient to be considered for intervention with disease-modifying anti-rheumatic drugs (DMARDs) (Aletaha *et al*., 2010; Venables, 2014).

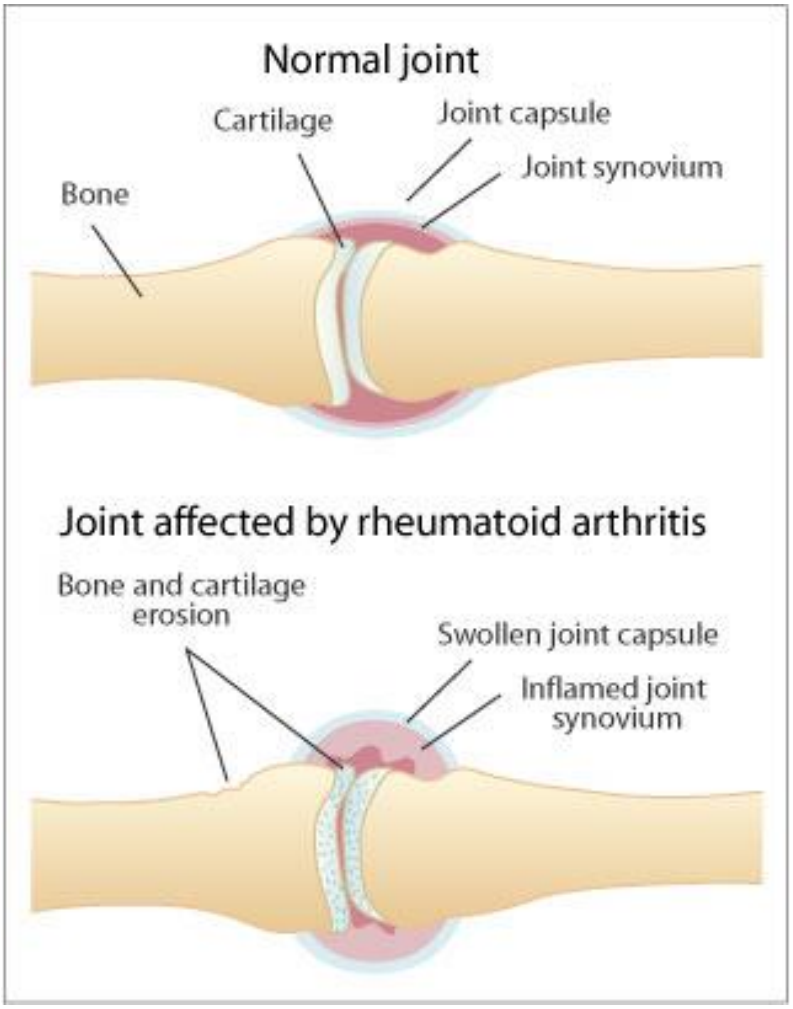

Figure 1.1: Normal vs Affected Joints

Using the 2010 ACR/EULAR classification criteria for RA, classification as definite RA is based upon the presence of synovitis in at least one joint, the absence of an alternative diagnosis that better explains the synovitis, and the achievement of a total score of at least 6 (of a possible 10) from the individual scores in four domains (Venables, 2014).

1.2. Treatment Options in RA

Several biologic classes are now approved for use in RA, including the tumor necrosis factor (TNF) blockers (i.e. adalimumab, etanercept, infliximab, golimumab, and certolizumab pegol), and agents targeting the interleukin (IL)-6 receptor (tocilizumab), IL-1 receptor (anakinra), cytotoxic T-lymphocyte antigen (CTLA)-4-mediated costimulation (abatacept), and B-cell depletion (rituximab). It is generally standard practice to start biologic therapy with a TNF blocker added to background Disease-modifying antirheumatic drugs (DMARDs) therapy. However, approximately 30–40% of patients do not respond to TNF blockers, and even among patients who do respond initially, few achieve complete remission and many lose their responses over time (Aaltonen *et al*., 2012; Alonso-Ruiz *et al*., 2008). Patients with inadequate responses to initial TNF blocker therapy are often switched to a second TNF blocker or a biologic with an alternative mechanism. Although some patients respond, few achieve major durable responses (Salliot *et al*., 2011). This clinical situation underscores the need for new biologics with novel mechanisms that can provide greater and longer lasting treatment responses. IL-17A has emerged as an attractive therapeutic target in RA.

1.3. Factors Involved in RA Pathogenesis

The cause of rheumatoid arthritis is unknown, and the prognosis is guarded. However, advances in understanding the pathogenesis of the disease have fostered the development of new therapeutics, with improved outcomes. The current treatment strategy, which reflects this progress, is to initiate aggressive therapy soon after diagnosis and to escalate the therapy, guided by an assessment of disease activity, in pursuit of clinical remission.

Genetic factors accounts to 50% of the risk of RA. If one member of a pair of identical twins has RA then the other member has a 15% chance of developing the disease. This is substantially higher than the risk in the general population, which is approximately 0.8%. Since identical twins have identical genes, this high degree of what is called 'concordance' points to a major genetic contribution to the cause of RA. The fact that the concordance is not 100% means that other non-genetic or "environmental" factors also play a part (Symmons, 2013). The higher prevalence of RA in women, especially during childbearing years and frequent disease improvement during pregnancy has led to the identification of the possible hormonal role in disease susceptibility (Nelson *et al*., 1993).

Genetic studies in autoantibody positive RA among subjects have identified multiple major histocompatibility complex (MHC) region alleles and 25 confirmed RA risk alleles in 23 non-MHC loci (Barton *et al*., 2009; Eyre *et al*., 2009; Kurreeman *et al*., 2007; Raychaudhuri *et al*., 2008; Raychaudhuri *et al*., 2009; Suzuki *et al*., 2003). One important assignment for RA genetic studies in Asian populations is the functional dissection of the role of the HLA-DRB1*0901 allele. The association of HLA-DRB1*0901 with the risk of developing RA is moderate, but has been reported repeatedly in Asian populations (Lee *et al*., 2004; Wakitani *et al*., 1998). Raychaudhuri and colleagues have established a sophisticated model that could explain the association of HLA-DRB1 with RA by using amino-acid sequences (Raychaudhuri *et al*., 2012). HLA-DRB1*0901 is involved in autoimmunity to citrullinated proteins with a mechanism differing from that of classical shared epitope alleles. Therefore, further functional analyses at the molecular level are needed to dissect the role of the HLA-DRB1*0901 allele in RA (Yamamoto*et al*., 2015).

Many other identified risk alleles in rheumatoid arthritis consistently aggregate functionally with immune regulation implicating nuclear factor κB (NF-κB)–dependent signaling (e.g., TRAF1–C5 and c-REL) and T-cell stimulation, activation, and functional differentiation (e.g., PTPN22 and CTLA4) (Begovich *et al*., 2004; Kurreeman *et al*., 2007; Plenge *et al*., 2007).

1.4. Epigenetic Factors playing their role in RA

Although there is no 'carved-in-stone' definition for epigenetics, it is broadly defined as the study of heritable changes in gene activity that do not involve any alterations in the primary DNA sequence (Virani *et al*., 2012). Epigenetics originally focused on DNA methylation and various histone modifications, but recently expanded to the field of non-coding RNAs. Each cell of the body inherits the same genetic information. What makes each cell unique is that, during ontogenesis, different sets of genes are turned on and off (Glant, Mikecz, & Rauch, 2014). Early studies found widespread DNA hypomethylation in RA synovial fibroblasts, including hypomethylation of the promoter of the CXCL12 gene (Karouzakis *et al*., 2011) and the LINE1 retrotransposons (Neidhart *et al*., 2000) that are repetitive elements normally repressed by DNA methylation. In these cases, loss of the repressive DNA methylation signal results in increased gene expression. A recent genome-wide study on RA synovial fibroblasts revealed a number of differentially (hypo- and hyper-) methylated genomic regions (Nakano, Whitaker, Boyle, Wang, & Firestein, 2013).

Most of the affected genes appear to be involved in inflammation, matrix remodeling, leukocyte recruitment and immune responses (Nakano *et al*., 2013). Another study found that the HAT to HDAC activity ratio in arthritic joints was shifted towards HAT

dominance, favoring histone acetylation (Huber *et al*., 2007), ultimately leading to an increase in gene transcription (Glant *et al*., 2014).

1.5. Role of Chemokines and Cytokines in RA Pathogenesis

Over the years, increasing numbers of cytokines have been involved in RA pathology, further to those used as target of cytokine-blocking therapies which emerged from the hypothesis that the most abundant cytokines present in the joint were more likely to be pathogenic. A large number of cytokines are detected at the disease site (through both mRNA and protein quantification) in both synovial tissue and fluid, where they have a role in perpetuating inflammation, cartilage destruction, and bone remodelling associated with RA. Several methods of detection (ELISA, immunohistochemistry) identified TNF-alpha and IL-1 as major players in the network of cytokines, notably directly expressed at the disease site in joint tissue or fluid. IL-6 and IFN-gamma are also present as well as GM-CSF and LIF. More recently, other cytokines were added to this list (IL-7, IL-15, IL-17, IL-18, IL-21, and MIP-1 notably) together with cytokines with activities targeted towards fibroblasts (TGF-betas notably) and finally several growth factors (PDGF, EGF, and VEGF) (Hetland *et al*., 2008) and chemokines (IL-8, SDF-1, RANTES, and MCP-1). Cytokines favouring survival of infiltrating cells have also been detected (such as the pairs between IL-7 and T cell or BAFF and B cells). However, if proinflammatory cytokines (TNF-alpha, IL-1, and IL-6) are abundant in all patients, cytokines classically defined as anti-inflammatory and regulatory (IL-4, IL-10, IL-13, and TGF) (Miossec, 1993) as well as antagonist receptors (IL-1RA, or soluble IL-2R, or TNF-R) are also present. Most of these cytokines have dual roles with anti- and proinflammatory aspects depending on the context and the network they form; hence, studying their roles and actual effects is particularly complex. The redundancy and synergy between the effects of all cytokines in such an intricate network may

further explain the inadequate response to single blockade therapy notably in established disease (Chabaud *et al*., 1998).

Interleukin 17A (IL17A), also known as IL-17, is the signature T helper 17 (Th17) effector cytokine (Korn *et al*., 2009). IL17A leads to the induction of many factors, including tumor necrosis factor-alpha (TNF-α), IL-6, and IL-1β, which are important in inflammatory processes (Ruddy *et al*., 2004). Interleukin 17 is elevated in synovial fluids of RA patients and in inflamed joints of experimental arthritis mouse models (Chabaud *et al*., 1999; Kim *et al*., 2005). IL-17 is a proinflammatory cytokine produced predominantly by T-helper-17 (Th17) cells (Harrington *et al*., 2005; Park *et al*., 2005). The Th17 cells are likely to be the major source of IL-17A in the circulation and, possibly, in synovial fluid, but mast cells are the major IL-17A source in rheumatoid tissue (Kellner, 2013). IL17A is also produced by many other cell types, including CD8+ T cells and γδ T cells, and is increased locally in the skin and joint diseases in human (Kirkham *et al*., 2014).IL-17 is an important regulator of immune and inflammatory responses, including the induction of other proinflammatory cytokines and osteoclastic bone resorption (Simpson *et al*., 2010). This cytokine is found in synovial fluids and synoviocytes of RA patients and is produced by both Th17 cells and RA synoviocytes (Simpson *et al*., 2010). IL-17 induces inflammatory cell infiltration into the synovium, enhances collagen degradation and decreases collagen synthesis in cartilage, reduces proteoglycan synthesis by chondrocytes, and increases bone resorption and erosion (Shahrara *et al*., 2010). Thus, inhibiting IL-17 might provide important therapeutic benefits in halting the joint destruction that occurs in RA.

CHAPTER 2

LITERATURE REVIEW

2.1. Rheumatoid Arthritis (RA)

Rheumatoid arthritis was first reported in 19th century and its pedigree was developed in 20th century. Even though its name was made known in the 1850s, classification criteria were merely developed 50 years back (Storey *et al*., 1994; Ropes *et al*., 1959; Arnett *et al*., 1988). These criteria are used in some observational studies which describe treated rheumatoid arthritis as a severe long-term disease having dominant extra-articular characteristics, inadequate treatment options as well as poor outcomes (Scott *et al*., 1987).

Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly affecting peripheral joints, resulting in the progressive damage of articular cartilage as well as juxta-articular bone (Benedetti and Miossec, 2014; Feldmann *et al*., 1996). During the initial stages of the disease cells of synovial lining which are known by the name synoviocytes, proliferate markedly leading to a synovium hyperplasia. Disorders in cytokine as well as chemokine signaling lead to permeation of the synovium with immune cells (Feldmann *et al*., 1996) which further lead to activation of various amplification mechanisms that propagate the primary immune response resulting in persistent inflammation and progressive destruction of the tissues.

2.2. RA Epidemiology

The existence of RA has many significant variations amongst countries and regions of the world. A declining trend has been reported in countries which are characterized by high rates of RA incidence as well as prevalence. On the other hand, the relatively low

number of studies for most parts of the world, their practical differences and the nonexistence of incidence studies aimed at the developing countries, bounds our understanding of universal RA epidemiology (Alamonas *et al*., 2006). During the last 2 decades, some incidence and prevalence studies of rheumatoid arthritis (RA) have been reported, signifying a great variation of the disease rate among different populations. Certain ethnic and racial groups reported an increased occurrence of the disease (Silman and Pearson, 2002; Gabrial, 2001).

Table 2.1: Prevalence rates of RA worldwide

S. No.	**Population**	**Countries**	**Prevalence Rates (%)**
1.	**North America**	General	0.9 – 1.1
		Native	5.3 – 6.0
2.	**South America**	Argentina	0.2
		Brazil	0.5
		Colombia	0.1
3.	**Asia**	Japan	0.3
		China	0.2 – 0.3
		Indonesia	0.2 – 0.3
		Pakistan	0.1
4.	**Middle East**	Egypt	0.2
		Israel	0.3
		Oman	0.4
		Turkey	0.5
5.	**Africa**		0 – 0.3

The occurrence of the disease seems to be lesser in developing countries. It has been reported that south European countries show a relative lower incidence of RA in

comparison to north European and North American countries (Alamonas, 2005). Some studies also show a decreasing tendency in the occurrence of RA, though it is challenging to confirm this trend (Silman, 1992). The annual prevalence rates of RA ranges between 20 and 50 cases for every 100,000 inhabitants in North American and North European countries (Riise *et al*., 2001; Gabriel *et al*., 1999). Approximately 0.5 – 1 % of the population is affected by RA globally (Silman and Hochberg, 2001). Around 0.1 – 0.2% of population is affected by RA in Pakistan (Baig, 2003). This disease can arise at any age however its occurrence increases at age amongst 40 – 60 years. The frequency of RA is seen three times more in women than men. The incidence rates of RA worldwide are précised in Table 2.1 (Alamonas, 2005; Tobon *et al*., 2010).

2.3. RA Pathogenesis

The beginning of RA is amalgamation of pre- determined genetic and random events. Vulnerability to RA is undoubtedly defined by a pattern of inherited genes, with the human leukocyte antigen (HLA) major histocompatability (MHC) genes as the most significant. Around 80% of diseased individuals carry the epitope of the HLA-DRB1*04 group, and affected individuals expressing two HLA-DRB1*04 alleles stand at high risk for nodular disease, major organ involvement along with surgery related to joint destruction (Smolen *et al*., 2007; Weyand *et al*., 1992). Additional RA-associated loci are PTPN22, TRAF1-C5, TRAF1-C5, STAT4, PADI4 and TNFAIP3, whereas non-MHC risk alleles may possibly represent only 3–5% of the genetic burden of this disease (Plenge, 2009). Some environmental factors, for instance smoking and infection, might also influence the growth, rate of progression and also the severity of RA (Klareskog *et al*., 2007; Getts and Miller, 2010).

A number of immune modulators for example cytokines and effector cells besides signalling pathways have been reported to be involved in the pathophysiology of RA (Smolen and Steiner, 2003). The multifarious interaction of certain immune modulators is accountable for the joint damage that starts at the synovial membrane and then covers most Induced Arthritis structures (Fig. 2.1) (Choy, 2012).

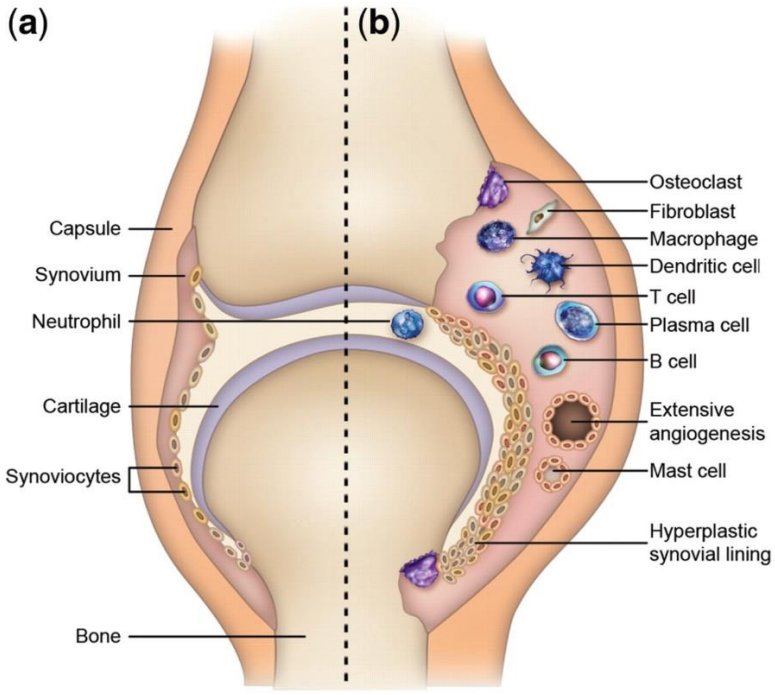

Figure 2.1: Schematic view of normal (a) and RA affected joint (b)

Synovitis is triggered by the influx or local activation or either both, of mononuclear cells including B cells, T cells, dendritic cells, plasma cells, macrophages and also the mast cells (Smolen and Steiner, 2003). The synovial lining turn out to be hyperplastic which leads to the expansion of synovial membrane and villi formation. The osteoclast-rich portion of the synovial membrane, or pannus, cause bone destruction, while enzymes secreted by neutrophils, chondrocytes and synoviocytes damage cartilage (Smolen and Steiner, 2003).

2.4. Risk Factors for RA

A risk factor is any factor which can be either genetic, environmental, or personal that rises the risk of emerging a disease (Alamonas, 2005). RA is defined as multifactorial disease which ususlly results from interactions amongst genetic and environmental factors (Alamonas, 2005; Tobon *et al*., 2010). Several environmental factors have been assumed and studied as it could be related to an increased risk of RA, as well as to a poorer or better prognosis of the disease. But, the impact of most of environmental factors on the risk of developing RA and the manifestation of the disease remains undefined. On the other hand, epidemiologic evidence have been reported which shows that genetic factors are associated to an increased risk of this serious disease. The nature and the effect of RA is becoming clearer through the recent years.

2.4.1. Genetics; The leading cause of RA

It has been reported that 50% of risk of emerging rheumatoid arthritis is because of genetic factors (van der Woude *et al*., 2009). Considerable advancement has been seen in identification of genetic regions marked by structural variation i.e. single nucleotide polymorphisms (SNPs); greater than 30 genetic regions are linked with rheumatoid arthritis (Barton and Worthington, 2009; Orozco *et al*., 2010; Stahl *et al*., 2010). Few gene polymorphisms linked with RA are summarized (Table 2.2).

2.4.2. Age and Gender affecting RA differently

The incidence of RA is greater in women as compared to men. The sex ratio differs in many studies reported from approximately 2:1 to around 3:1, which suggests an effect of reproductive and hormonal factors in the incidence of the disease. On the other hand, the results of some studies provide a conflicting picture on this concern. At the moment, it is not clear just how gender effects the occurrence of RA (Riise *et al*., 2009; Gabriel

et al., 1999; Aho *et al*., 1998). The stage of development of disease start presents a peak in the fifth decade of life conferring to the majority of epidemiological studies that have been conducted. Recent studies reports a later onset of the disease (Riise *et al*., 2009; Gabriel *et al*., 1999; Doran *et al*., 2002; Symmons *et al*., 1994).

Table 2.2: Polymorphisms Associated with Rheumatoid Arthritis According to Meta-Analyses

S. No.	Gene	Chromosome	Polymorphism	Ethnicity	Reference
1	CTLA4	2q33	rs231775 rs3087243	Asians Europeans	Lee *et al*., 2012 Plant *et al*., 2010
2	PTPN22	1p13.2	rs2476601	Overall	Lee *et al*., 2012
3	STAT4	2q32.2 - 32.3	rs7574865	Overall	Liang *et al*., 2012
4	IL-6	7p21	rs1800795	Europeans	Lee *et al*., 2012
5	IL-10	1q31 – 32	rs1800896 rs1800872	Overall	Lee *et al*., 2012
6	IL-2 / IL-21	4q26 – 27	rs6822844	Europeans	Plant *et al*., 2010
7	IL-1B	2q14	rs1143634 rs16944	Asians Europeans	Harrison *et al*., 2008 Lee *et al*., 2009
8	IRF5	7q32	rs2004640 rs739302	Europeans Asians	Lee *et al*., 2013
9	FCRL3	1q21–q22	rs7528684	Asians	Lee *et al*., 2010
10	CD40	20q12–q13.2	rs4810485	Europeans	Plant *et al*., 2010

2.4.3. Hormonal Factors and RA susceptibility

The higher incidence of RA in females in comparison to males suggests a probable part of hormonal factors in susceptibility to the disease. Moreover, estrogens are well-known to have a stimulatory effect on the immune system. Some epidemiological studies tried to inspect this association via studying the possible defensive role of pregnancy, the consumption of oral contraceptives in addition to the hormone replacement therapy after menopause (MacGregor and Silman, 2008; Silman and Hochberg, 2001). In male patients of RA, lower levels of testosterone have been stated (Cutolo *et al*. 2002). Hormone replacement therapy (HRT) may reduce RA susceptibility in women who have HLA-DRB1*01and/or*04 alleles (Salliot *et al*. 2009).

2.4.4. Environmental Factors Playing their Role in RA

The effects of some environmental factors on the risk in addition to outcome of RA have been reported. Environmental factors which affect RA may perhaps act many years before the disease develops clinically apparent.

2.4.4.1. Smoking is Associated with Increased Susceptibility

Among environmental factors, smoking has been reported with the strongest association with this disease. Smoking increases susceptibility to RA and poorly affects the clinical course of the disease too, as revealed by longitudinal and cross-sectional studies (Costenbader *et al*., 2008). The association seems to be dependent on the dose and is most clear for chain smokers. Smoking influences the severity and outcome of this disease, though it is not obvious which clinical physiognomies of RA are related to smoking. An increased risk for seropositive disease is associated to smoking habits (Albano *et al*., 2001; Papadopoulos *et al*., 2005).

2.4.4.2. Protective Role of Dietary Factors against RA

A diet rich in fish, olive oil, and cooked vegetables has been shown to protect against RA, an effect ascribed to the high content in these foods of omega 3 fatty acids. Some studies reports that individuals from southern Europe had milder forms of RA, having reduced extra-articular and radiological manifestations in comparison to other populations (Hagen *et al*., 2009; Rojahn, 2011). This variance may be ascribable in part to the diet in Mediterranean region. A high vitamin D consumption has been related with a lower risk of this disease. Vitamin K, which is present mainly in legumes and other vegetables, might inhibit the multiplication of fibroblast-like synoviocytes, thus lessening the severity of inflammation in RA (Hagen *et al*., 2009; Rojahn, 2011).

2.4.4.3. Pollution Increasing the Risk of RA

Pollutants may also affect the risk of emerging RA. By means of cigarette smoke, inhaled particulate matter might induce local lung inflammation as well as systemic inflammation. Indirect provision for this hypothesis originates from the established link amongst air pollution and diseases concerning pulmonary and systemic inflammation for instance asthma and chronic bronchitis, lung cancers, laryngeal cancers and cardiovascular disease. A latest study conducted in the Nurse's Health Study inspected the distance between the place of residence in 2000 and the nearest road, which assisted as an indicator of contact to traffic pollution. The necessary data were available for about 90,297 nurses. The statistical models were accustomed for age, race, calender year, cigarette smoking, lactation, parity, body mass index, menopausal status and hormone use, use of oral contraceptive, physical activity, and census-tract-level median income and house value. Females living by 50 m of a road had an elevated of RA (hazard ratio, 1.31; 95%CI, 0.98–1.74) as compared to **females** living 200 m or farther

from a road. Therefore, disclosure to traffic pollution in maturity may be a recently acknowledged environmental risk factor for RA (Puett *et al*., 2009).

2.4.4.4. The Effect of Urbanization on RA prevalence

Urbanization has been reported to be related with an increased incidence of RA (Tobon *et al*., 2010). For instance, in the tribe of South Africa (Xhosa), the prevalence of RA is reported to be higher in individuals who are living in an urban as compared to those living a rural environment. Related findings have also been gained in urban, suburban, and rural populations in another country, Taiwan (Chou *et al*., 1994). These findings suggest that environmental factors can also affect RA vulnerability in individuals who have the similar genetic background (Tobon *et al*., 2010).

2.5. Role of Epigenetics in RA

Epigenetics examines the inheritance of characteristics that are not set out in the DNA sequence. For Paro, epigenetic mechanisms form an additional, paramount level of information to the genetic information of DNA (Nave, 2014). Epigenetics originally focuses on DNA methylation and various histone modifications but recently expanded to the field of non-coding RNAs. Ab ovo, each cell of the body inherits the same genetic information. What makes each cell unique is that, during ontogenesis, different sets of genes are turned on and off. Epigenetic mechanisms establish the proper nuclear milieu for cell-specific gene expression and are responsible for the cellular memory that is, keeping and transmitting cell-specific gene expression patterns to daughter cells. Epigenetic factors can deposit, interpret and eliminate epigenetic information and, in this sense, they can be divided into distinct functional groups: epigenetic ‘writers’ or enzymes that modify DNA and histones; epigenetic ‘readers’ with specific protein

domains that recognize DNA or histone marks; and epigenetic 'erasers' that can delete the existing signals to make room for new modifications (Glant *et al*., 2014).

Findings from twin studies support a considerable role for environmental prompts in determining RA risk, as shown by high discordance rates among monozygotic twins (MacGregor *et al*., 2000). The best-understood epigenetic phenomena comprise posttranslational histone modifications and DNA methylation which have a profound effect on gene expression (Jaenisch and Bird, 2003).

Methylation of DNA cytosine residues at the carbon 5 position, generating 5-methylcytosine, arises principally in the setting of cytosine-guanine dinucleotides (CpGs). An unpredicted characteristic of the human genome is the relative scarcity of CpGs due to the frequent mutation of 5-methylcytosine to thymine (Bestor, 2000). Those regions of the genome having high CpG content are termed as CpG islands (CGI), are usually hypomethylated and are linked with the promoter regions of actively transcribed genes (Gardiner-Garden and Frommer, 1987; Saxonov *et al*., 2006). Methylation in regions up to 2kb far from CGIs (termed GpG island shores) can strongly effect gene expression also (Irizarry *et al*., 2009).

N-terminal tails of histone proteins subject to a varied range of diverse modifications comprising acetylation, phosphorylation, methylation then ubiquitylation. More than 60 different histone modification sites have been reported (Kouzarides, (2007). A mechanistic connection evidently exists amongst histone modifications and DNA methylation (Cedar and Bergman, 2009). For instance, the incidence of DNA methylation seemingly helps deacetylation of histone 4, also dimethylation of histone 3 on lysine 9 and inhibiting methylation of histone 3 at lysine 4, all of which are main modifications that prevent gene repression (Hashimshony *et al*., 2003).

Data for epigenetic phenomena in RA are presently limited, particularly in terms of study scale and power (Ballestar, 2011). Still, some interesting observations after studies of DNA methylation patterns are starting to emerge. Such as, examination of DNA methylation in T cells has discovered overall hypomethylation in cells taken from patients with RA in comparison to those from healthy controls (Richardson *et al*., 1990).

Exact study design will be critical uncertainty, when our understanding of the role of epigenetic modifications in RA is to expand. Retrospective case–control studies are promising and also include GWAS information, however care must be taken to make sure that detected differences reflect accurate epigenetic differences and not variance in, for instance, cell-type composition (Viatte *et al*., 2013).

Epigenetic alterations may be beneficial in the clinical setting as markers of disease progression and response to treatment. Likewise, epigenetic alterations offer new and main targets for the improvement of therapeutics in RA patients. Histone deacetylase inhibitors (HDACIs) are presently the best epigenetic therapeutic agents with proven results (Khan *et al*., 2008). The anti-inflammatory properties of HDACIs are reductions in the levels of cytokines such as IL-6, TNF and INF-γ (Leoni *et al*., 2005; Leoni *et al*., 2002). HDACIs possibly will represent, sooner or later, a suitable therapeutic choice for the management of autoimmune diseases for instance RA as they orally active and well tolerated at low doses (De Santis and Selmi, 2012).

2.6. Role of IL-17 in RA

Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly affecting peripheral joints, resulting in the progressive damage of articular cartilage as well as juxta-articular bone (Benedetti and Miossec, 2014; Feldmann *et al*., 1996). During the initial stages of the disease cells of synovial lining which are known by the name

synoviocytes, proliferate markedly leading to a synovium hyperplasia. Disorders in cytokine as well as chemokine signaling lead to permeation of the synovium with immune cells (Feldmann *et al*., 1996) which further lead to activation of various amplification mechanisms that propagate the primary immune response resulting in persistent inflammation and progressive destruction of the tissues. The proinflammatory cytokine interleukin 17 (IL-17), secreted by a range of immune and nonimmune cells together with CD4+ Th17 T cells, has been reported to be a significant contributor to the pathogenesis of few autoimmune diseases, like RA (Zhu anf Qian, 2012). IL-17 is now mentioned as IL-17A, is part of the IL-17 family including six members — from IL-17A to IL-17F. The main family members are IL-17A and IL-17F , both bind then act via two receptors i.e IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC) (Miossec and Kolls, 2012). Some studies in patients and animal models revealed the inference of IL-17 in RA. IL-17 was existing in high concentrations in supernatants obtained from the synovium of RA patients but not from osteoarthritis (OA) patients then these elevated IL-17 levels were positively associated with the severity of the RA in patients (Chabaud *et al*., 1999; Metawi *et al*., 2011). IL-17 is a primary initiator of inflammation. CD4+ T cells were the IL-17-producing cells which were first identified in synovium of RA patients, which were later acknowledged as a distinct lineage from Th1 and Th2 cells and then named as Th17 cells (Harrington *et al*., 2005). Some other cell types may possibly produce IL-17, while it remains to be established at a single cell level. A role for IL-17 was reported throughout all stages of the disease in mouse models of arthritis. Long-term intra-articular administration of this chemo cytokine stimulated collagen arthritis having pathological signs like RA (Lubberts *et al*., 2002) The stimulation of collagen-induced arthritis (CIA) was evidently inhibited in IL-17-deficient mice and arthritis no longer advanced in mice

deficient, both IL-1 receptor antagonist and IL-17 (Nakae *et al*., 2003; Nakae *et al*., 2003). In the acute streptococcal cell wall (SCW)-induced arthritis mouse model, IL-17 signaling was compulsory to change to chronic destructive synovitis (Lubberts at al., 2005). Moreover, antibody-mediated inhibition of IL-17 after the onset of CIA decreased and decelerated the progression of the disease (Lubberts at al., 2004). So, IL-17 is a significant orchestrator of the chronicity of RA by backing to both induction and progression of RA. Three major events are there that initiate IL-17-regulated chronicity. Quite a few epigenetic events, enhanced in patients suffering from RA, cause the augmented production of IL-17. IL-17 further encourages a continuous inflammation in the diseased synovium and then activates anti-apoptotic pathways in the synoviocytes and inflammatory cells which in turn leads to an increased amount of these cells in the joints. Moreover, these IL-17-driven events also initiates many positive feedback-loop mechanisms which leads to increased growth of Th17 cells and thus increased production of IL-17 (Benedetti and Miossec, 2014).

IL-17 prompts a cascade of events producing chronic and worsened inflammation in the synovium. IL-17 persuades the production of several inflammatory mediators by macrophages, mesenchymal and epithelial cells which, consecutively synergize with IL-17 and synergistically produce further immune mediators (Fig. 2.2A). In synoviocytes of RA patients, epigenetic events regulating IL-17 expression in Th17 cells are hypothetically upregulated which further leads to increased IL-17 production (Fig. 2.2B). It has been reported that IL-17 induces the proliferation and survival of the synoviocytes causing synovial hyperplasia. It promotes the proliferation, survival, and maturation of neutrophils and T and B lymphocytes, which arrange themselves in follicular-like structures having germinal centers. The proliferative synoviocytes participates in the T-cell and neutrophil maturation also, by encouraging the production

of particular chemotactic along with anti-apoptotic molecules (Fig. 2.2C) which in turn leads to an higher number of immune cells present in the synovial fluid and the synovium and further adds to the prolongation of inflammation (Fig. 2.2D). Lastly, a number of positive feedback-loop mechanisms lead to the development of Th17 cells and the increased production of IL-17, in addition, establish an IL-17-mediated vicious circle.

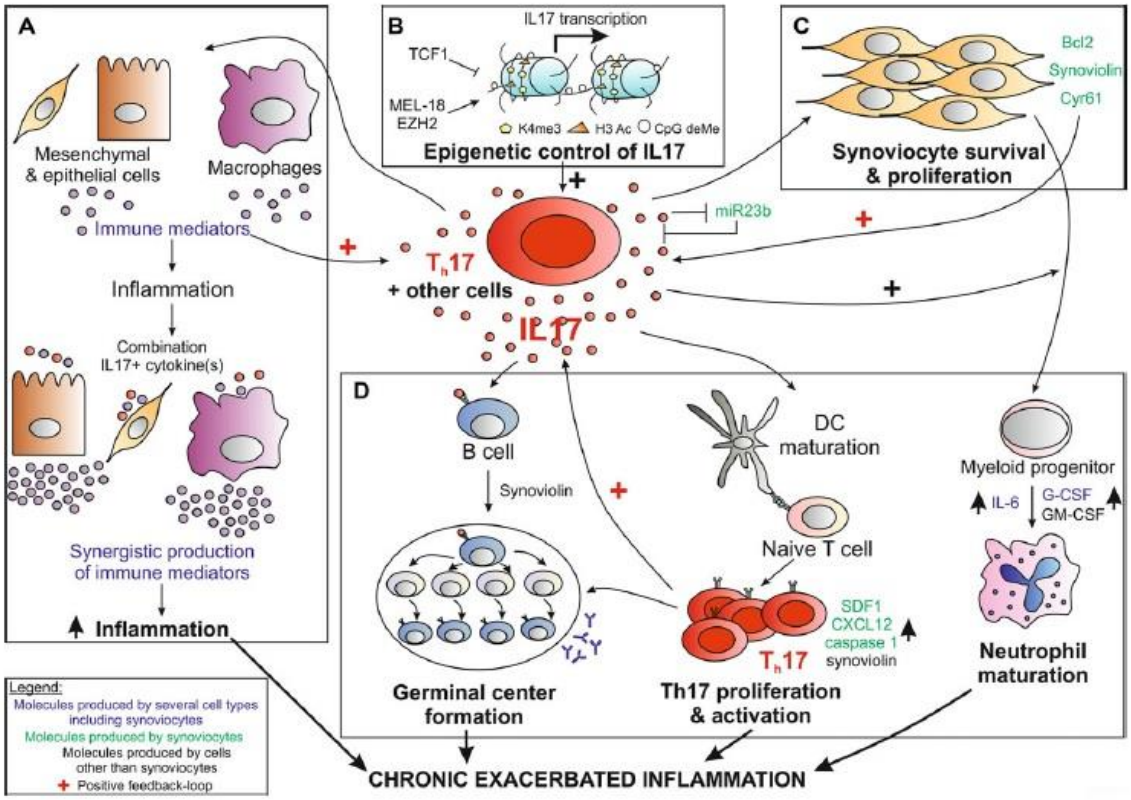

Figure 2.2: Mechanisms regulated by IL-17 leading to RA chronicity

Aims of the Study:

The current study was designed with the following aims.

- To investigate the role of IL-17A polymorphisms in RA pathogenesis.
- To assess the DNA methylation status of IL-17 promoter in RA patients.

CHAPTER 3

MATERIALS AND METHODS

3.1. Genotyping

Genotyping is the process of determining differences in the genetic make-up (genotype) of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.

3.1.1. Study Subjects

The study was a case control based study. Patients were recruited from Rehmat Noor Clinic, Rawalpindi and Military Hospital, Rawalpindi. The patients 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

Blood samples were collected from a total of 125 RA patients and 125 healthy controls for this study. Venous blood for patients and healthy controls were collected in EDTA tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA) using 5 ml 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. Blood samples collected in the vacutainers were stored on ice and dispatched to Immunogenetics Laboratory (IGL), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad. They were then stored at 4^{o}C before further processing.

3.1.3. Genomic DNA Extraction Solutions

DNA was extracted using Phenol-Chloroform extraction method. Four main extraction solutions are used in this method. Composition of these solutions is given in Table 3.1. Components of Solution A were prepared, autoclaved. Solution A was prepared fresh each use. Solution B was prepared and refrigerated.

Table 3.1: Composition of Solutions used in Phenol-Chloroform Extraction of Genomic DNA

S. No.	Solution A	Solution B	Solution C	Solution D
1	0.32M Sucrose	10mM Tris (pH7.5)	Phenol	Chloroform
2	10mM tris (pH7.5)	400mM NaCl		Isoamyl Alcohol (24:1)
3	5mM $MgCl_2$	2mM EDTA (ph 8.0)		
4	1% Triton X-100			

Table 3.2: Purpose of different solutions in Phenol - Chloroform Extraction of DNA

S. No.	Solutions	Function
1	**Solution A**	Lysis of white blood cells (WBCs)
2	**Solution B**	DNA precipitation and separation of proteins
3	**Solution C**	Separation of DNA
4	**Solution D**	Forms the aqueous layer which separates the DNA

3.1.3.1. 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, 11.4 ml glacial acetic acid and 20ml 0.5M EDTA (pH 8.0) was added to distilled water and dissolved then raised to make 1 liter 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 $M_1V_1 = M_2V_2$.

3.1.4. Genomic DNA Extraction

DNA was extracted using phenol-chloroform extraction method. This is a cost effective method, high yielding and a two days protocol.

750µl of blood was transferred to a 1.5 ml Eppendorf tube and Solution A was added in equal amount (750µl). 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 37ºC 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.

3.1.5. Primer Designing

The two IL-17A SNPs rs8193036 and rs2275913 were selected for this study via restriction digestion. Primer sequences were taken from literature that amplified a specific region of IL-17A 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 (http://www.basic.northwestern.edu/biotools/oligocalc.html). The primer sequences, Annealing temperatures and product sizes are given in Table 3.2.

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

Isolation of sufficient DNA for RFLP analysis is time consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA to the levels required for RFLP analysis. An alternative name for the technique is Cleaved Amplified Polymorphic Sequence (CAPS) assay.

PCR was carried out to amplify a specific region on the IL-17A gene, the PCR product was later digested with restriction enzymes for genotyping.

Table 3.3: PCR Primers and Amplification Conditions

S. No.	dbSNP	Change	Primer Sequences	Annealing Temp. (°C)	Product Size (bp)
1	**rs8193036**	C > T	**Forward:** *GAGAAAAGAACCGCTAACTCC* **Reverse:** *TACTCAAGTTCCCATCATATAAG*	47° C	350
2	**rs2275913**	A > G	**Forward:** *GCTCAGCTTCTAACAAGTAAG* **Reverse:** *AAGAGCATCGCACGTTAGTG*	48° C	338

The reaction mixture having a total volume of 20 µl was prepared in 0.2 mL tubes (Biologix, USA). 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). For rs8193036, the thermocycling conditions consisted of initial template denaturation at 95^{o} C for 5 minutes followed by 35 cycles of PCR amplification. Each amplification step further consisted of 3 steps: denaturation at 95^{o} C for 45 seconds, annealing of primers to the template at 47^{o} C for 45 seconds and 1 minute at 72^{o}C for extension of complementary strands of DNA. These amplification cycles were then followed by a final extension step for 7 minutes at 72^{o}C.

For rs2275913, the thermocycling conditions consisted of initial template denaturation at 95^{o} C for 5 minutes followed by 35 cycles of PCR amplification. Each amplification step further consisted of 3 steps: denaturation at 95^{o} C for 30 seconds, annealing of primers to the template at 47^{o} C for 30 seconds and 40 seconds at 72^{o}C for extension of complementary strands of DNA. These amplification cycles were then followed by a final extension step for 7 minutes at 72^{o}C. The PCR products were kept at 4^{o}C until loading on agarose gel.

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

3.1.7. Restriction Digestion

The SNPs located at restriction site can be identified through restriction digestion as any change at the restriction site will change the digestion pattern of the PCR product by restriction enzyme producing fragments of different lengths that can be observed on agarose gels.

MboII enzyme (Thermo Scientific, EU, Lithuania) was used to genotype the SNP rs8193036. *MboII* restriction enzyme recognizes GAAGA (8/7)^ sites and cuts best at 37°C in B buffer (Fig. 3.1). Restriction digestion was carried out in 0.2 ml PCR tubes by adding 2 µl Blue Buffer (Thermo Scientific, EU, Lithuania) and 0.25 µl *MboII* enzyme to 10 µl of PCR product and incubated at 37ºC overnight.

Figure 3.1: Restriction Site of *MboII*

Eam1104I (*EarI*) enzyme (Thermo Scientific, EU, Lithuania) was used to genotype the SNP rs2275913. *Eam1104I* (*EarI*) restriction enzyme recognizes CTCTTC(1/4)^ sites and cuts best at 37°C in Tango buffer (Fig. 3.2).

Figure 3.2: Restriction Site of *Eam1104I* (*EarI*)

Restriction digestion was carried out in 0.2 ml PCR tubes by adding 2 µl Tango Buffer (Thermo Scientific, EU, Lithuania) and 0.25 µl *EarI* enzyme to 10 µl of PCR product and incubated at 37ºC overnight. After the overnight incubation enzymes were denatured to stop the reaction. Enzymes were denatured through incubation at 65º C for 20 minutes. The fragment sizes are given in Table 3.4.

3.1.8. Gel Electrophoresis

Gel electrophoresis was used to determine the purity and analyze DNA qualitatively, to confirm the amplification of desired region of the PCR product and to visualize the fragments of PCR products after restriction digestion.

Table 3.4: Restriction Enzymes and Fragment sizes

S. No.	SNP	Product Size (bp)	Restriction Enzyme	Fragment Size (bp)
1	rs8193036	350	*MboII*	**TT:** 257 + 93 **CC:** 350 **CT:** 350 + 257 + 93
2	rs2275913	338	*Eam1104I* (*EarI*)	**AA:** 259 + 79 **GG:** 338 **AG:** 338 + 259 + 79

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.

2 µl DNA or 5 µl PCR product was mixed with 3 µl loading dye (3.9 ml glycerol, 500 µl 10% (w/v) SDS, 200 µl 0.5 M EDTA, 0.025 g bromophenol blue in final volume of 10ml with distilled water). The mixture was carefully loaded in the wells. 50 bp GeneRuler (Thermo Scientific, EU, Lithuania) was run to measure the length of PCR product or the restriction digestion fragments.

Electrophoresis was done at 100 Volts for 35 minutes in 1X TAE buffer. The gel was analyzed on Gel Documentation System (Dolphin Doc).

3.1.9. Statistical Analysis

Statistical analysis was performed using Graph pad Prism for windows (Version 5.01). Chi-squared (χ^2) test and Fisher's exact tests were used for association analysis. Hardy–Weinberg equilibrium (HWE) was tested using chi-squared test to compare the observed genotype frequencies to the expected frequencies among controls.

3.2. Methylation Analysis

3.2.1. DNA Quantification

DNA was quantified using Thermo Scientific Nanodrop 2000 Spectrophotometer. Double clicked on the desktop NanoDrop™ 2000 software icon and selected the application of interest i.e. nucleic acids and further dsDNA. Followed all the prompts for instrument initialization. Established a Blank using the TE buffer as sample DNA to be quantified was earlier resuspended in TE buffer. Pipetted 1-2 µl of the blanking buffer onto the bottom pedestal, lowered the arm and clicked the Blank button. Wiped away the blank and enter the sample ID in the appropriate field on PC.

Pipetted 1-2 µl of sample and hit Measure. After a measurement, wiped both measurement pedestals using a dry tissue paper and the above steps were repeated to obtain 2nd and 3rd readings. Average was then calculated.

3.2.2. Bisulfite Conversion

For, 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 are supplied in the form of dry mixtures or concentrated form. They need to be dissolved or diluted before use.

3.2.2.1.1. Preparation of Modification Reagent

Modification Reagent is supplied as a dry mixture and it needs to be dissolved before use. The amount of Modification Reagent in each vial provided is sufficient for 10 DNA conversion reactions. For 10 conversion reactions: Added 0.9 mL of molecular biology grade water, 200 µl of Modification Solution I and 60 µl of Modification Solution II to one vial of dry Modification Reagent. Dissolved it by inverting for about 10 min.

3.2.2.1.2. Preparation of Wash Buffer

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

3.2.2.1.3. Preparation of Desulfonation Buffer

Desulfonation buffer is supplied in concentrated form and it was prepared to use through dilution using 10 ml of 96-100% ethanol.

3.2.2.2. Protocol of Bisulfite Conversion

Added 500 ng of purified genomic DNA into a PCR tube. Made the volume of DNA sample up to 20 µl using Nuclease Free Water (NF water). 120 µl of prepared Modification Reagent solution was then added to this PCR tube. Mixed the sample by pipetting up and down, then centrifuged the reaction mixture to the bottom of the tube. Placed PCR tubes into a thermal cycler and proceeded with the following Protocol to perform denaturation and bisulfate conversion of DNA (Fig. 3.3).

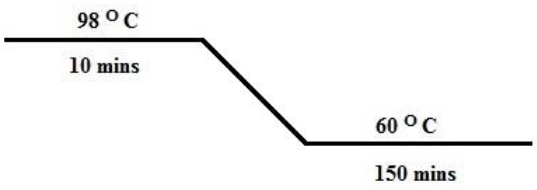

Figure 3.3: PCR Profile for Bisulfite Conversion of Genomic DNA

Added 400 ul of Binding Buffer to the DNA Purification Micro Column placed into the collection tube. Loaded the converted DNA sample into the Binding Buffer in the column, mixed completely by pipetting. Centrifuged the micro column placed into the collection tube at 12,000 rpm for 30 seconds. Discarded the flow-through. Placed the micro column into the same collection tube. Added 200 ul of Wash Buffer, prepared with ethanol, to the micro column and centrifuged at 12,000 rpm for 30 seconds. Discarded the flow-through. Placed the micro column into the same collection tube. Added 200 µl of Desulfonation Buffer, prepared with ethanol, to the micro column and let the column stand at room temperature for 20 min. Centrifuged the micro column placed into the collection tube at 12,000 rpm for 30 seconds. Discarded the flow-through. Placed the micro column into the same collection tube. Added 200 µl of Wash Buffer, prepared with ethanol, to the micro column and centrifuged at 12,000 rpm for 30 seconds. Discarded the flow-through. Place a micro column into the same collection tube. Added an additional 200 µl of Wash Buffer, prepared with ethanol, to the micro column and centrifuged at 12,000 rpm for 60 seconds. Place the column into a clean 1.5 ml microcentrifuge tube. Added 10 µl of Elution Buffer to the micro column. 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

Logged on to Ensembl Genome Browser (http://www.ensembl.org/index.html). Chose "Human" as desired specie and searched for IL-17A. Clicked on the best matched search on search result page and Gene summary page was opened. On the left, under "Gene Summary", clicked "Sequence", 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. Clicked "Configure this page" in the lower left column, a popup window opened allowing to input the size of 5' Flanking sequence (upstream). Desired size of upstream region i.e. 1000 bp was entered in the box and then saved the configuration. Just to ensure that the selected region was promoter sequence, copied the promoter sequence. 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. The promoter sequence was entered in the "source sequence" box, checked "Pick MSP Primers" and leaving all the other entries to default settings, clicked "Submit". On the result page, selected the primers covering maximum number of CpG islands for this study.

Two sets of primers were used for analysis for methylation status. One pair for methylated DNA and other for unmethylated DNA. The primers sequences are given in Table 3.5.

Table 3.5: Methylated Specific Primers for amplification of Bisulfite Converted DNA

S. No.	Primer	Sequence (5' – 3')	Product Size (bp)
MSP 1	Left Methylated-Specific primer	TTTTGAATTTATTGCGATACGTTAC	163
	Right Methylated-Specific primer	ATAAAATCTTCCCAAAAATCATCG	
	Left Unmethylated-Specific primer	TTTGAATTTATTGTGATATGTTATGT	161
	Right Unmethylated-Specific primer	TAAAATCTTCCCAAAAATCATCATT	
MSP 2	Left Methylated-Specific primer	TTTTGAATTTATTGCGATACGTTAC	163
	Right Methylated-Specific primer	ATAAAATCTTCCCAAAAATCATCG	
	Left Unmethylated-Specific primer	TTTGAATTTATTGTGATATGTTATGT	160
	Right Unmethylated-Specific primer	AAAATCTTCCCAAAAATCATCATT	

MSP = Methylation Specific Primers; Left Methylated- Specific Primer = Forward Primer; Right Methylated- Specific Primer = Reverse Primer;

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 IL-17A. For each sample, two reactions were run. One containing Methylated-Specific primer pair while other containing Unmethylated-Specific primer pair. Reaction

mixture (20 µl) was prepared in 0.2 ml PCR tubes (Biologix, USA) contained 2 µl of Bisulfite converted DNA, 2 µl of Left and Right Methylated or Unmethylated-specific primers, 4 µl NF 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 35 cycles of PCR amplification. Each amplification step further consisted of 3 steps: denaturation at 95^{o} C for 1 minute, annealing of primers to the template at 55^{o} C for 1 minute and 1 minute at 72^{o}C for extension of complementary strands of DNA. These amplification cycles were then followed by a final extension step for 10 minutes at 72^{o}C. The PCR products were kept at 4^{o}C until loading on agarose gel.

3.2.5. Gel Electrophoresis

Gel Electrophoresis was carried out to analyze the methylation status of the IL-17A promoter. 2% agarose gel was prepared by melting 1.6 g of agarose in 80 ml of 1x TAE buffer in microwave oven for 2 minutes. 2 µl Ethidium bromide (0.5 µg/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 µl 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:

Case – control study is comprised of examination of affected and control samples for the presence of polymorphisms. In this study, polymorphisms were examined through RFLP. A specific region of IL-17A including the SNP was amplified through PCR and was then digested using specific restriction endonucleases that recognises and cut the amplified PCR product at SNP site indicating its presence or absence in the sample DNA. Two IL-17A SNPs rs2275913 and rs8193036 were included in this study.

4.1.1. Study Subjects:

A total of 250 human DNA samples were used in the study. 125 of the total were RA patients while the remaining 125 were healthy control subjects. All the RA patients recruited in this study were tested for Anti-CCP and RA factor. The patients who were positive for both the tests were then recruited in the study. 82.4% of RA patients included in the study were females and remaining 17.6% were males. The control group had 72.8 % female and 27.2 % male samples. The average age of RA patients was 43.74 years while 37.09 years in case of controls subjects.

The characteristics of RA patients and control subjects included in this study are summarized in the Table 4.1.

Table 4.1: Characteristics of RA patients and control subjects included in study

S. No.	Characteristics	Cases		Controls
1.	**Anti – CCP* (Positive)**	125		0
2.	**RA Factor (Positive)**	125		0
3.	**Females (%)**	82.4		72.8
4.	**Males (%)**	17.6		27.2
5.	**Age (Years ± SD**)**	43.74 ± 14		37.09 ± 13.36
6.	**Disease Duration**	>6 months	31.5 %	
		>2 Years	14.6 %	
		>4 Years	53.9 %	

*Anti - CCP: Anti-Cyclic Citrullinated Peptide Antibody Test

**SD: Standard Deviation

4.1.2. Association Analysis of IL-17A SNP rs8193036:

SNP rs8193036 is located in promoter region of IL-17A. A total of 250 individuals including 125 control and 125 control subjects were examined for this SNP. The extracted genomic DNA was in less quantity, so it has to be amplified in order to get a quantity that is suitable for RFLP analysis. After PCR amplification, the products were run on 2% agarose gel to confirm the amplification (Fig. 4.1).

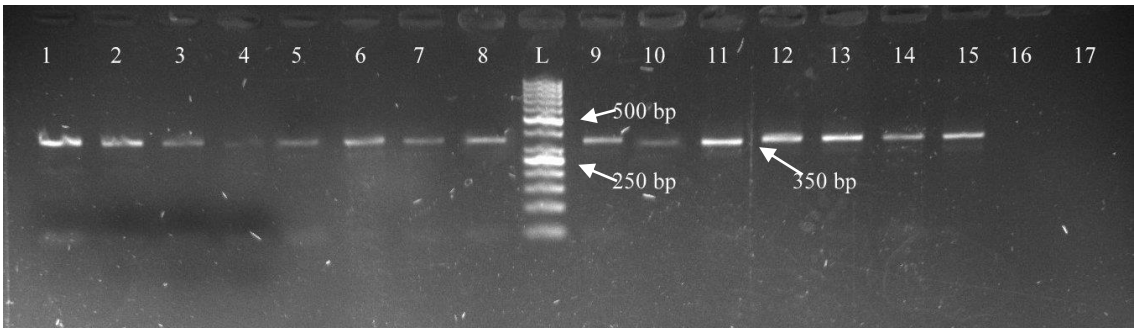

Figure 4.1. 2% Agarose Gel image showing amplified PCR product containing the SNP rs8193036

(Lane 1 – 15 = amplified PCR Product for different DNA samples (Product size = 350 bp). "L" denotes 50 bp Ladder. Lane 16 – 17 Negative controls)

These PCR products were then digested using restriction enzymes. All the results obtained from experiments through PCR amplification followed by restriction digestion and finally visualized on agarose gel were carefully analysed for presence or absence of the SNP (Fig 4.2).

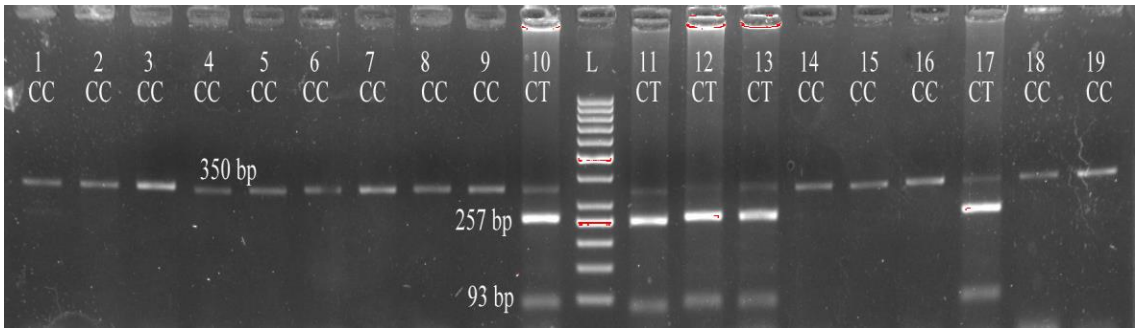

Figure 4.2: Restriction Fragment Length Polymorphism (RFLP) analysis of amplified PCR product of IL-17A Promoter region (350 bp) containing SNP rs8193036 subsequently digested by *MboII*.

(The restriction enzyme *MboII* cuts the PCR product only if "T" allele is present at SNP site. Lane 1 – 9 = No restriction is observed, as only a single fragment of 350 bp can be seen, so we analysed that the individual is homozygous for the allele present at SNP site that is not restriction site for the enzyme i.e. CC. In Lane 10 = three bands can be seen corresponding to 350 bp, 257 bp and 93 bp indicating the individual to be heterozygous for the allele at SNP site i.e. CT. "L" denotes 50 bp Gene Ruler. **Note:** This representative gel does not contain any individual with the homozygous TT alleles, in that case fragments of 257 and 93 bp will be observed).

Genotype and allele frequency was calculated using traditional counting techniques. Genotype distribution among affected and control subjects was carefully calculated and matched. The data was then analysed statistically for significance evaluation. GraphPad Prism for windows, version 5.01 was used for statistical analysis. Chi squared ($\chi 2$) test was performed to evaluate any association between SNP rs8193036 and RA. The association value was 1.299 and the *p value* for the genotype data for SNP rs8193036 obtained through experimentation was 0.5223 (Table 4.2). The values obtained showed no statistically significant association of the SNP rs8193036 to RA. The data is graphically represented in Fig. 4.3.

Table 4.2: IL-17A rs8193036 Genotype Distribution among Cases and Controls Subjects

S. No.	Cases / Controls	Genotype Count (%)			$\chi 2$	D*f*	P value (<0.05)
		CC	CT	TT			
1	**Cases**	65 (52)	44 (35.2)	16 (12.8)	**1.299**	**2**	**0.5223**
2	**Controls**	57 (46.6)	47 (37.6)	21 (16.8)			

Allele frequencies were also calculated for the SNP rs8193036. The data obtained was statistically analysed through chi squared ($\chi 2$) test and also validated through Fisher's exact test. Odd ratios with 95% confidence interval (95% CI) were calculated. The statistical analysis does not showed any significant drift between allele frequencies among RA cases and healthy control individuals (p = 0.2163). The data is given in Table 4.3 and graphically represented in Fig. 4.4.

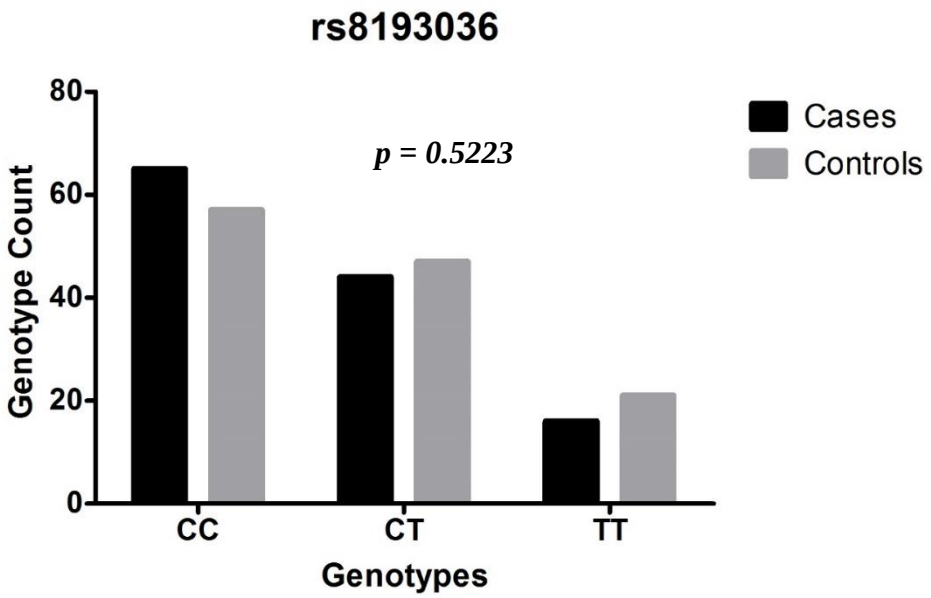

Figure 4.3: Graphical Representation of IL17A rs8193036 Genotype Distribution

Table 4.3: Allele Frequency Distribution of SNP rs8193036 among Cases and Control Subjects

S. No.	Cases / Controls	Allele Count (%)		OR (95% CI)	χ^2	P value (<0.05)
		C	T			
1	**Cases**	174 (69.6)	76 (30.4)	1.266 (0.8709 - 1.839)	1.529	**0.2163**
2	**Controls**	161 (64.4)	89 (35.6)			

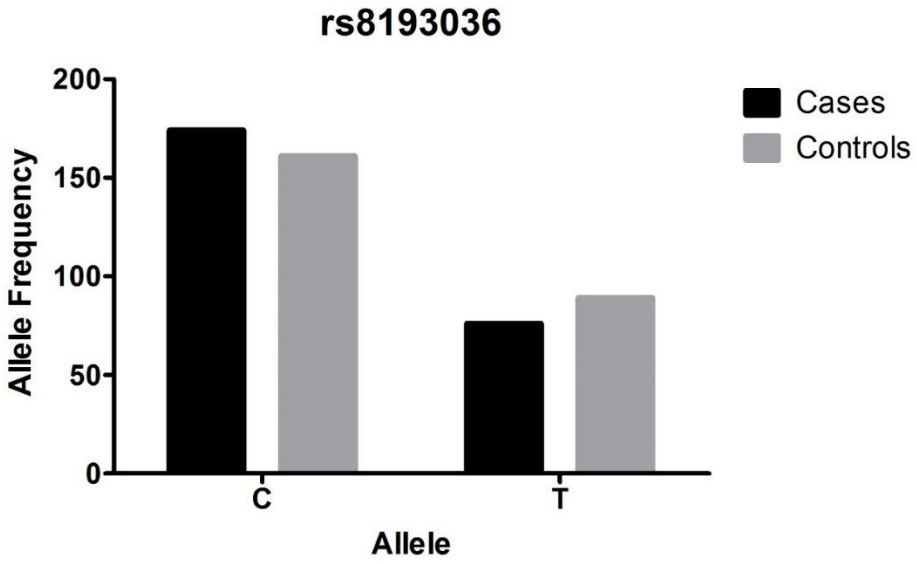

Figure 4.4: Graphical Representation of Allele Frequency Distribution of IL17A rs8193036

Hardy Weinberg Equilibrium (HWE) calculations were made for both RA cases and controls to check the deviation of observed genotype frequencies from that of expected genotype frequencies estimated by HWE equation. There was no significant difference between the observed and expected genotypes of RA cases (p = 0.4654) and between observed and expected genotypes of control patients (p = 0.3933). The observed genotype frequencies for the polymorphism rs8193036 in the RA cases and healthy control samples were in HWE. All the possible observed and expected genotypes for polymorphism rs8193036 are summarized in Table 4.4.

Table 4.4: Hardy Weinberg Equilibrium Calculations of the Study Population for SNP rs8193036

S. No.	Cases / Controls	Observed Genotype Count			Expected Genotype Count			$\chi 2$	P value (<0.05)
		CC	CT	TT	CC	CT	TT		
1	**Cases**	65	44	16	60.6	52.9	11.6	**1.530**	**0.4654**
2	**Controls**	57	47	21	51.8	57.3	15.8	**1.867**	**0.3933**

4.1.3. Association Analysis of IL-17A SNP rs2275913:

SNP rs2275913 is located in promoter region of IL-17A. A total of 250 individuals including 125 control and 125 control subjects were examined for this SNP. For increase the quantity of genomic DNA, it was amplified with the specific pair of primers to amplify the region including SNP rs2275913 (Fig. 4.5). For confirmation, PCR products were run on 2% agarose gel.

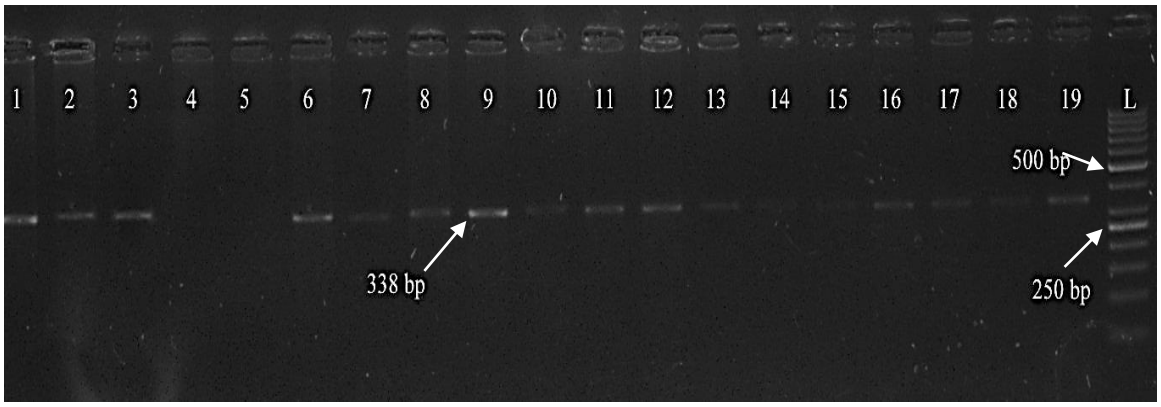

Figure 4.5. 2% Agarose Gel image showing amplified PCR product containing the SNP rs2275913

(Lane 1 – 19 = amplified PCR Product for different DNA samples (Product size = 338 bp). "L" denotes 50 bp Ladder)

The products were then digested using restriction enzymes and were analysed on 3 % agarose gel (Fig. 4.6).

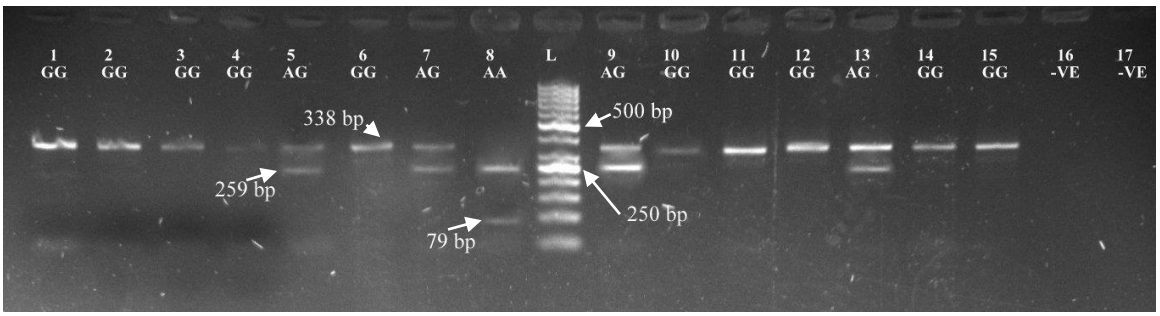

Figure 4.6: Restriction Fragment Length Polymorphism (RFLP) analysis of amplified PCR product from IL-17A promoter region including SNP rs2275913 subsequently digested by restriction enzyme *Eam1104I* (*EarI*) and visualized on 3% agarose gel

(The restriction enzyme *Eam1104I* (*EarI*) cuts the PCR product only if "A" allele is present at SNP site. Lane 1 – 4 = No restriction is observed, as only a single fragment of 338 bp can be seen, so we analysed that the allele present at SNP site will be GG. In Lane 5 = a restriction fragment of 259 bp can be seen along with a fragment of 350 bp, therefore, we concluded that this subject would be heterozygous for the SNP and contains both the alleles i.e. AG. In Lane 8, one fragments of 259 bp and a very faint band of 79 bp can be seen, therefore, the analysis revealed it to be homozygous for the SNP i.e. AA. "L" denotes 50 bp Gene Ruler. "-ve" refers to negative controls).

Traditional counting method was used for genotype and allele counting. Genotype distribution among affected and control subjects was carefully calculated and matched. The data was then analysed statistically for significance evaluation. GraphPad Prism for windows, version 5.01 was used for statistical analysis. Chi squared ($\chi 2$) test was performed to evaluate any association between SNP rs2275913 and RA. The association valve was 8.760 and the *p value* for the genotype data for SNP rs2275913 obtained through experimentation was found to be 0.0125 (Table 4.5). The values obtained showed significant association of the SNP rs2275913 to RA. The data is graphically represented in Fig. 4.7.

Table 4.5: IL-17A rs2275913 Genotype Distribution among Cases and Controls Subjects

S. No.	Cases / Controls	Genotype Count (%)			$\chi 2$	d*f*	P value (<0.05)
		AA	AG	GG			
1	**Cases**	22 (17)	52 (42)	51 (41)	**8.760**	**2**	**0.0125**
2	**Controls**	20 (16)	32 (26)	73 (58)			

Allele frequencies were also calculated for the SNP rs2275913. The data obtained was statistically analysed through chi squared ($\chi 2$) test and also validated through Fisher's exact test. Odd ratios with 95% confidence interval (95% CI) were calculated. The statistical analysis does not showed any significant drift between allele frequencies among RA cases and healthy control individuals. The data is given in Table 4.6 and graphically represented in Fig. 4.8.

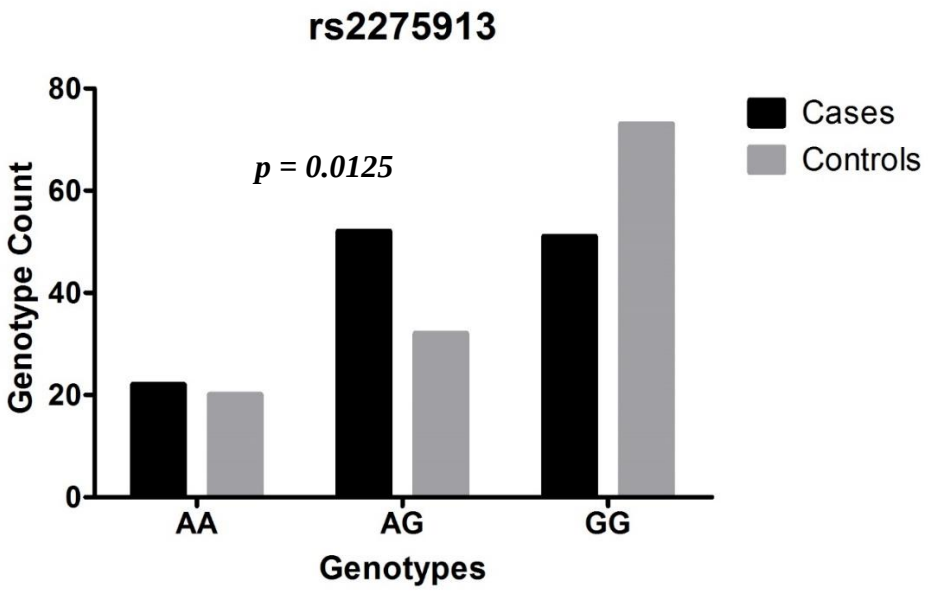

Figure 4.7: Graphical Representation of IL17A rs2275913 Genotype Distribution

Table 4.6: Allele Frequency Distribution of SNP rs2275913 among RA Cases and Control Subjects

S. No.	Cases / Controls	Allele Count (%)		OR (95% CI)	$\chi 2$	P value (<0.05)
		A	G			
1	**Cases**	96 (38.4)	154 (61.6)	1.576 (0.8695 - 2.857)	2.261	**0.1326**
2	**Controls**	72 (28.2)	178 (71.2)			

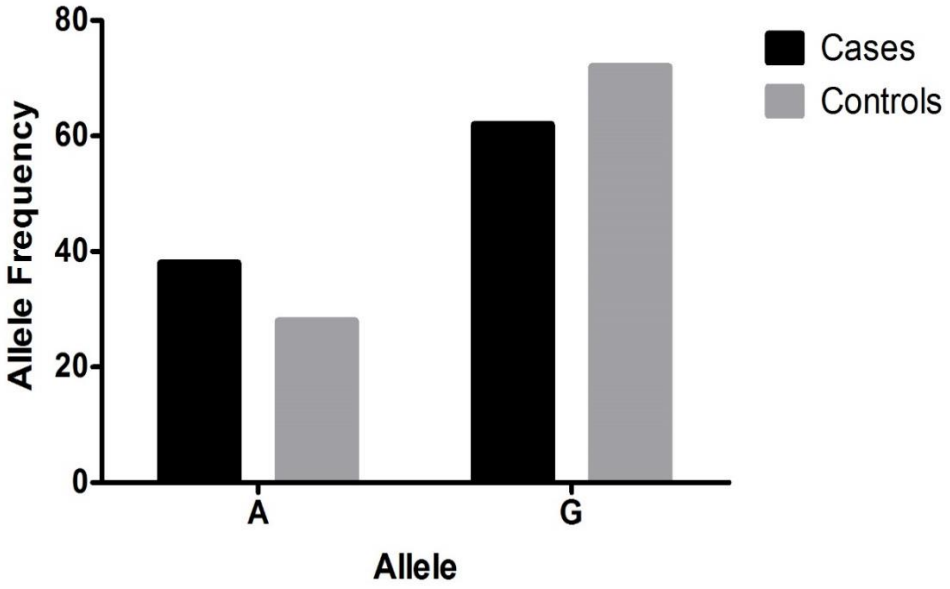

Figure 4.8: Graphical Representation of Allele Frequency Distribution of IL17A rs2275913

Hardy Weinberg Equilibrium (HWE) calculations were made for both RA cases and controls to check the deviation of observed genotype frequencies from that of expected genotype frequencies estimated by HWE equation. There was no significant difference between the observed and expected genotypes of RA cases (p = 0.6063). While a significant difference was found in observed and expected genotypes of control patients (p = 0.0149). The observed genotype frequencies for the polymorphism rs2275913 in the RA cases was in HWE but not in controls. All the possible observed and expected genotypes for polymorphism rs2275913 are summarized in Table 4.4.

Table 4.7: Hardy Weinberg Equilibrium Calculations of the Study Population for SNP rs2275913

S. No.	Cases / Controls	Observed Genotype Count			Expected Genotype Count			χ^2	P value (<0.05)
		AA	AG	GG	AA	AG	GG		
1	**Cases**	22	52	51	18.4	59.1	47.4	**1.001**	**0.6063**
2	**Controls**	20	32	73	10.4	51.3	63.4	**8.414**	**0.0149**

4.2. Methylation Status of IL-17A promoter:

PCR amplification of the CpG islands encompassing the promoter region of IL-17A by use of methylation-specific and unmethylation-specific primers revealed that this region was consistently unmethylated in healthy controls. However, in RA patients the high percentage of partially methylated individuals was observed. The gel electropherogram of the methylation-specific and unmethylation-specific PCR products in given in Fig. 4.9. Table 4.8 summarizes the individual methylation status of RA cases and healthy controls, as examined by methylation-specific and unmethylation-specific PCR. The results are graphically represented in Fig. 4.10.

The results obtained from methylation and unmethylation-specific PCR were evaluated for statistical significance through chi squared ($\chi 2$) test. The results shows significance association of methylation status to RA (p = <0.0001). It can be interpreted that the promoter region in RA patients were hyper-methylated as compared to healthy controls.

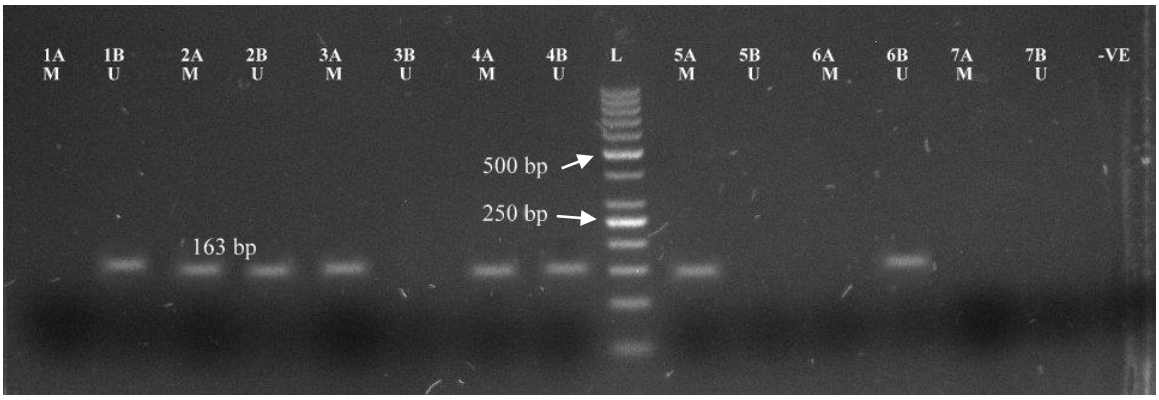

Figure 4.9: Methylation Status of IL-17A promoter Region, as determined by Methylation-specific and Unmethylation-specific PCR

(M = Methylation-Specific Primers; U = Unmethylation Specific Primers; Numbers 1 – 7 denotes DNA samples; L = 50 bp Gene Ruler)

Table 4.8: Methylation Status of IL-17A promoter regions as determined by Methylation Specific Polymerase Chain Reaction

S. No.	Cases / Controls	Methylation Status (%)			χ^2	P value (<0.05)
		Methylated	Partially Methylated	Unmethylated		
MSP1	Cases	30	50	20	78.86	< 0.0001
	Controls	0	20	80		
MSP2	Cases	20	60	20	40.00	< 0.0001
	Controls	20	20	60		

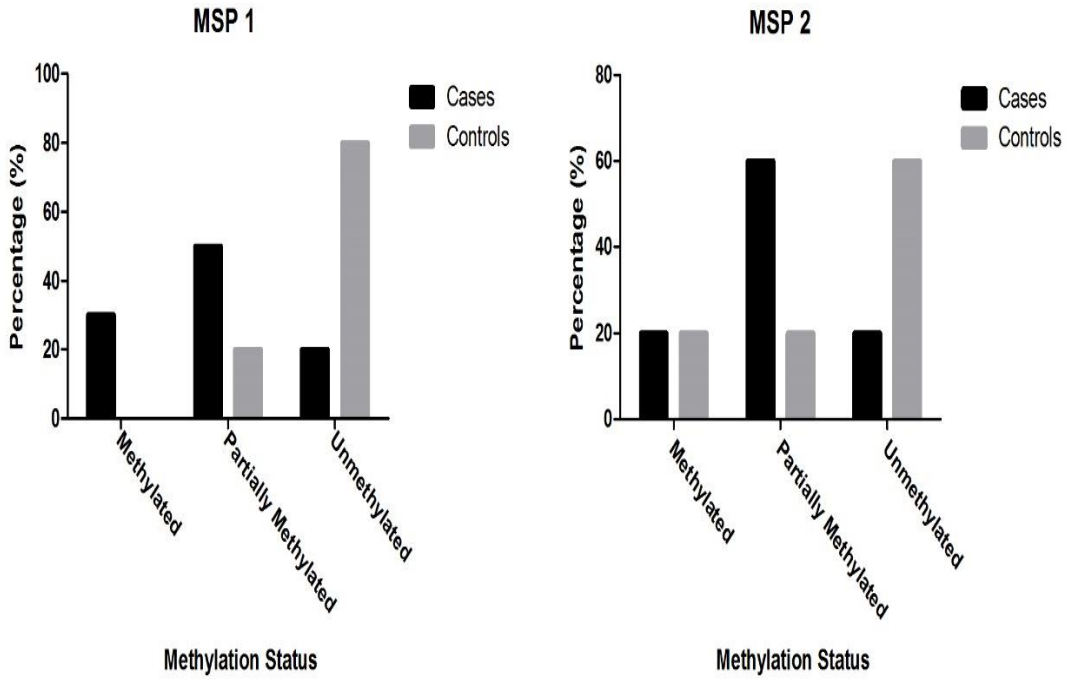

Figure 4.10: Graphical Representation of Methylation Status of IL-17A Promoter

CHAPTER 5

DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by hypertrophy, hyperplasia and angiogenesis of synovial tissue leading to inflammatory joint destruction and it affects 1% of the adults worldwide. IL-17A is a proinflammatory cytokine which affects several different cell types to increase the production of other proinflammatory molecules and several evidences have shown its involvement in pathogenesis of RA (Hueber *et al*., 2010; Zrioual *et al*., 2009; Hot and Miossec, 2011; Ahmed *et al*., 2015; Carvalho *et al*., 2015; Bogunia-Kubik *et al*., 2015). The IL17A gene is located on Chromosome 6, and the association between its SNPs and certain diseases has been studied recently.

In this study, the association between the IL17A rs2275913 (A>G) and rs8193036 (C>T) polymorphisms and the RA risk in a Pakistani population. No significant association was found between IL17A rs8193036 SNP in this study (p = 0.5223). Shen *et al*. reported association between IL17A rs8193036 and a significantly increased RA risk in younger patients (Shen *et al*., 2015). No significant association between RA risk and IL17A SNP rs8193036 was reported in Caucasian populations of Norway and New Zealand (Nordang *et al*., 2009).

A significant association between IL17A rs2275913 and RA was found. It was found that SNP A>G decreases the risk for RA as the percentage of genotype “GG” was significantly higher in healthy controls (58%) against RA patients (41%). Similar results are reported in a recent study stating that IL17A rs2275913 variant alleles decrease the risk of RA (Shen *et al*., 2015). An association between IL17A SNPs and

RA was established in Caucasian populations of Norway and New Zealand (Nordang *et al*., 2009). Their study showed a fair association between RA prevalence and IL17A SNP rs2275913 among Norwegian population but their results were not replicated in population from New Zealand (Nordang *et al*., 2009). IL17A polymorphisms are found to be associated with an increased risk of gastric cancer, breast cancer and cervical cancer in several studies (Shibata *et al*., 2009; Quan *et al*., 2012; Wang *et al*., 2012; Han *et al*., 2014). In a recent study, Han *et al*. showed that polymorphisms of IL17A G-197A may be closely associated with susceptibility to osteoarthritis in the Korean population (Han *et al*., 2014).

Methylation status of the IL17A promoter region was also evaluated. Methylation status was scored as methylated, partially methylated and unmethylated. The results obtained showed significant difference among RA patients and healthy controls (p = <0.0001). The promoter region was predominantly unmethylated in healthy controls while partially methylated among RA cases in this study. The data suggests that the methylation status of CpG islands in IL17A promoter region may affect IL17 cytokine production and may play a role in pathogenesis of RA. Nile *et al*. reported the potential role of CpG islands methylation in pathogenesis of RA (Nile *et al*., 2008). The CpG Island in the DR3 gene promoter was specifically methylated to down-modulate the expression of DR-3 protein in rheumatoid synovial cells, which may provide resistance to apoptosis in RA synovial cells (Takami *et al*., 2006).

There are several limitations of this study should be properly addressed. First, the sample pool employed in this study case - control study cannot fully represent the general population. Secondly, the genetic variability of any gene cannot be viewed comprehensively on the basis of two or three polymorphisms, therefore, IL17A cannot be viewed comprehensively on the basis of polymorphisms investigated in this study

alone. That ask for further studies in the future. Thirdly, the sample size of this study was not sufficiently large to evaluate the low penetrance effect of the SNPs. Fourth, the observed genotype frequencies for IL17A rs2275913 A>G polymorphisms in the healthy controls did not establish Hardy Weinberg Equilibrium.

Conclusion:

Rheumatoid Arthritis (RA) is a chronic polyarthritic autoimmune condition characterized by severe joint destruction as a consequence of synovial hyperplasia. In conclusion, the present study demonstrated that the IL17A SNP rs2275913 (A>G) significantly decreases the risk of RA. While no association was found between IL17A SNP rs8193036 (C>T) and risk of RA. Methylation status of the IL17A promoter region in significantly associated with the disease. However, these results were obtained from only a moderate-sized sample population. Replication of these findings in larger populations from diverse ethnicity are required to confirm these findings.

Future Prospects:

The limitations of the current study should be addressed in future. The study should be performed on a larger populations and diverse ethnicities for confirmation and better understanding of the current results. More polymorphisms of IL-17A should be studied to evaluate its genetic variability. Methylation analysis should be verified through a more advanced technique i.e. Bisulfite Sequencing.

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