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GENETIC, PHARMACOGENETIC AND EPIGENETIC OF EARLY RHEUMATOID ARTHRITIS

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TABLE OF CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

Abstract

Background: Undifferentiated arthritis (UA) is a common inflammatory type of arthritis which is known by joint swelling, pain, and stiffness and is not classified as a specific rheumatologic disease. It is estimated that 32% of UA patients will develop rheumatoid arthritis (RA) that is a chronic, inflammatory, autoimmune disease which could cause joint damage and unchangeable disability. RA has heterogeneous presentations and affects 0.5 to 1.0% in white populations. Several risk factors have been reported for RA development. These include genetic, sex, and environmental factors such as slilica exposure, infectious agents, smoking, deficiency of vitamin D, obesity, and microbiota changes. Genetic factors play a significant role in rheumatoid arthritis development, and probably account for 50–60% of disease susceptibility which are classified as Human Leukocyte Antigen (HLA) and non-HLA genes. Methotrexate (MTX) is a first-line treatment in rheumatoid arthritis management. It is the most important drug in combination therapies, and is considered as a gold standard among RA therapies. Epigenetics as a promising and growing research field in rheumatoid arthritis not only contributes to the RA development but also could be involved in disease management and therapy.

Objectives: The current study aimed in **a)** evaluating the association of well-known single nucleotide polymorphisms (SNPs) belonging to human leukocyte antigen (HLA) and non-HLA genes with early rheumatoid arthritis (ERA) development among UA diagnosed patients; **b)** assessing the response to therapy of ERA patients according to genetic background based on SNPs involved in MTX/folate pathway, and **c)** studying long interspersed transposable element 1 (LINE1) methylation status among healthy control, ERA and RA cases and also its methylation changes in response to MTX therapy among ERA patients.

Subjects, Material and Methods: A total of 471 patients with UA have been recruited. Genomic DNA from white blood cells (WBC) was extracted and genotyping for 7 polymorphisms belonging to HLA genes including rs1233334, rs1063320, 14 bp Insertion/deletion, rs660895, rs6910071, rs9275595 and rs10807113 and another 7 SNPs belonging to non-HLA genes including PTPN22 rs2476601, PADI4 rs2240340, STAT4 rs7574865, CTLA4 rs231775, TRAF1 rs3761847, IL-10 rs1800871 and IL-6 rs1800795 have been performed. Regarding response to the therapy, 6 SNPs involving in MTX/Folate pathway including ATIC rs2372536, MTRR rs1801394, SHMT1 rs1979277, SLC19A1 rs1051266, MTHFR rs1801133 and MTHFR rs1801131 had been genotyped. In respect of epigenetics, LINE1 methylation status of synovial cells and WBC was evaluated. Besides, the methylation status of 50 healthy controls, 50 ERA and 30 RA patients were compared. Finally, LINE1 methylation of ERA patients according to the response to therapy have been investigated. Statistical analyses for genetic association, gene-gene interaction and methylation level has been carried out.

Results: Regarding the association of HLA variants with ERA development, no significant association was found. With respect to non-HLA SNPs there was not significant association with ERA

development, too. After stratification according to sex, there was a significant association of TRAF1 rs3761847 GA heterozygous in males ($p<0.05$). The association was under dominant model. Multifactorial Dimensionality Reduction (MDR) analysis also revealed that there was an association between smoking and anti-Citrullinated Protein Antibody (ACPA) with ERA development. Regarding association of MTX/folate-related SNPs, there was a significant association of CC homozygous genotype of SLC19A1 rs1051266 in ACPA-positive patients with response to the therapy ($p<0.02$). In addition, MDR analyses has shown that there was an association of SHMT1 rs1979277 SNP and smoking with response to the therapy. LINE1 methylation study has demonstrated that there was no significant differences between methylation level of synovial cells and WBC. In addition, there was no significant difference between LINE1 methylation level among either healthy controls, ERA and RA patients or ERA patients and response to the therapy.

Conclusion: Considering the heterogeneity in RA development and discrepancy in observed results, further genetic and epigenetic studies are recommended in order to provide more detailed information as the present study revealed potential interactions between different factors. This approach will shed light on more efficient and fruitful personalized medicine in RA management and treatment.

Chapter 1

Introduction

Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease which affects joints and known by antibodies against immunoglobulin G (IgG). RA can result in accumulating joint damage and unchangeable disability. RA has heterogeneous presentations and pathogenetic pathways among cases with identical diagnosis or different disease phases. In spite of having autoantibodies as an important feature of RA (seropositive RA), some cases do not have autoantibodies (seronegative RA). Rheumatoid arthritis has complicated pathology that by involving environmental parameters, initiates disease in individuals with susceptible genetic (Malmstrom *et al.,* 2017).

During the last two decades, there have been considerable progresses for RA classification criteria that resulted from RA cases with early rheumatoid arthritis (ERA) to recently identified autoantibodies. New advancements in disease evaluation and treatment policies along with development and approval of different therapies have been presented. Overall, significant improvement in the field has facilitated prognoses of many patients. Despite lack of complete cure of RA, remission is currently a reachable aim. Nevertheless, remission for all cases is not possible and further work and studies are required to provide each patient with the advantages of treatment success (Smolen *et al.,* 2018).

Epidemiology

Due to lack of comprehensive epidemiological reports, the prevalence of rheumatoid arthritis is limited; however, its rate seems similar across different regions (Myasoedova *et al.,* 2010). Most of RA prevalence reports belong to Western countries, indicating a rate of 0.5 to 1.0% in white populations (Myasoedova *et al.,* 2010; Tobon *et al.,* 2010). Approximately, similar prevalence has been reported in Kinshasa, Democratic Republic of the Congo, 0.6% in black individuals with 0.9% in cases above 18 years old (Malemba *et al.,* 2012). RA prevalence is different among various races, as a higher range, 5 to 6%, had been presented for Native Americans (Peschken *et al.,* 1999). The prevalence for African-American, Asian and Hispanic females are 1.02%, 0.69%, and 0.45%, respectively (Kawatkar *et al.,* 2012). According to some limited reports, geographical differences in RA prevalence has been observed; for instance, northern Europe has higher prevalence than southern Europe (Tobon *et al.,* 2010).

Risk Factors

Several risk factors have been reported for rheumatoid arthritis development. These include genetic, sex, and environmental factors such as slilica exposure, infectious agents, smoking, deficiency of vitamin D, obesity, and microbiota changes (Smolen *et al.,* 2018).

Genetic. Twin studies have shown an approximate 60% heritability among anti-citrullinated protein antibodies (ACPAs) positive rheumatoid arthritis patients (MacGregor *et al.,* 2000; Stahl *et al.,* 2012). However, this value is lower among seronegative patients (Padyukov *et al.,* 2011). On the other hand, 12 to 15% of disease concordance among identical twins highlights the role of environmental factors in RA development (Smolen *et al.,* 2018).

Sex. Female are 2 to 3 times more prone than men to be affected by rheumatoid arthritis (Ngo *et al.,* 2014). The risk of developing adult RA among males and females is 1.7% and 3.6%, respectively (Crowson *et al.,* 2011). This can be explained by stimulatory influence of oestrogen on the immune system; although, complete effect of hormones on developing RA is yet to clarified (Alpizar-Rodriguez *et al.,* 2017). In female patients, it has been shown that nulliparity contributes to RA development while pregnancy can result in disease remission; on the other hand disease flares during postpartum period. Rheumatoid arthritis usually presents signs about middle age or during menopause in women; however, its onset begins later in men who possibly are RF positive with higher titres of ACPAs (Alamanos *et al.,* 2006).

Smoking. Tobacco smoking gradually increases the risk of RA by double in smokers with a 20-pack per year history comparing to nonsmokers (Sugiyama *et al.,* 2010; Vesperini *et al.,* 2013). This association is often stronger in ACPA positive patients who carry at least one copy of the shared epitope of Human Leukocyte Antigen (HLA) gene (Kallberg *et al.,* 2011). Actually, the risk of developing RA in smokers with shared epitope is 20-fold higher compared to nonsmokers without shared epitope (Kallberg *et al.,* 2011). Currently-smokers may have elevated levels of pro-inflammatory cytokines and subsequently higher disease activity (Sokolove *et al.,* 2016). Epigenetic modifications could intermediate high risk of RA with smoking; as smokers show hypomethylation of DNA while disease-modifying antirheumatic drugs (DMARDs) cause DNA hypermethylation (Svendsen *et al.,* 2016). It has been reported that non-nicotine inhaled components of cigarette contribute to RA but not tobacco components (Jiang *et al.,* 2014). However, the contribution of smoking to rheumatoid arthritis development is still controversial as there are conflicting reports (Vesperini *et al.,* 2013; Naranjo *et al.,* 2010).

Dust inhalation. It has been reported that silica exposure can be an environmental factor for RA (Stolt *et al.,* 2005) as after 2001 World Trade Center collapse in New York, US, an increased risk of RA had been reported among emergency responders. The dust composed of silica, asbestos, glass fibers, cement and other components (Webber *et al.,* 2015). Among Malaysian woman who exposed to textile dust, there was an increased risk of RA development, which was regardless of ACPA (Too *et al.,* 2016).

Microbiota. There are some evidence that periodontal disease shares similarities with chronic inflammation and inflammatory bone erosions (Hajishengallis *et al.,* 2015). These similarities originate

partially from oral microbiota such as *Porphyromonas gingivalis* (Kharlamova *et al.,* 2016) and *Aggregatibacter actinomycetemcomitans* (Konig *et al.,* 2016).

It is shown that gut microbiota is decreased in patients with RA and rare taxa like Actinobacteria are spread in these patients and other frequent taxa is somehow removed (Chen *et al.,* 2016) It seems *Prevotella copri* is more common in untreated early rheumatoid arthritis patients than in cases with established RA or healthy individuals (Scher *et al.,* 2013). Recently, peptides of two new autoantigens of HLA-DR molecules from RA patietns have been identified which were very similar to peptides of *Prevotella* and other gut bacteria species (Pianta *et al.,* 2017). These observations, make connection between autoimmunity, disease and environment. Among viruses, Chikungunya virus can cause RA-like disease (Gasque *et al.,* 2016); however, the role of parvovirus B19 infection is still unclear (Naciute *et al.,* 2016). On the other hand, the role of Epstein-Barr virus (EBV) infection in RA development has been shown since last decades (Tan *et al.,* 2016).

Other Factors. Changeable lifestyle parameters such as obesity has been reported to be associated with rheumatoid arthritis development (Ljung *et al.,* 2016). An association between long-term moderate alcohol consumption and decreased RA risk has been reported (Lu *et al.,* 2014). High symptomology of post-traumatic stress disorder (Lee *et al.,* 2016) and low socioeconomic status (Camacho *et al.,* 2012) can increase the risk of rheumatoid arthritis.

Mortality

The most frequent early death in RA patients is resulted from cardiovascular disease. In these individuals the rate of diabetes mellitus, hyper lipidaemia, obesity and hypertension is 6.0%, 9.9%, 4.4% and 18.6%, respectively (Radner *et al.,* 2017). In order to identify the patients at high risk of cardiovascular disease, genetic and serological factors could play a role (Lopez-Mejias *et al.,* 2016). It has been reported that total mortality of female patients due to respiratory and cardiovascular diseases is increase in cases with RA compared to healthy controls. The mortality risk in seropositive RA female patients comparing to control individuals without RA is 3 times (Sparks *et al.,* 2016). Although, considering present treatments, premature death does not occur anymore (Markusse *et al.,* 2016).

Pathophysiology

Preclinical Rheumatoid Arthritis. In most of cases, the RA pathogenesis starts some years before clinical manifestations (Masi, 1983). Therefore, RA continues developing with a susceptibility stage considering genetic background and goes on through preclinical RA before articular inflammation

(early RA) develops. Environmental parameters contribute to this process. Finally, established rheumatoid arthritis develops in non-self-resolved cases (Figure 1.1).

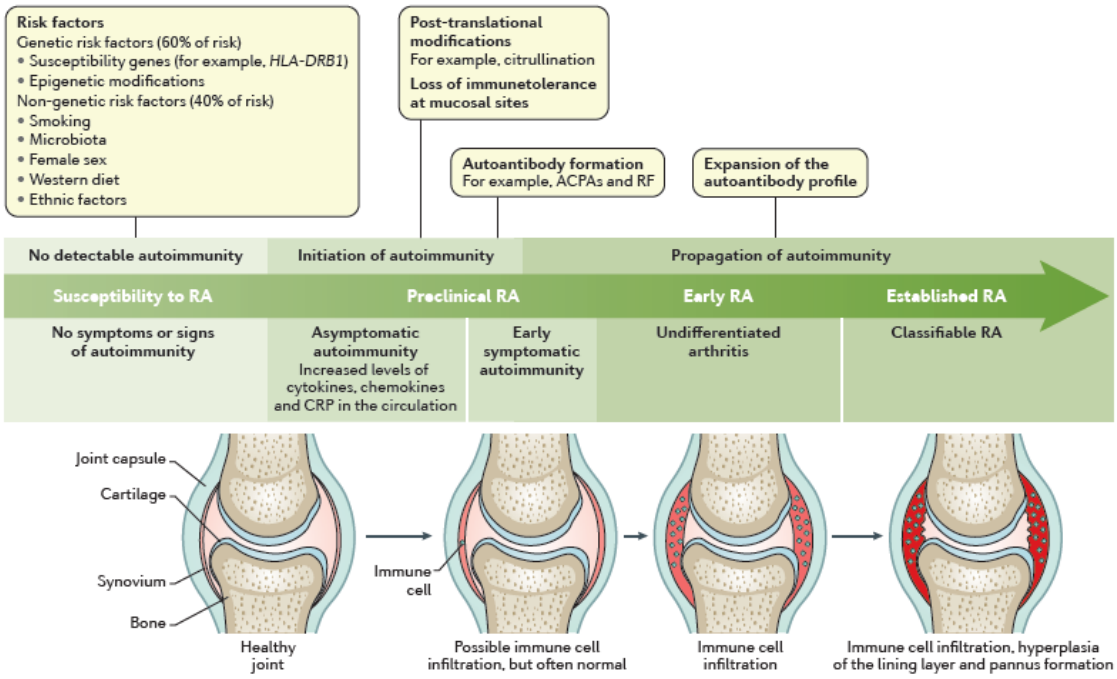

Figure 1.1. Development and progression of RA. Both genetic and non-genetic factors are required to pass immune threshold in order to initiate RA. Later, autoimmunity begins to propagate that can happen before inflammation of the synovium and clinical symptoms. ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; RF, rheumatoid factor (Smolen *et al.*, 2018)

Rheumatoid arthritis is defined by a genetic background in which a crosstalk between environmental and genetic factors ends in the inflammatory and destructive synovial response (Figure 1.2). The contribution of environmental factors in this process is not fully understood, but it is thought that stressor such as smoking can affect cells in mucosal sites and stimulate post-translational modifications of the amino acid arginine to citrulline within intracellular proteins such as histones or matrix proteins like collagen, fibrinogen, fibronectin, enolase and vimentin through activity of peptidyl arginine deiminases which is called citrullination (i.e. deamination) (Makrygiannakis *et al.*, 2008). Microbiota like *P.gingivalis* may cause citrullination and therefore produce ACPA (Dissick *et al.*, 2010). Beside, *A. actinomycetemcomitans* by releasing a toxin that cause calcium influx, may contribute to citrullination and subsequently RA development (Konig *et al.*, 2016).

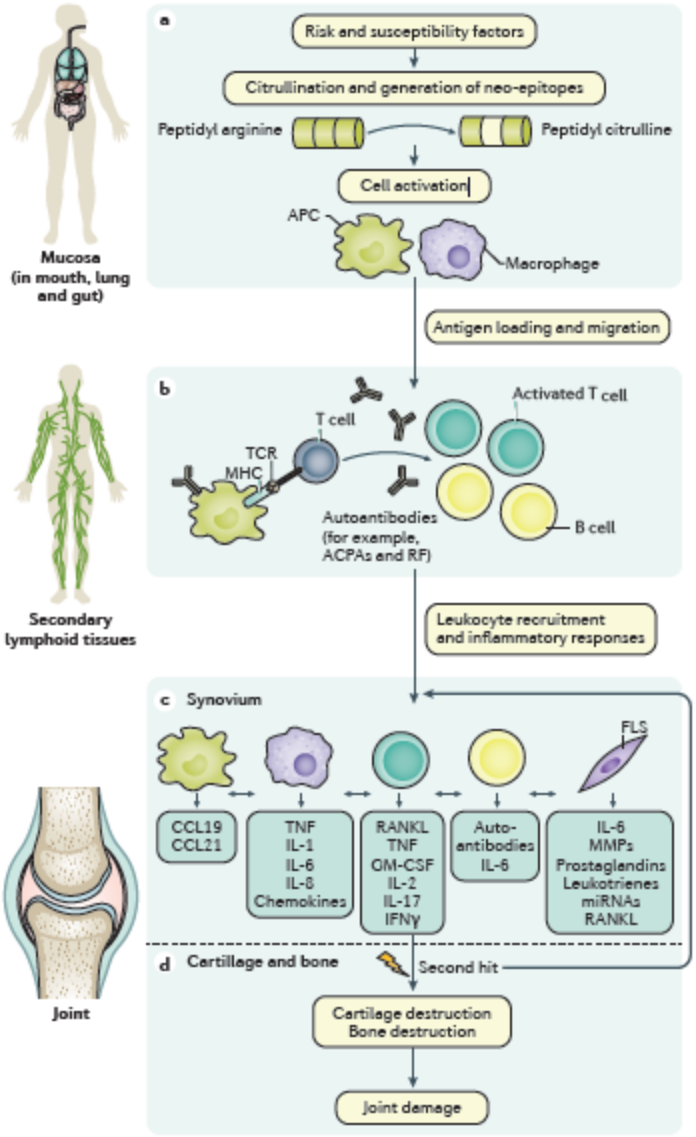

Figure 1.2. Involved mechanisms in rheumatoid arthritis development. a) Post-translational changes create immune system-recognizable new epitopes. b) These modified peptides are presented by APCs and induce autoantibody creation. c) Stromal cells, APCs and macrophages can release inflammatory factors. Synovial inflammation can start, potentially by a second hit. d) Cytokines with steady immune responses can cause cartilage and bone destruction. APCAs, anti-citrullinated protein antibodies; CCL19, CC-chemokine ligand 19; CCL21, CC-chemokine ligand 21; GM-CSF, granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex; miRNA, microRNA; MMP. Matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RF, rheumatoid factor; TCR, T cell receptor; TNF, tumor necrosis factor. (Smolen *et al.*, 2018)

After citrullination or other post-translational changes like acetylation or carbamylation, the modified peptides attach to Major Histocompatibility Complex (MHC) protein heterodimer, which may have shared epitopes, cause antigen presentation to T cells and subsequently provoke synthesis of autoantibodies such as RF and ACPAs in B cells (Holers *et al.*, 2013; Muller *et al.*, 2015). This process

could be assumed as normal immune response but not autoimmunity. Other pathways of protein changes like acetylation or enzyme-free carbamylation can change self-proteins to targets for autoimmunity (Trouw *et al.,* 2013).

The existence of ACPAs, RF, cytokines and chemokines could be detected 10 years before disease begins that indicates activation of immune system in preclinical phase. ACPAs and RF together cause a more severe disease that could be used as prognostic and diagnostic markers (Aletaha *et al.,* 2010; Nielen *et al.,* 2004; Aletaha *et al.,* 2015; Laurent *et al.,* 2015; Sokolove *et al.,* 2014).

Although APCAs are not homogeneous, their identical profile of peptide recognition is not able to predict the clinical course (van Beers *et al.,* 2013; Deane *et al.,* 2010).Usually, synovial biopsy of seropositive patients is normal; however, synovial infiltration may occur in absence of clinical symptoms (Kraan *et al.,* 1998). Accompanying of ACPAs with immune complex formation, complement activation, or microvascular insult may probably needed in order to begin clinical synovitis (Arend *et al.,* 2012).

Early and established Rheumatoid Arthritis. Mononuclear cell infiltration mainly by $CD4^+$ T cells and macrophages, and early activation of stromal cell lead to synovial inflammation which is characteristic of early rheumatoid arthritis (ERA). Matrix-degrading enzymes are detectable one week after disease onset in the synovial intimal lining, and beside ACPAs, RF, glucose-6-phosphate isomerase, type 2 collagen, proteoglycans and nuclear antigens contribute to pathogenesis (Steiner *et al.,* 2007).

Comparing early RA with established RA, shows that synovium changes are mainly stable with only minor differences which make ERA as "window of opportunity" (Kinslow *et al.,* 2016). During ERA, ACPA profile is almost similar and further modifications do not occur during establishing phase which highlights autoantibodies' role in pathogenesis. The same situation happens regarding RF and other autoantibodies like anti-carbamylated peptides (Shi *et al.,* 2014). Levels of RF in response to appropriate treatment show strong changes compared with ACPA, indicating flexibility and/or different source of RF (Bohler *et al.,* 2013). Beside, plasmablasts presence during ERA shows the role of mucosal sites in disease trigger (Kinslow *et al.,* 2016). On the other hand, detection of T cells during established RA due to be polyclonal is challenging (Klarenbeek *et al.,* 2012). The role of macrophages and fibroblasts are considered significant during synovitis in established RA. Comparing DNA methylation of fibroblast-like synoviocytes (FLS) from ERA and established RA shows differences which mainly originate from cell differentiation, adhesion and proliferation (Ai *et al.,* 2015).

Pathogenesis

The synovium. In rheumatoid arthritis different immunological phenomena at mucosal surfaces and lymphoid tissues occur; however, the synovium is the main area and has two important homeostasis functions: a) provides lubricant for cartilage to lower friction and b) provides nutrients to cartilage. A normal synovium with an intimal lining made-up of fibroblast- and macrophage-like synoviocytes, a sublining composed of adipocytes, fibroblasts, blood vessels and dispersed immune cells. The intimal lining due to lack of basement membrane and tight junctions is permeable and allows cells and proteins to enter into synovial fluid (Castor, 1960).

Two important pathogenic events during RA occurs: a) Intimal lining expansion due to synoviocytes presence (Mclnnes and Schett, 2011) and production of cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF). At this point, FLS also produces MMPs, prostaglandins and leukotrienes, (Bartok *et al.,* 2010) and microRNAs (Stanczyk *et al.,* 2008; Philippe *et al.,* 2013); and by taking invasive feature propagate disease from joint to joint (Lefevre *et al.,* 2009). b) Infiltration of adaptive immune cells into the synovial sublining (Ziff, 1974). $CD4^+$ memory T cells make approximately 50% of the sublining cells; these T cells can infiltrate the tissue or contribute in proliferation, differentiation and antibody production of B cells in about 15% to 20% of patients. At this phase, plasma cells, plasmablasts and B cells also exist that some of them release RF or ACPAs. Some studies reported that cells which produce autoantibody (e.g. IgM, IgG and IgA) experience affinity maturation in the tissue which indicates immunity against modified or native peptides is continuous (Humby *et al.,* 2009; Randen *et al.,* 1992). Since the major part of affinity maturation happens before clinical signs of the disease, the exact pathogenic role of these synovial pathways is yet to define (Catrina *et al.,* 2014). Other cells including follicular dendritic APCs, mast and macrophages are also present in the synovial sublining; however, neutrophils are interestingly absent. There are reports indicating the synovial histology is related to response to targeted agents or clinical phenotype (Orr *et al.,* 2017).

Joint damage. The main sign of rheumatoid arthritis is the damage of cartilage and bone caused by synovial invasion. Different mechanisms between $ACPA^+$, $ACPA^-$ or having other autoantibodies in RA patients are proposed. Neutrophils, mast cells and macrophages cause the damage to the joints by producing MMPs and cytokines. The most damaging cells for cartilage are cadherin-11-positive FLS (Kiener *et al.,* 2009) that release proteases like collagenases and stromelysins. It has been shown that protein level of these enzymes are very high in RA patients comparing to other diseases like osteoarthritis; and this high expression occurs in the pannus. Although, inhibitors of MMP are present, they are not enough to prevent bone damage (Keyszer *et al.,* 1998).

Aggressiveness of fibroblast-like synoviocytes in RA causes matrix destruction (Bottini and Firestein, 2013), which can continue for several months. It is reported that FLS from RA patients are able to damage cartilage explants in mice (Muller-Ladner *et al.,* 1996). However, the mechanism of this

observation is less-known, and probably sentrin-specific protease 1 (*SENP1*), tumour suppressor p53 (*TP53*), and phosphatase and tensin homologue (*PTEN*) genes are involved (Tak., *et al.,* 2000). Genes functioning in RA-related features, like cytokine signaling, cell migration and cell adhesion, are reported to have different methylation profile comparing to non-RA diseases (Ai *et al.,* 2016). These changes can be different according to the joint and explain various responses to targeted therapies (Smolen *et al.,* 2018).

Osteoclasts are bone-resorbing cells and get maturated and activated by RANKL which is created by T cells. These osteoclasts together with IL-1, IL-6 and TNF cause bone erosions (Schett *et al.,* 2012). In an acidic environment, osteoclasts release proteases, like cathepsin K, and degrades bone matrix (Redlich and Smolen, 2012). It was reported that maturation and activation of osteoclasts occur by interaction of ACPAs with citrullinated peptides that starts articular damage. Theses interactions can happen before synovial inflammation onset and provide new mechanisms by which autoantibodies contribute to inflammation and remodeling of tissues (Harre *et al.,* 2012; Krishnamurthy *et al.,* 2016). Although, severe joint damage and extra osteoclast activation have been reported in animals with TNF-driven arthritis lacking autoantibodies (Hayer *et al.,* 2007).

Cytokine and signaling networks. Pro-inflammatory and tissue-damaging activities in the cells during synovitis are modulated by networks of cytokine. Advent of anti-TNF therapies highlighted the role of TNF in RA pathogenesis contributing to MMP production, leukocyte activation, angiogenesis, and inducing pain. Other reports on different cytokines like IL-6 demonstrated the diversity of cytokines during RA (Smolen *et al.,* 2018).

Paracrine and autocrine actions of cytokines produced by synovial cells can promote and perpetuate inflammation in rheumatoid arthritis. For instance, cytokines produced by macrophages can activate T cells, FLS and dendritic cells which in turn by producing other cytokines can activate other cells next to the joint. IL-8 which is induced by ACPA and produced by osteoclasts may have a role in early disease by bringing neutrophil to the synovial fluid and activating subsequent reactions (Krishnamurthy *et al.,* 2016). Therefore, recruitment of new cells, cell activation, immunity, and decreases apoptosis are continued. Locally, neutrophils, macrophages and/or fibroblasts produce inhibitors like soluble TNF receptor, IL-10, IL-35 and IL-1 receptor antagonist protein, however their level is not enough to decrease inflammatory response (Nakano *et al.,* 2015).

Targeting cytokines resulted partially in successful therapies by IL-6 and TNF. However, focusing on IL-1 and IL-17 inhibitors was not promising that demonstrates the challenge in finding appropriate cytokine. Although antibodies that neutralize granulocyte–macrophage colony-stimulating factor are reported successful in clinical studies on RA, they use are not confirmed (Behrens *et al.,* 2015; Smolen *et al.,* 2016).

Cytokines such as IL-6, IL-15 and IL-7, send signal by Janus kinases (JAKs) after binding to their receptors. Their inhibitors suppress transcription factors of the signal transducer and activator of transcription (STAT) in the synovium and are shown to be successful in RA treatment (Genovese *et al.,* 2016). Some reports demonstrated that tofacitinib decreases phosphorylation of STAT1 and STAT3, which involve IL-6 signaling, and helps RA treatment (Boyle *et al.,* 2015). Several studies suggested different signaling pathways such as MAPK/ERK kinase (MEK), p38 mitogen-activated protein kinases (MAPKs), Bruton tyrosine kinase, spleen tyrosine kinase, and phosphoinositide 3-kinase1, however targeting them was partially successful (Genovese, 2009; Genovese *et al.,* 2014).

Therefore, the mechanisms that contribute to RA development provide many opportunities for treatment intervention. The differences of pathways and responses to targeted agents propose that RA has final common pathway, and comprehensive knowledge about all pathways will actualize personalized treatment (Smolen *et al.,* 2018)

Clinical Presentation

Rheumatoid arthritis is a disease which affects multiple joints symmetrically. An individual suffering from RA usually experience pain and swelling in the joints of the hands and feet. The first swelling mainly happens in the wrists and metacarpophalangeal, metatarsophalangeal, and proximal interphalangeal joints. Patients experience joint stiffness in the morning which last 30 minutes to several hours. Due to synovitis and effusion, the swelling is "soft" dissimilar to the "hard" swellings of osteoarthritis. During involvement of fingers, swelling occurs around the joint (fusiform) not the whole digit (sausage digit) that happens in psoriatic arthritis. Although the distal interphalangeal joints hardly involved, small joints including the metacarpophalangeal, proximal interphalangeal, metatarsophalangeal, wrist joints and large joints including the elbow, knee, ankle, and shoulder joints could be affected. (Aletaha and Smolen, 2018).

In case rheumatoid arthritis is not treated efficiently, other manifestations could develop. The most common are rheumatoid nodules that cause subcutaneous lumps near bony bulges like the elbow. A more dangerous presentation is rheumatoid vasculitis that mainly involves skin, vasa nervorum, and sometimes arteries from other organs (Aletaha and Smolen, 2018).

During RA physical abilities, work efficacy, and quality of life are limited (Sokka *et al.,* 1999). If RA is not managed well, 80% of cases will have dysfunctional joints and 40% will lose work ability in 10 years from disease onset (Sokka *et al.,* 1999; Wolfe, 1996). Compared to cardiovascular disease and diabetes, RA patients have similar or worse quality of life assessment as indicated by the 36-Item Short Form Health Survey (Matcham *et al.,* 2014). RA influence all everyday life activities (Radner *et al.,* 2011). In long term, , accumulation of irreversible joint damage cause disability in inefficiently treated RA and patients with irreversible joint damage will experience physical dysfunction, even if clinical remission is finally acquired; and most effective treatments would not reverse joint damage (Aletaha *et*

al., 2008). The recent radiographic observations show joint changes from minimal abnormalities to severe destructive damages as bony erosions and limited joint space, which demonstrate cartilage changes that associated more with irreversible disability than bony damage (Figure 1.3) (Aletaha *et al.*, 2011).

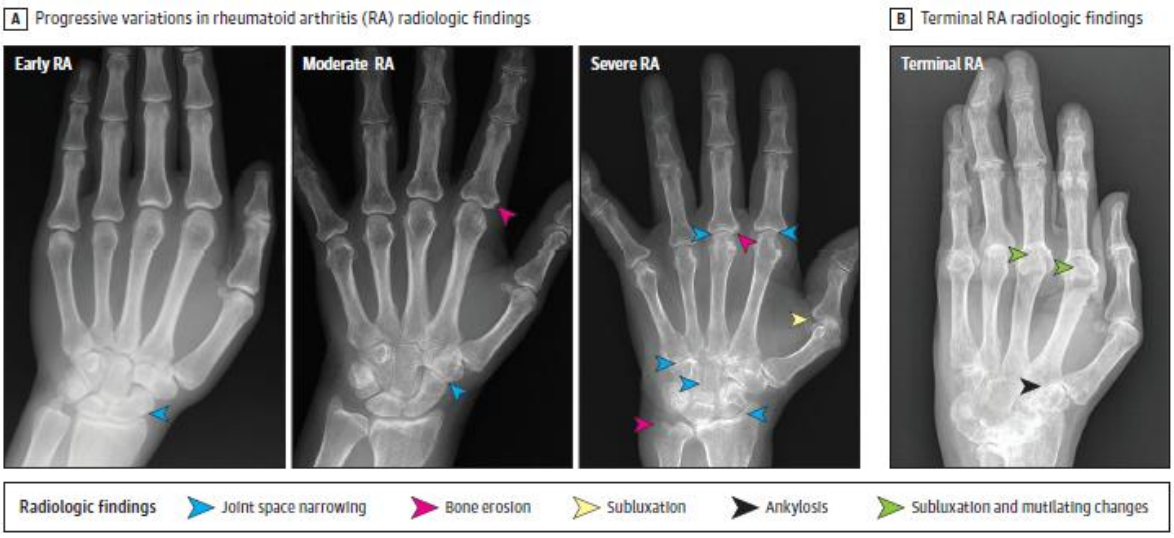

Figure 1.3. Structural Phenotypes of Rheumatoid Arthritis. In early disease there is no or less bony or cartilage damage. In severe established RA joint damage progresses in affected joints and spreads to additional joints, cartilage (joint space narrowing) and bone damage (erosions); can also be seen. In terminal RA, joint damage has severely involved most joints (Aletaha and Smolen, 2018).

Diagnosis and Assessment

In early rheumatoid arthritis only one or a few joints could involve that can accompanied by tendon inflammation (tenosynovitis). The tenosynovitis and subclinical synovial inflammation could be monitored by imaging with color Doppler sonography or gadolinium-enhanced magnetic resonance imaging (Aletaha and Smolen, 2018).

Although there is no diagnostic criteria for RA, the 2010 classification criteria which designed based on identification of homogenous RA patients, may assist clinical diagnosis (Aletaha *et al.*, 2011; Radner *et al.*, 2014). The RA classification needs at least one swollen joint and a minimum of 6 out of 10 points from a scoring system (Table 1.1) (Aletaha *et al.*, 2010) Joint involvement according to imaging by MRI or ultrasound or physical examination provide 5 points; elevated levels of RF, ACPAs, or both contribute to 2 more points (or 3 points with levels more than 3-fold the upper limit of normal); and elevated acute phase reactant (APR) condition, like high CRP level or erythrocyte sedimentation rate (ESR), and duration of signs (6 weeks) add 1 extra point each. These 2010 criteria present a sensitivity

of 82% and specificity of 61%. Specificity of the present classification criteria was 4% lower and sensitivity 11% higher than the 1987 criteria (Radner *et al.*, 2014).

Classification	Points
Joint Distribution (0-5 points)	
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints (large joints not counted)	3
>10 joints (≥1 small joint)	5
Serology (0-3 points)	
Negative RF and negative ACPA	0
Low positive RF or low positive ACPA	2
High positive RF or high positive ACPA	3
Symptom Duration (0-1 point), weeks	
<6	0
≥6	1
Acute Phase Reactants (0-1 point)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1

Table 1.1. Classification and Follow up of Rheumatoid Arthritis. Abbreviations: ACPA, anticitrullinated peptide antibodies; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor (Aletaha and Smolen, 2018

Because of 90% prevention of joint damage in early diagnosis and treatment of early RA patients, (Goekoop-Ruiterman *et al.*, 2005) it is critical to identify RA cases as soon as possible. Particular signs that may demonstrate possible RA include morning stiffness of finger joints lasting 30 minutes or longer, articular swelling and pain in metacarpophalangeal joints, metatarsophalangeal joints or both, and autoantibody positivity (Emery *et al.*, 2002).

Primary evaluation needs examination of the joints and serologic testing for autoantibodies and APRs. Assessment of APRs, joint evaluation, and considering of patient-based reports like global assessment of disease activity followed by evaluation of physical function are important for follow-up. Combinational measures that consider number of tender and swollen joint counts provides the most optimal way to assess RA disease activity in both practice and trials, because they collect the most important disease features in a single score. These scores, which called the disease activity score using 28 joint counts (DAS28), the simplified disease activity index (SDAI), or the clinical disease activity index (CDAI), connect the results like damage progression and functional impairment (Aletaha *et al.*, 2005; van der Heijde *et al.*, 1992). All these scores help monitoring disease activity based on various aspects for better treatment. Treatment aims in remission, which means no disease activity, and low disease activity, defined as low risk of damage progression; these two situations therefore contrast with

moderate and high disease activities, which means uncontrolled disease could progress over time (Smolen *et al.,* 2006). The CDAI is the most convenient indices to perform. It is a plain composite measure of four variables: swollen and tender joints (using 28 joint counts), evaluator global assessment, and patient global assessment. It ranges from 0 to 76, in which higher scores means disease progression (Aletaha *et al.,* 2005).

The CDAI, as the first evaluation tool, should be applied to monitor treatment using the "treat-to-target" approach (Smolen *et al.,* 2016). This guideline includes treating, and adapting therapy whenever required, to meet a progression in a disease activity index with a minimum of 50% in 3 months and therefore to reach a 50% probability to obtain low disease activity or remission at 6 months. The treatment aims to meet clinical remission in early RA or low disease activity in established RA in case remission is not reachable (Aletaha *et al.,* 2015). Clinical remission that is measued by CDAI or SDAI is a condition in which physical function is highly improved and further joint damage does not happen (Aletaha and Smolen, 2011) The European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) have determined remission criteria based on a Boolean approach or according to indices such as CDAI and SDAI. (Felson *et al.,* 2011) Treatment should result in clinical remission based on these indices and not according to improvement in subclinical inflammation as for instance measured by ultrasound. There is no prove that continuous therapy after clinical remission improves results; thus, it should not be followed (Dale *et al.*,2016).

Treatment

Disease-Modifying Antirheumatic Drugs

Considering that rheumatoid arthritis is not curable, current therapeutic procedure leads to achievement of great disease control. RA patients have to be treated with disease-modifying antirheumatic drugs (DMARDs). A DMARD deals with signs and symptoms of RA, promotes physical situation, and prevents progression of joint damage. Symptoms-decreasing therapies, like nonsteroidal anti-inflammatory drugs or pain medications, do not decrease damage progression and irreversible disability. These medications are not considered as DMARDs and should only be prescribed before or along with DMARDS.

DMARDs are classified into synthetic and biologic agents (Table 1.2) (Smolen *et al.,* 2014). The synthetic includes conventional synthetic and targeted synthetic DMARDs. Conventional synthetic DMARDs resulted from empiric experience, have been applied for more than 50 years, and their molecular targets are not recognized yet. On the other hand, targeted synthetic DMARDs are known to interfere with a specific molecule or enzymes like Janus kinases (JAKs) which are intracellular signal transduction molecules.

Subgroup and Type[a]	Molecular Target	Structure	Selected Adverse Events[b]	Efficacy (ACR70 Response Rates)[c]
Synthetic DMARDs				
Conventional[d]				
Methotrexate (10-25 mg/wk)	Unknown	Small chemical molecules (oral)	Nausea, stomatitis, liver enzyme level increase, bone marrow suppression, pneumonitis, teratogenicity	20-40%[49,50]
Sulfasalazine (2-4 g/d)	Unknown		Hypersensitivity reactions (mainly cutaneous), nausea, diarrhea, agranulocytosis, drug-induced lupus, azoospermia	No RCT data for 3 g daily; little modern data at all 8% at 2 g[51]
Leflunomide (20 mg/d)	Dihydroorotate dehydrogenase		Diarrhea, hypertension, hypersensitivity reactions, liver enzyme level increase, leukocytopenia, teratogenicity	10%[51]
(Hydroxy-) chloroquine (Hydroxychloroquine: 400 mg/d; chloroquine: 250 mg/d)	Unknown		Retinopathy	Unavailable
Targeted[d]				
Tofacitinib (10 mg/d)	JAK 1,2,3	Small chemical molecules (oral)	Infections, reactivation of tuberculosis, herpes zoster, cytopenias (including anemia), hyperlipidemia, CPK level increases	20% (methotrexate insufficient responders)[52] 14% (TNF inhibitor insufficient responders)[53]
Baricitinib (2-4 mg/d)	JAK 1,2			24% (methotrexate insufficient responders)[54] 17% (TNF inhibitor insufficient responders)[55]
Biologic DMARDs				
Originator biologic[e]				
Etanercept (50 mg/wk)	TNF	Receptor construct	Infections, reactivation of tuberculosis, psoriasiform skin changes, exacerbation of demyelinating diseases, drug-induced lupus, nonmelanoma skin cancer, injection site or infusion reactions	20% (methotrexate insufficient responders) 12% (TNF inhibitor insufficient responders)[56]
Infliximab (3-10 mg/kg every 8 wk)	TNF	Chimeric monoclonal antibody		
Adalimumab (40 mg every 2 wk)	TNF	Human monoclonal antibodies		
Golimumab (50 mg/mo)	TNF	Human monoclonal antibodies		
Certolizumab (200 mg every 2 wk)	TNF	Fab' fragment of humanized monoclonal antibody		
Tocilizumab (162 mg/wk)	IL-6 receptor	Humanized monoclonal antibody	Infections, reactivation of tuberculosis, bowel perforation, hypersensitivity reactions, neutropenia, injection site reactions, hyperlipidemia	22% (methotrexate insufficient responders)[57] 12% (TNF inhibitor insufficient responders)[58]
Sarilumab (150 mg-200 mg every 2 wk)		Human monoclonal antibody		
Rituximab 1000 mg every 6 mo	CD20 (B-cell)	Chimeric monoclonal antibody	Hypersensitivity reactions, reactivation of hepatitis B, leukocytopenia	22% (methotrexate insufficient responders)[59] 12% (TNF inhibitor insufficient responders)[60]
Abatacept (125 mg/wk)	CD80/86 (costimulation)	Receptor construct	Infections, reactivation of tuberculosis, leukocytopenia, injection site reactions	22% (methotrexate insufficient responders)[61] 10% (TNF inhibitor insufficient responders)[62]
Biosimilar[e]				
Etanercept	TNF	Receptor construct	See above	Similar to originator data[63]
Infliximab	TNF	Chimeric monoclonal antibody		
Adalimumab	TNF	Human monoclonal antibody		
Rituximab	CD-20 (B cell)	Chimeric monoclonal antibody		

Table 1.2. Currently Approved Disease-Modifying Antirheumatic Drugs (United States, Europe, or Both). Abbreviations: ACR, American College of Rheumatology; CPK, creatine phosphokinase; DMARD, disease-modifying antirheumatic drug; IL, interleukin; JAK, Janus kinase; RCT, randomized clinical trial; TNF, tumor necrosis factor (Aletaha and Smolen, 2018).

Methotrexate (MTX) is the most important conventional DMARDs that is being prescribed for more than 50 years (Hoffmeister, 1983) with an optimal dose of 25 mg per week (van Ede *et al.*, 2001). Intolerant patients for this dose could improve by a lower dose and due to adverse effects, about 5% of patients should not continue therapy with methotrexate.

Methotrexate is important since about 25% to 40% of patients significantly improve with its monotherapy, and when it is combined with glucocorticoids approximately 50% of patients show low disease activity or remission in early RA (Emery *et al.*, 2017; Nam *et al.*, 2014). In addition, its adverse effects including nausea, hair loss, stomatitis, and hepatotoxicity are well-known and by folate prescription can be avoided (van Ede *et al.*, 2001). Beside, when methotrexate prescribed with synthetic, targeted or biologic DMARDs their efficacy increased (Nam *et al.*, 2017).

Other conventional synthetic DMARDs include sulfasalazine, leflunomide. and hydroxychloroquine that the efficacy of the latter is lower than of the other two (van der Heijde *et al.*, 1990). EULAR and ACR suggested that each newly diagnosed patient should be treated by methotrexate combined with short-term (up to 3-4 months) glucocorticoids and a treat-to-target approach (Smolen *et al.*, 2017; Singh *et al.*, 2016). Combinations of conventional synthetic DMARDs have not led to better therapy compared with methotrexate monotherapy due to more adverse effects and drug discontinuation (Smolen *et al.*, 2017).

In case of methotrexate and glucocorticoids inefficacy, prognostic markers have to be considered. Early joint damage, the presence of autoantibodies, and high disease activity as poor prognostic markers indicate fast disease progression which could be lessen by adding a targeted synthetic DMARD (JAK inhibitor) or biologic DMARD instead of another conventional synthetic DMARD (Smolen *et al.*, 2006; Vastesaeger *et al.*, 2009). When 2 or more conventional synthetic DMARDs fails, achieving the treatment target does not seem promising (Kiely *et al.*, 2011). In the presence of poor prognostic markers, EULAR suggest starting a targeted synthetic DMARD or preferentially any biologic DMARD beside methotrexate, biologic DMARDs are preferred due to long-term experience with efficacy and safety profiles. Treating patients based on prognostic markers is not widely evident. However, once the first biologic DMARD (or targeted synthetic DMARD) failed, any other biologic DMARD or targeted synthetic DMARD even targeting the same pathway could be used. There is evidence that for instance switching to another TNF inhibitor could be as efficacious as using a drug with different target, like IL-6 inhibitors or of other pathways (Nam *et al.*, 2017; Smolen *et al.*, 2016). Treat-to-target therapy could be limited due to comorbidities or patient preferences, and a patient-involved process should be considered (Smolen *et al.*, 2016).

When biologic DMARDs or targeted synthetic DMARDs are prescribed with methotrexate or other conventional synthetic DMARDs, their efficacy increases (Emery *et al.*, 2015; Burmester *et al.*, 2016; Kaneko *et al.*, 2016; Fleischmann *et al.*, 2017). Thus, combining biologic DMARDs and targeted

synthetic DMARDs with methotrexate or other conventional synthetic DMARDs is recommended by EULAR. Although, comparing to anti-TNF monotherapy, monotherapies of IL-6 receptor antibodies, and maybe likewise JAK inhibitors, seems act more efficiently (Gabay *et al.,* 2013, Burmester *et al.,* 2017). In case of conventional synthetic DMARDs failure, IL-6R antibodies and JAK inhibitors will be promising options (Smolen *et al.,* 2017).

Genetic

Genetic factors play a significant role in rheumatoid arthritis development, and probably account for 50–60% of disease susceptibility (MacGregor *et al.,* 2000). Several studies reported the correlation between human genome variation and RA phenotypes that resulted in identification of some genetic variants associated with RA susceptibility. The first risk locus was introduced in 1980 by highlighting the function of *HLA-DRB1* alleles in the major histocompatibility (MHC) locus (Gregersen *et al.,* 1987). Later in 2000s, the International HapMap Project unified the variations of human genome from different populations (International HapMap consortium, 2003) that contributed in unbiased screening of genetic variants associated with human phenotypes (Ozaki *et al.,* 2002). As a result of early RA GWAS, *PADI4* was recognized as the first non-MHC RA risk locus in Japanese population (Suzuki *et al.,* 2003). Then, large-scale GWA studies were performed for RA (Plenge *et al.,* 2007a; Plenge *et al.,* 2007b; remmers *et al.,* 2007).

When early RA GWASs were carried out separately for each population, association of RA risk loci had been replicated among the different cohorts (Plenge *et al.,* 2005; Ikari *et al.,* 2005). Since 2010, several collaborations have been started with the aim of managing data from different RA GWASs that meta-analyses of these data provided a number of RA risk genes (Stahl *et al.,* 2010; Okada *et al.,* 2012; Eyre *et al.,* 2012).

The overall results of these RA GWAS meta-analysis introduced evidence of a common genetic background among RA cases from different populations and reported multiple genetic loci that result in RA risk in multiple ancestry such as reidentification of *PADI4* as a risk locus in Europeans (Eyre *et al.,* 2012). It was also mentioned that GWA study based on single-population were mainly underpowered, and integrating data from different ethnic increased statistical power to identify the disease-related loci and to reveal disease aetiology. These observations resulted in strong motivation for a transethnic study to integrate multiple populations (Stahl *et al.,* 2010; Okada *et al.,* 2012; Eyre *et al.,* 2012; Kochi *et al.,* 2011; Kurreeman *et al.,* 2012; Kim *et al.,* 2016; Okada *et al.,* 2016; and Sakaue *et al.,* 2018) which up to date identified >100 RA risk genetic loci that in figure 1.4 these genes are listed according to chromosomal position and are classified as Human Leukocyte Antigen (HLA) and non-HLA.

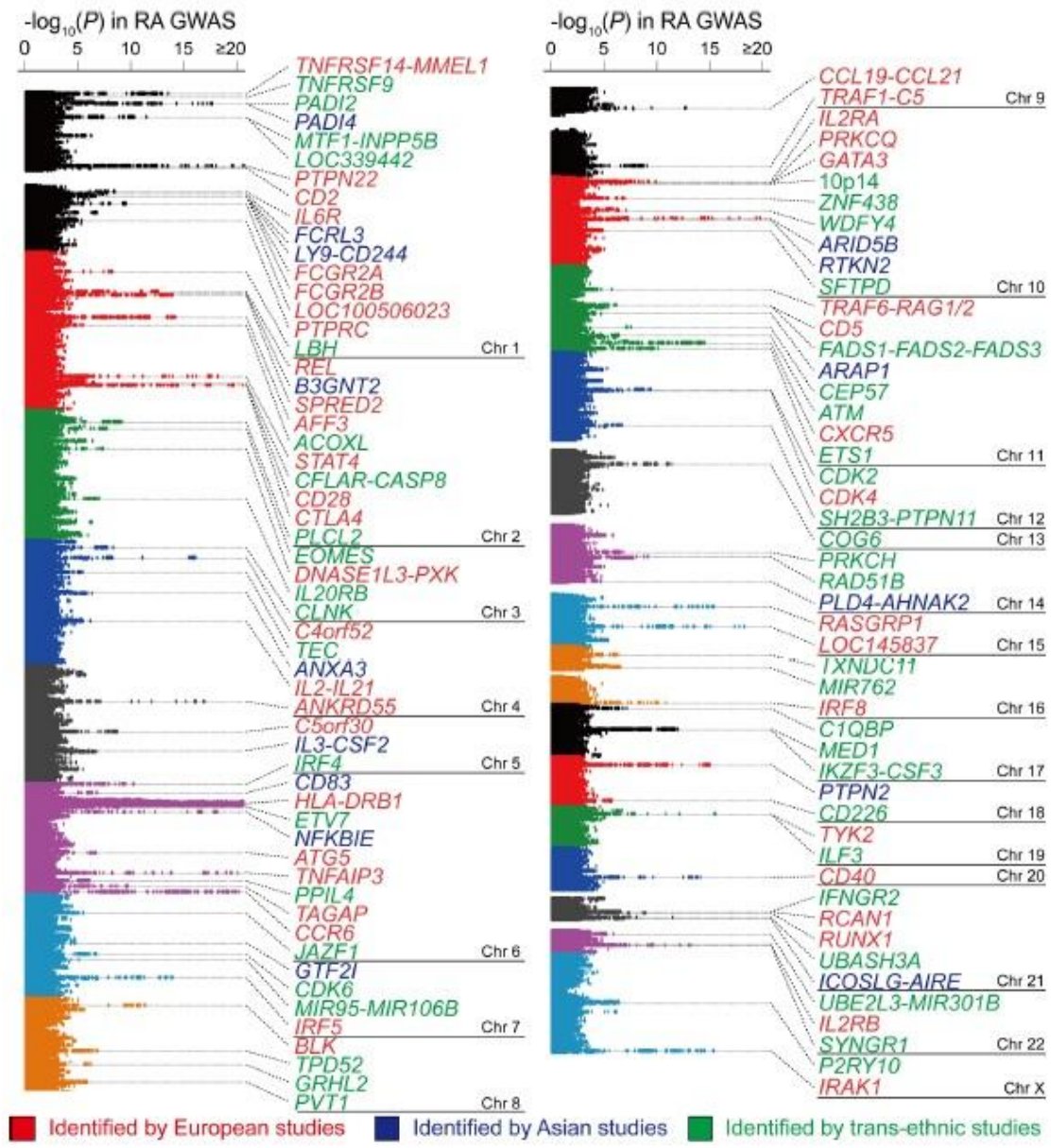

Figure 1.4. Current catalogue of rheumatoid arthritis (RA) risk gene loci.

The Major Histocompatibility Complex

The human major histocompatibility complex lies on the short arm of chromosome 6. There are three classes (I, II, III) within MHC that among which class I and class II are the most polymorphic loci (Rhodes and Trowsdale, 1999).

The Class I genes include *HLA-A*, *-B*, and *-C* and the non-classical MHC-Ib genes *HLA-E*, *-F*, and *-G*. The expression level of *HLA-E*, *-F*, and *-G* are lower than the classical genes, and are not as polymorphic as the *HLA-A*, *-B*, and *-C* genes; besides they have fewer rules in the immune system. There are 18 closely linked loci among class II genes which encode for the α and β chains of the class II molecules.

The most clinically relevant of these are the *DRA*, *DRB1*, *DQA1*, *DQB1*, and *DPA1*, *DPB1* genes encoding the *DR*, *DQ*, and *DP* heterodimers, respectively. The Class I and the Class II genes belong to the immunoglobulin gene family and have been originated from gene duplication and divergence from a common ancestor over time. They share significant sequence and some of them have closely related pseudogenes (Williams, 2001).

About 30 years after introducing HLA alleles as a risk factor for RA, non-HLA genes within the MHC have also been assessed for association with RA. Since 2007, a large number of RA susceptibility genes has been identified and approved in well-powered cohorts (Begovich *et al*. 2004; Bowes and Barton 2008; Coenen and Gregersen 2009; Kochi *et al*. 2009; Plant *et al*. 2010). After discovery of peptidylarginine deiminase type 4 (*PADI4*) gene in 2003 as a second risk factor for RA (Suzuki *et al*. 2003), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) was discovered in 2004 (Begovich *et al*. 2004; Carlton *et al*. 2005; Gregersen 2005). After one year, in 2005, cytotoxic T lymphocyte-associated antigen 4 (*CTLA4*) was introduced by a candidate gene study (Plenge *et al*. 2005). In 2007, a new genetic risk factor was recognized in the 9q33 region of the genome including *TRAF1/C5* gene (Kurreeman *et al*. 2007) which was also detected simultaneously in a genome wide study (Plenge *et al.* 2007a, b). In 2007, the signal transducer and activator of transcription (*STAT4*) gene region on chromosome 2q gene was reported as RA risk (Remmers *et al*. 2007). SNPs in Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were also found to be associated with RA risk (Marinou *et al*. 2007).

Methotrexate

The effectiveness and cytotoxicity of methotrexate, folate antagonists, are because of their suppression of DNA and RNA synthesis. The main target of MTX is folate metabolism (Figure 1.5). Although, the precise mechanism of anti-inflammatory effect of MTX in RA is still unknown. It is suggested that maybe the anti-inflammatory influence caused by adenosine release is more important than the antiproliferative effects (Cutolo *et al.*, 2001; Chan and Cronstein, 2002). MTX enters the cells through reduced folate carrier 1 (RFC1) or SLC19A1. In the cell, MTX is converted to active form, MTX polyglutamates, (MTXPGs) mediated by folypolyglutamate synthase (FPGS) through adding glutamate residues. MTXPGs suppress target enzymes of the folate pathway including dihyrofolate reducatse (DHFR), thymidylate synthase (TS) and subsequently inhibit important enzymes involved in de novo purine synthesis like 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) and GART. This leads to accumulation of adenosine that has anti-inflammatory activity (Chan and Cronstein, 2002). Other folate enzymes including serine hydroxymethyltransferase 1 (SHMT), and mehthylenetetrahydrofolate reductase (MTHFR), enzymes in the one carbon metabolism like methionine synthase reductase (MTRR) and methionine synthase (MS) are not affected by MTX directly, however their level of expression could lead to the antifolate effects of MTX through minor

modifications in the folate pools (Kremer, 2004). Genetic variations in the genes that encode these MTX transporters and metabolizing enzymes may affect their efficiency or activity that in turn could influence the overall therapeutic effect of the drug (Ghodke-Puranik *et al.*, 2015).

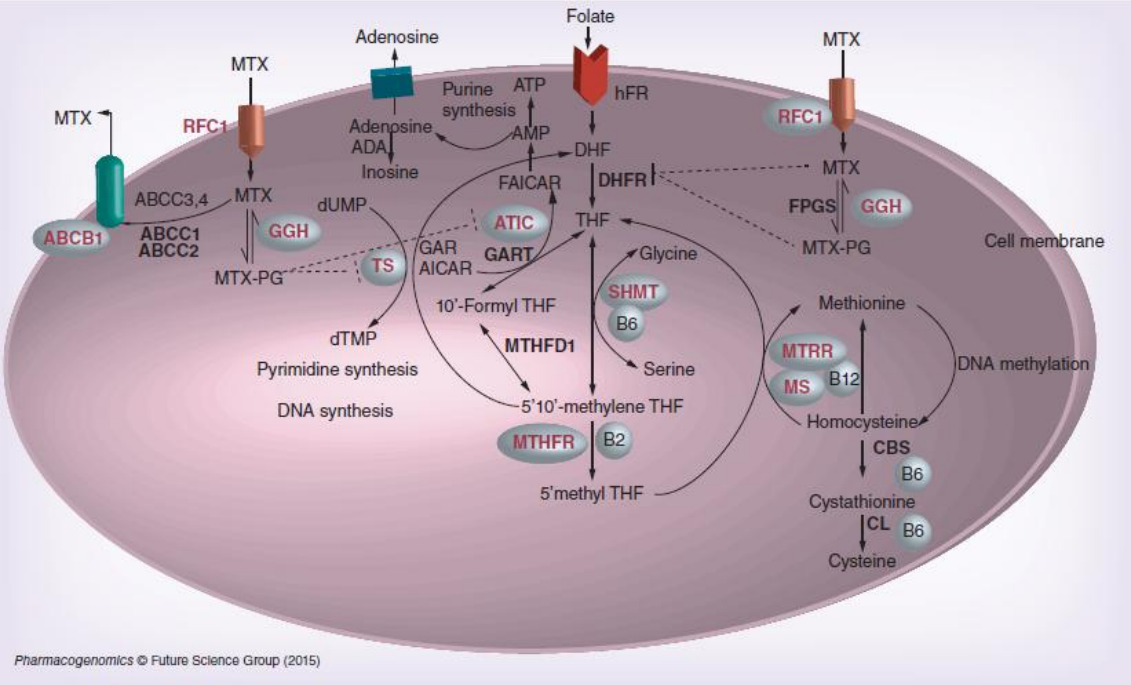

Figure 1.5. **Intracellular methotrexate metabolic pathway.**
Transporters: ABCB1 and ABCC1–4: Adenosine triphosphate–binding cassette (ABC) transporters; hFR: Human folate carrier; RFC-1: Reduced folate carrier1. Enzymes: ADA: Adenosine deaminase; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP cyclohydrolase; CBS: Cystathionine-β-synthase; CL: Cystathionine lyase; DHFR: Dihydrofolate reductase; FPGS: Folylpolyglutamyl synthase; GART: Glycinamide ribonucleotide formyltransferase; GGH_γ: Glutamyl hydrolase; MS: Methionine synthase; MTHFR: Methylenetetrahydrofolate reductase; MTHFD1: Methylenetetrahydrofolate dehydrogenase; MTRR: Methionine synthase reductase; SHMT: Serine hydroxymethyltransferase; TS: Thymidylate synthase. Metabolites: ADP: Adenosine diphosphate; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CH3: Methyl group; DHF: Dihydrofolate; dTMP: Deoxythymidine-5′-monophospate; dUMP: Deoxyuridine-5′-monophospate; FAICAR: 10-formyl-AICAR; IMp: Inosine monophosphate; GAR: Glycinamide ribonucleotide; MTX: Methotrexate; MTXPG: methotrexate polyglutamates; THF: tetrahydrofolate. Vitamins: B2, B6 and B12 are cofactors in the pathway (Ghodke-Puranik *et al.*, 2015).

Epigenetic

Epigenetics is a promising and growing research field in rheumatoid arthritis that can be described as the study of heritable modifications in gene expression which do not involve changes to the underlying DNA sequence (Baba *et al.*, 2018). DNA methylation, histone modification, and RNA changes play a critical role in epigenetic changes (Portela and Esteller, 2010). DNA methylation is a biochemical phenomenon of adding a methyl group to the cytosine of ring carbon at position 5 to form 5-methylcytosine (5-mC). DNA methylation happens preferentially in CpG dinucleotides in a gene either

as single dinucleotide or gathered into CpG-islands close to the gene promoters. Hypermethylation of the promoters is a mark of dense heterochromatin conformation that inhibits the binding of transcription factor to DNA and results in inactivation of gene transcription. On the other hand, the hypomethylation is correlated with open chromatin conformation and gene expression (Eden and Cedar, 1994). DNA methyltransferases (DNMTs) process and maintain CpG methylation by transfering a methyl group from S-adenosylmethionine (SAM) to cytosine (Goll and Bestor, 2005). Three DNMTs have been identified in mammals including DNMT1 that maintains the methylation patterns during DNA replication whereas DNMT3a and DNMT3b are responsible for de novo CpG methylation (Bird, 2002). CpG dinucleotides are largely and variably distributed across the human genome, and ususally happen at a rate of approximately one per 80 dinucleotides. However, CpG islands comprise 1% to 2% of the human genome. It is estimated that about 45,000 CpG islands are located next to the promoter regions of different genes (Baba *et al.*, 2018).

Changes in global DNA methylation has a critical role in pathogenesis of diseases. Growing evidence demonstrate that DNA hypo/hypermethylation of repetitive sequences such as short interspersed transposable elements (SINEs or Alu elements) or long interspersed transposable elements (LINEs) leads to chromosomal modifications of cells that results in genetic instability (Gaudet *et al.*, 2003). These chromosomal instabilities contribute to disease development and progression. By considering that LINE-1 constitutes a significant proportion, approximately 17%, of the human genome (Cordaux and Batzer, 2009), the level of LINE-1 methylation is considered as a surrogate marker of global DNA methylation. Currently, aberrant epigenetic profiles of rheumatoid arthritis is being investigated. It has been found that a global DNA methylation in T-cells and monocytes of RA patients compared to healthy individuals was decreased (de Andres *et al.*, 2015). Microarray analysis showed that genome-wide methylation in B-cells of early RA patients who have not started treatment compared to healthy donors is different (Glossop *et al.*, 2016). Some studies reported global and gene-specific hypomethylation of LINE-1 (Neidhart *et al.*, 2000) and *CXCL12* (Karouzakis *et al.*, 2011) in synovial fibroblasts. Beside, Nakano *et al.* have found that RA patients have a significant differently methylated regions thorough their genome (Nakano *et al.*, 2013). It has been reported that even different methylation pattern of a single CpG site in the *IL-6* gene could result in the inflammation related to RA (Nile *et al.*, 2008). Another investigation has reported that lack of DNMT1 function contributing to aberrant CpG methylation at the *MMP13* gene leads to elevated *MMP13* expression and finally collagen degradation in the cartilage (Bui *et al.*, 2012). Although it is interesting to study the epigenetic modifications that cause diseases like RA, the use of current DNA methylation-profiling technologies could likewise be applied in patient treatment.

Rational and objectives of the thesis

Rheumatoid arthritis is an autoimmune disease with unknown etiology which affects about 1% of the world's population. Current management of RA is focused on quick induction of disease remission to prevent joint damage and subsequent disability. Its onset is unknown; however, medication is able to restrain disease activity and permanent joint damage. Despite increase of therapeutic agents, the folate antagonist MTX remains the most prescribed first-line therapy. However, up to 40% of treated patients do not efficiently respond to therapy and require to change to expensive biological ones after 3 to 6 months of therapy, or discontinue due to severe adverse events. On the other hand, genetic heterogeneity does not explain all the features of RA. Thus, investigation of epigenetic factors and mechanisms related to the progression of the disease and response to therapy is increasingly demanding. Therefore, new biomarkers during different stages of the disease are required to monitor disease progression and distinguish non-responders before any treatment is initiated. As such, the current study aimed to assess:

Association of seven polymorphisms in human leukocyte Antigen (HLA) gene with early rheumatoid arthritis development among patients with undifferentiated arthritis.

Association of seven single nucleotide polymorphisms in non-HLA genes with early rheumatoid arthritis development among patients with undifferentiated arthritis.

Association of six single nucleotide polymorphisms in genes involved in methotrexate/folate pathway with response to the therapy.

Intra-patient modification of long interspersed transposable element 1 (LINE1) methylation in rheumatoid arthritis patients, by focusing on synovial and white blood cells.

LINE1 methylation among healthy controls, early rheumatoid arthritis and rheumatoid arthritis patients.

LINE1 methylation in response to the therapy among early rheumatoid arthritis patients.

Chapter 2

Subjects, Materials and Methods

Undifferentiated Arthritis cases

In this study 471 patients recruited in a regional project entitled "Prognostic value of a combined panel of soluble and genetic biomarkers in patients with Early Arthritis". (Project code: RF-2010-2317168). The study design was reviewed and approved by ethical board and written informed consent was taken from all recruited patients.

Patient enrolment and peripheral blood sample collection was performed in the following centers:

Immunorheumatology and Tissue Regeneration Lab, Orthopedic Institute Rizzoli, Bologna, Italy
Rheumatology Unit, Sant' Anna University Hospital, Ferrara, Italy
Rheumatology Unit, S. Maria Nuova Hospital, Reggio Emilia, Italy
Rheumatology Unit, Catholic University of Sacred Heart, Roma, Italy
Inclusion criteria for patients with Undifferentiated Arthritis (UA) or Early Rheumatoid Arthritis (ERA) was according to 2010 ACR/EULAR as presented in Figure 2.1 and Table 2.1.

The disease duration was ≤6 months, and clinical evaluation (Table 2.2) was performed at first visit (T0) and after 6 months (T6) along with collecting peripheral blood samples for genetic and epigenetic analyses. Freshly obtained samples after being aliquoted were stored at -20 °C for further analyses.

Rheumatoid Arthritis cases

30 patients after meeting 2010 ACR/EULAR criteria for rheumatoid arthritis were recruited in Rheumatology Unit, Sant' Anna University Hospital, Ferrara and Gaetano Pini, CTO Hospital, Milan, Italy. Written informed consent was taken from all recruited patients. Peripheral blood samples from all patients and synovial tissue samples available for 6 patients were collected for epigenetic study.

Healthy control cases

DNA of healthy control cases after matching the study design was selected and used from available biobank in laboratory of medical genetic, university of Ferrara, Italy.

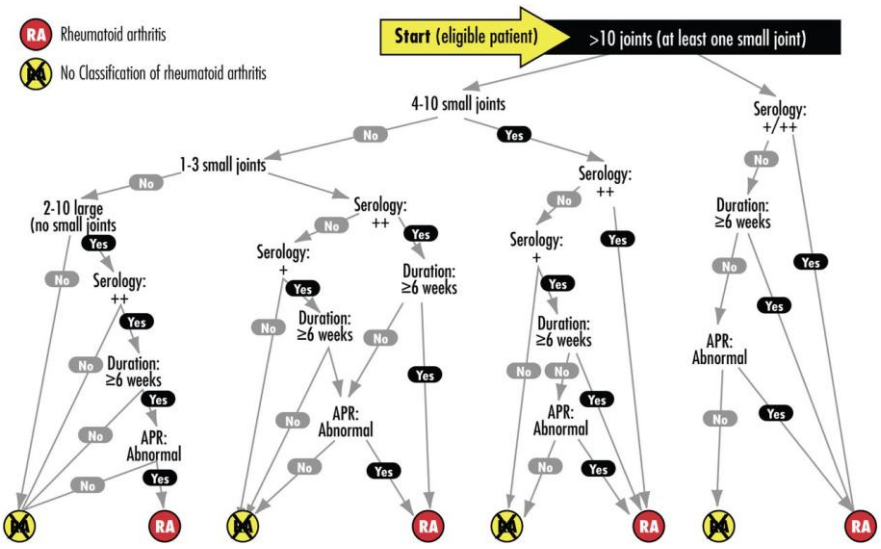

Figure 2.1. Tree algorithm for classifying define RA (red circles) or for excluding its current presence (yellow circles) (Kay and Upchurch, 2012).

Domain	Category	Point score
A	**Joint involvement (0-5 points) [a]**	
	1 large joint	0
	2-10 large joints	1
	1-3 small joints (large joints not counted)	2
	4-10 small joints (large joints not counted)	3
	>10 joints including at least one small joint	5
B	**Serology (at least one test needed for classification; 0-3 points) [b]**	
	Negative RF and negative ACPA	0
	Low positive RF or low positive ACPA	2
	High positive RF or high positive ACPA	3
C	**Acute-phase reactants (at least one test needed for classification; 0-1 point) [c]**	
	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
D	**Duration of symptoms [d]**	
	<6 weeks	0
	≥6 weeks	1

Table 2.1. 2010 ACR/EULAR RA classification criteria: domains, categories and point scores. The points from each of domains A through D are added and the sum is considered to be the total score. A total score of ≥6 is needed to classify a patient as having defined RA. [a] Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. DIP joints, first CMC joints and first MTP joints are excluded from assessment. Large joints refers to shoulders, elbows, hips, knees and ankles. Small joints refers to MCP joints, PIP joints, second through fifth MTP joints, thumb IP joints and wrists. [b] Negative means less than or equal to the upper limit of normal (ULN); low positive means >ULN; high positive means >3xULN. [c] Normal and abnormal are determined by local laboratory standards. [d] Duration of symptoms as per patient's self-report (Kay and Upchurch, 2012).

Clinical Parameters
TJC 28 (Tender Joint Counts on 28 joints)
SJC 28 (Swollen Joint Counts on 28 joints)
DAS 28 (Disease Activity Score on 28 joints)
CDAI (Clinical Disease Activity Index)
SDAI (Simplified Disease Activity Index)
VAS (Visual Analogue Scale)
HAQ (Health Assessment Questionnaire)
GH (General Health Score)

Table 2.2. Clinical parameters to assess patients' status.

Genomic DNA extraction

All drawn blood samples were collected and shipped to the genetic laboratory in EDTA treated vacutainer (BD, United States). The synovial tissue samples were collected and shipped in lysis buffer. Genomic DNA (gDNA) was extracted from 1.5 ml of fresh or frozen white blood cells and lysate of synovial tissue using Nucleon^{TM} DNA extraction kit (Amersham Biosciences, part of GE Healthcare Europe, CH) according to the manufacturer's instructions (Box 2.1)

BOX 2.1. Genomic DNA extraction using Nucleon^{TM} DNA extraction kit

1.5 ml of blood/tissue lysate was mixed with 6 ml of Reagent A.
The mix was shaken for 5 min at 50 rpm at room temperature.
The mix was centrifuged for 5 min at 3000 rcf.
Supernatant was discarded very slowly without disturbing the pellet.
350 µl of Reagent B was added and mixing was done by vortex.
The mix was transferred into a new tube and 100 µl of 5M sodium perchlorate was added and shook for 5 min at 50 rpm at room temperature.
The sample was incubated for 5 min at 65°C.
580 µl of cold (-20°C) chloroform was added and shaken for 15 min at 50 rpm at room temperature.
The mix was centrifuged for 1 min at 3000 rpm.
Upper phase of the mix (≈450µl) was transferred into a new tube.
Cold (-20°C) absolute ethanol (≈900µl) was added and inversion was done until observing DNA skein.
DNA skein transferred into a new tube and dissolved in 150 µl H_2O.

For blood samples with less than 1 ml volume, gDNA extraction was conducted using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer's instruction as described in box 2.2.

BOX 2.2. Genomic DNA extraction using QIAamp® DNA Blood Mini Kit
20 µl of proteinase K (PK) was added into a tube.
200 µl of blood/ 25 mg tissue was added to the PK-containing tube
200 µl of Buffer AL was added to the sample, and mixed by pulse-vortexing for 15s.
The mix was incubated at 56°C for 10 min.
200 µl of cold absolute ethanol was added to the sample, and mixed by pulse-vortexing for 15s.
All mix was transferred into QIAamp mini spin column and centrifuged at full speed for 1 min.
Supernatant was discarded and 500 µl of Buffer AW1 was added and centrifuged at full speed for 1 min.
Supernatant again was discarded and 500 µl of Buffer AW2 was added and centrifuged at full speed for 3 min.
Supernatant was discarded and the column was embedded inside a new tube and 200 µl of Buffer AE was added and finally centrifuged at 10000 rpm for 1 min.
The column was discarded and DNA was diluted in Buffer AE.

Genomic DNA titration and normalization

All extracted gDNA samples were titrated using Qubit® dsDNA BR Assay Kit (Life Technologies Oregon, USA). After quantification, all samples were stored in 2D-Barcoded Matrix Tubes (Thermo Fisher Scientific) and the DNA-Biobank were kept at -80°C freezer with limited-authorized access. For each DNA sample, 10 ng/µl and 1 ng/µl working dilution were prepared for subsequent use.

Genotyping

Genotyping for several variations belonging to HLA and non-HLA regions and also folate metabolism, which are listed in Table 2.3, was carried out using TaqMan 5’ discrimination assay by ABI 7300 Real-Time thermocycler (Thermo Fisher Scientific, Foster City, CA, USA) or by Polymerase Chain Reaction (PCR) followed by Polyacrylamide Gel Electrophoresis (PAGE).

Gene Family	Genetic Variant	Allelic form	Comparison groups
HLA GENES	HLA-G rs1233334	G>A	ERA vs. non-ERA
	HLA-G rs1063320	C>G	
	HLA-G 14bp	Insertion/Deletion	
	HLA-DRB1 rs660895	A>G	
	HLA-DRB1 rs6910071	A>G	
	HLA-DQA2 rs9275595	T>C	
	HLA-DQB2 rs10807113	C>A	
NON-HLA GENES	PTPN22 rs2476601	G>A	ERA vs. non-ERA
	PADI4 rs2240340	T>C	
	STAT4 rs7574865	G>T	
	CTLA4 rs231775	A>G	
	TRAF1 rs3761847	G>A	
	IL-10 rs1800871	G>A	
	IL-6 rs1800795	C>G	
FOLATE GENES	ATIC rs2372536	C>G	Responder vs. non-Responder
	MTRR rs1801394	A>G	
	SHMT1 rs1979277	C>T	
	SLC19A1 rs1051266	C>T	
	MTHFR rs1801133	A>G	
	MTHFR rs1801131	T>G	

Table 2.3. List of candidate genetic polymorphisms belonging to HLA, non-HLA and folate pathway genes.

Real-Time PCR

Allelic discrimination for SNPs were performed by Real-Time PCR using 1 unit of 2x TaqMan Universal PCR Master Mix (Appliedbiosystems, Warrington, UK), 1 unit of 1 ng/µl of gDNA and 1 unit of 40x corresponding TagMan SNP assay (Applied Biosystems, Foster City, CA). The PCR program was as follow: 50°C for 2 min, 95°C for 10 min and 50 cycles of: 95°C for 15s and 60°C for 1min, ended in 22°C for 30 min. (Figure 2.2.) The results of genotypings were obtained using 7300 System SDS software of the instrument (Figure 2.3).

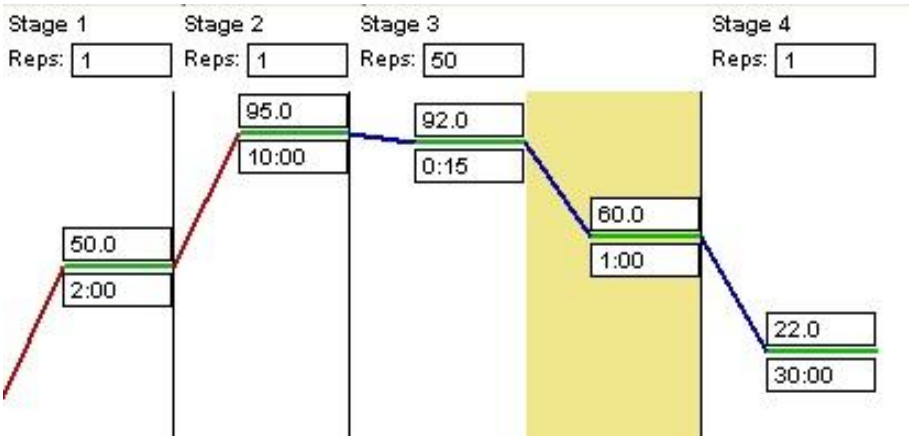

Figure 2.2. Real-Time PCR Program.

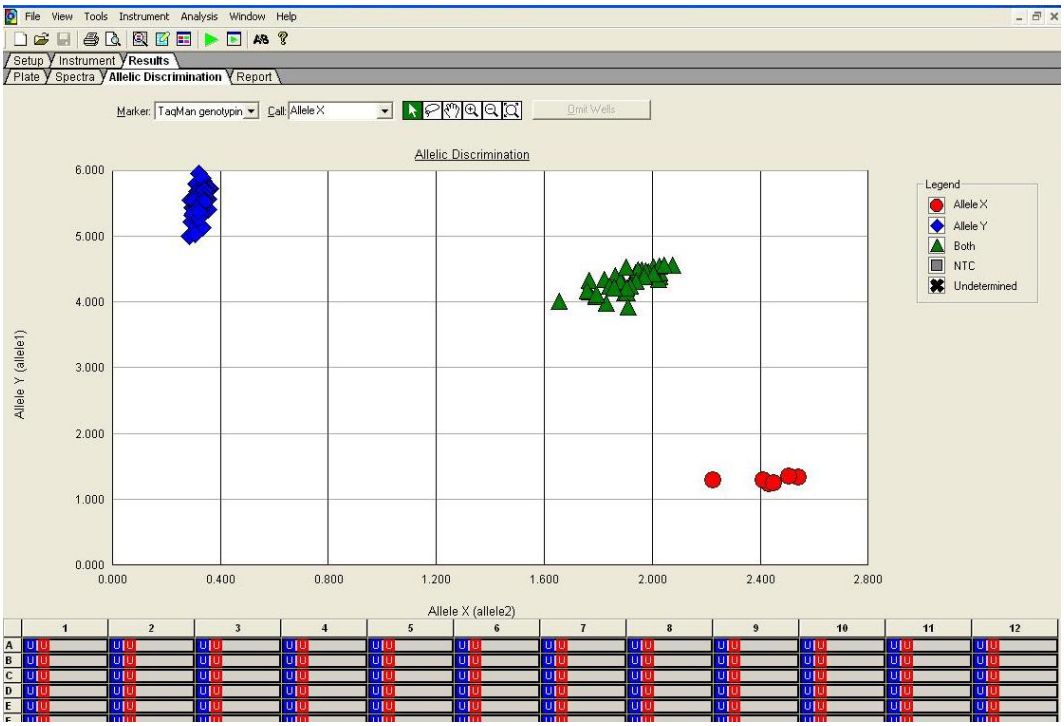

Figure 2.3. Results of allelic discrimination obtained by 7300 System SDS software

PCR-PAGE

HLA-G 14 bp insertion-deletion polymorphism was detected by PCR as described by (Castelli *et al.*, 2014). The polymorphic region of interest was amplified using forward primer: 5'-GTGATGGGCTGTTTAAAGTGTCACC-3' and reverse primer: 5'GGAAGGAATGCAGTTCAGCATGA-3' (Wisniewski *et al.*, 2015). The amplification was carried out by Applied Biosystem 2700 Thermocycler (Applied Biosystems, Foster City, CA) in a 15µl reaction containing 5ul of 1ng/µl gDNA, 1.50µl of 10X PCR Buffer, 0.45µl of 50mM $MgCl_2$, 0.25µl dNTPs, 0.5µl of 20ρmol primers and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR program included initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 15s, annealing at 64°C for 30s and extension at 72°C for 30s, then 25 cycles of denaturation at 94°C for 15s, annealing at 63°C for 30s and extension at 72°C for 30s, and final extension at 72°C for 5 min. The PCR product was run in 8% polyacrylamide gel and followed by silver staining. The product size was 224bp for Insertion/Insertion (I/I), 210bp for Deletion/Deletion (D/D) and 224bp/210bp for Insertion/Deletion (I/D) genotypes.

Bisulfite conversion of DNA

10 ng/µl of gDNA was bisulfite converted using EZ DNA Methylation-Lightning™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction as described in box 2.3.

BOX 2.3. Bisulfite conversion of gDNA using EZ DNA Methylation-Lightning™ Kit
130 µl of Lightning conversion reagent was added to 20 µl of 10 ng/µl of gDNA and placed in thermocycler.
Following steps were performed:
98°C for 8 minutes
54°C for 60 minutes
600 µl of M-Binding buffer was added to Zymo-Spin IC column and the sample from step 2 was added.
Centrifuged at full speed (> 10,000 xg) for 30 seconds and the flow-through was discarded.
100 µl of M-Wash buffer was added and centrifuged at full speed for 30 seconds.
200 µl of L-Desulphonation Buffer was added and incubated at room temperature for 20 min and followed by centrifugation at full speed for 30 seconds.
200 ul of M-Wash Buffer was added and centrifuged at full speed for 30 seconds
Stpe 7 was repeated
The column was placed into a 1.5 ml tube and 20 µl of M-Elution Buffer was added and centrifuged at full speed for 30 seconds.
The DNA was stored at -20° for later use.

Pyrosequencing

Line-1 methylation level was measured for all the study subjects with pyrosequencing on PyroMark Q96 ID using PyroMark Gold reagents (Qiagen). First LINE-1 amplification was carried out using forward primer: 5'-TTTTGAGTTAGGTGTGGGATATA-3' and reverse primer: /5Biosg/AAAATCAAAAAATTCCCTTTC-3'. The PCR was performed by Applied Biosystem 2700 Thermocycler (Applied Biosystems, Foster City, CA) in a 25µl reaction containing 2.5ul of 10 ng/µl bisulfite converted gDNA, 2.50 µl of 10X PCR Buffer, 0.75µl of 50 mM $MgCl_2$, 1.25 µl dNTPs, 1.25 µl of each 20 ρmol primer and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR program was shown in table 2.4.

1 Cycle			10 Cycles			27 Cycles			2 Holds	
98.0°	62.0°	72.0°	95.0°	61.0°	72.0°	94.0°	60.0°	72.0°	72.0°	4.0°
0:30	0:20	0:30	0:15	0:20	0:30	0:15	0:30	0:30	10:00	∞

Table 2.4. PCR Program for LINE1 amplification

The PCR product was run in 8% polyacrylamide gel and followed by silver staining for controlling success of PCR. The product size was 147bp. The residual PCR product was transferred in PCR plate with each well containing 20µl of the product, 20µl of RNase free distilled water, 3µl of sepharose beads containing streptavidin and 37 µl of binding buffer; i.e. total volume of 80µl of the mixture was placed on the thermo-mixture. Following this, the PCR product was made single-stranded to act as a template in a pyrosequencing reaction by washing with ethanol 70% and denaturation buffer using a Pyrosequencing Vacuum Prep tool (Bio-Stage). Pyrosequencing run was performed for each PCR reaction using AQ mode in a total volume of 40µl per well, including 38µl of annealing buffer and 1.6µl of 10 pM sequencing primer (AGT TAG GTG TGG GAT ATA GT) with suspended beads containing the sample DNA. The assays was created according to manufacturer's instruction. The nucleotide dispensation order was: ACT CAG TGT GTC AGT CAG TTA GTC TG.

Statistical Analyses

Genetic analysis for RA development: the analyses were carried out considering the group of patients who diagnosed with UA at first visit (T0). These patients were followed up to 6 months (T6) and then after meeting ACR/EULAR classification criteria for early rheumatoid arthritis were considered as ERA group and undefined conditions were considered as non-ERA group.

Genetic analysis for response to therapy: all enrolled patients were under pharmacological treatment of steroid (Medrol) and first line therapy, methotrexate, according to the patient's clinical condition. Response to therapy was evaluated according to DAS28 changes after 6 months follow up. EULAR criteria for response to therapy classifies patients in three groups: Good Responders, Moderate Responders, and Non-Responders (Table 2.5). In this study, in order to evaluate the response to the therapy, the overall responders (good responders plus moderate responders) versus non-responders were considered.

EULAR Response Criteria			
Value of composite measure at endpoint *(Present DAS28 score)*	Improvement in composite measure from baseline *(Improvement in DAS28 score)*		
	> 1.2	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2	Good		
> 3.2 and ≤ 5.1		Moderate	
> 5.1			None

Table 2.5. EULAR response criteria according to DAS28 changes (Bentley *et al.*, 2010)

Epigenetic analysis between different tissues: methylation profile of white blood cells and synovial cells from 6 patients were compared. Further, the differences between methylation level of white blood cells in 50 ERA patients (T0 and T6) and 30 RA patients and 50 healthy controls were compared. Finally, the variation in methylation of white blood cells in response to the therapy among non-responder, moderate responder and high responder ERA patients were assessed.

All corresponding odds ratios (ORs) and 95% confidence intervals (95% CIs) for the association of 1) ERA development, and 2) response to therapy of ERA patients with related polymorphisms (Table 2.3) and subsequently for stratification by sex, age, BMI, smoking, Rheumatoid Factor (RF) and anti- Citrullinated Peptide Antibodies (ACPA) were calculated. Bonferroni correction for multiple comparisons was performed. Different genetic models (dominant and recessive) were evaluated.

Average LINE-1 methylation level was calculated as the mean of the proportions of C (%) at the 3 CpG sites which were located at positions +319, +322 and +329.The differences of LINE-1 methylation level was analyzed by applying unpaired and paired student's t-test according to the comparison models.

A two-tailed p-value less than 0.05 was considered statistically significant. All statistical analysis was performed by Microsoft Excel (Microsoft, 2013).

Chapter 3

Results

Early Rheumatoid Arthritis Development

The first part of the present study focused on genetics of undifferentiated arthritis patients who developed rheumatoid arthritis. 174 patients had been recruited that after 6 months follow up according to ACR/EULAR classification criteria, 35 of them developed early rheumatoid arthritis (ERA), 53 resolved to other autoimmune disease that were excluded from our analyses and 86 remained as unresolved group (non-ERA). The mean ± standard deviation (SD) regarding the age, body mass index (BMI), and diseasy activity indexes and percentage of female sex, smokers, rheumatoeid factor positive and anti-citrullinated protein antibody positive of non-ERA and ERA patients were presented in Table 3.1.

Parameter	non-ERA (86)	ERA (35)	*p*
Age	58±14	58±17	0.83
BMI Kg/m^2	25.84±5.18	26.77±6.30	0.46
Female (%)	72	66	0.50
Smokers (%)	31	46	0.15
RF+ (%)	23	31	0.38
ACPA+ (%)	16	24	0.39
TJC/28	3.56±4.16	4.2±3.80	0.43
TJC/44	3.06±4.32	3.37±3.88	0.70
SJC/28	2.37±2.50	3.31±3.41	0.14
SJC/44	2.08±2.58	2.80±3.62	0.29
GH	43.57±24.40	46.29±26.19	0.60
HAQ	0.67±0.62	0.78±0.60	0.38
CDAI	13.65±9.71	16.55±9.10	0.12
SDAI	14.37±9.32	17.65±8.95	0.08
VAS	42.78±26.98	40.91±27.83	0.74
DAS28	3.73±1.17	4.04±0.94	0.13

Table 3.1. Characteristics of recruited undifferentiated arthritis patients. BMI: Body Mass Index; **RF**: Rheumatoid Factor; **ACPA**: Anti-Citrullinated Protein Antibody; **TJC28/44**: Tender 28/44 Joints Count; **SJC 28/44**: Swollen 28/44 Joints Count; **GH**: General Health Score; **HAQ**: Healthy Assessment Questionnaire; **CDAI**: Clinical Disease Activity Index; **SDAI**: Simplified Disease Activity Index; **VAS**: Visual Analogue Scale; **DAS28**: Diseasy Activity Score on 28 Joints.

Fourteen single nucleotide polymorphisms belonging to HLA or non-HLA genes were studied in order to invesitgate the association of SNP between non-ERA and ERA groups. The number of successful genotyping, Hardy Weinberg Equilibrium and rare allele frequency for each SNP were presented in Table 3.2. All SNPs except STAT4 rs7574865 (p=0.02) were in Hardy Weinberg Equilibrium.

Gene variant	Genotype Count						HW (p)	Minor allele Frequency	
	non-ERA			ERA					
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3		non-ERA	ERA
HLA-G rs1233334 G>A	72	16	0	31	4	0	0.62	9.1%	5.7%
HLA-G rs1063320 C>G	35	39	15	13	15	6	0.69	38.8%	39.7%
HLA-G 14bp In/del	26	38	26	10	15	10	0.24	50%	50%
HLA-DRB1 rs660895 A>G	68	18	2	27	7	1	0.69	12.5%	12.9%
HLA-DRB1 rs6910071 A>G	67	19	3	26	9	0	0.94	14%	12.9%
HLA-DQA2 rs9275595 T>C	61	23	4	24	10	1	0.74	17.6%	17.1%
HLA-DQB2 rs10807113 C>A	35	40	13	13	18	3	0.97	37.5%	35.3%
PTPN22 rs2476601 A>G	80	9	0	31	4	0	0.83	5.1%	5.7%
PADI4 rs2240340 T>C	26	48	12	8	22	5	0.14	41.9%	45.7%
STAT4 rs7574865 T>G	50	26	13	25	9	1	0.02	29.2%	15.7%
CTLA4 rs231775 A>G	48	32	9	17	14	4	0.54	28.1%	31.4%
TRAF1 rs3761847 G>A	36	39	13	19	12	4	0.65	36.9%	43.2%
IL-10 rs1800871 A>G	48	32	10	21	9	5	0.10	28.9%	27.1%
IL-6 rs1800795 C>G	40	38	8	17	14	2	0.93	31.4%	27.3%

Table 3.2. Genotyping data, Hardy Weinberg Equilibrium and rare allele frequency of non-ERA and ERA patients. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous; **HW**: Hardy Weinberg Equilibrium; ***p:*** p-Value.

Regarding HLA-G rs1233334 SNP, there was no significant association between heterozygous genotype and ERA (OR=0.58, 95% CI=0.18-1.88, *p*= 0.37); however due to lack of AA homozygous patients, association investigation was not possible (Figure 3.1).

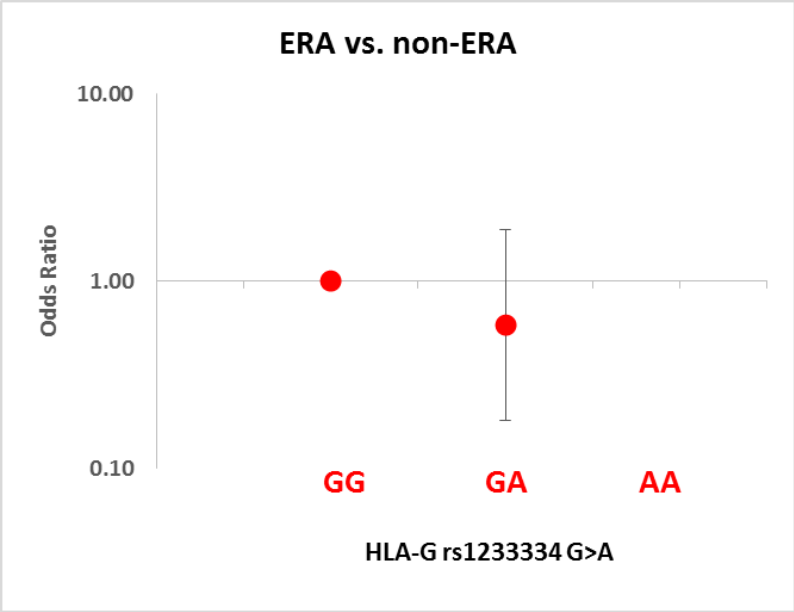

Figure 3.1. Association of HLA-G rs1233334 SNP with ERA

As shown in figure 3.2, regarding HLA-G rs1063320 SNP, the association of both CG heterozygous and GG homozygous patients in ERA development was almost null (OR=1.04, 95% CI=0.43-2.48, *p*=0.94; OR=1.08, 95% CI=0.34-3.37, *p*=0.91, respectively).

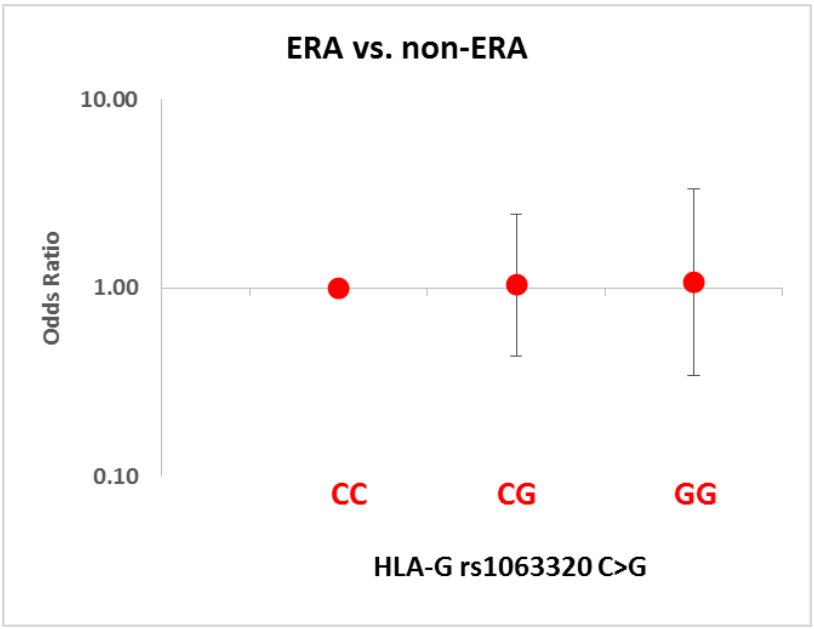

Figure 3.2. Association of HLA-G rs1063320 SNP with ERA

As shown in figure 3.3, the association of HLA-G 14bp In/del in both DI heterozygous and II homozygous groups in ERA development was almost null, likewise (OR=1.03, 95% CI=0.40-2.63, *p*=0.96; OR=1.00, 95% CI=0.36-2.80, *p*=1.00, respectively).

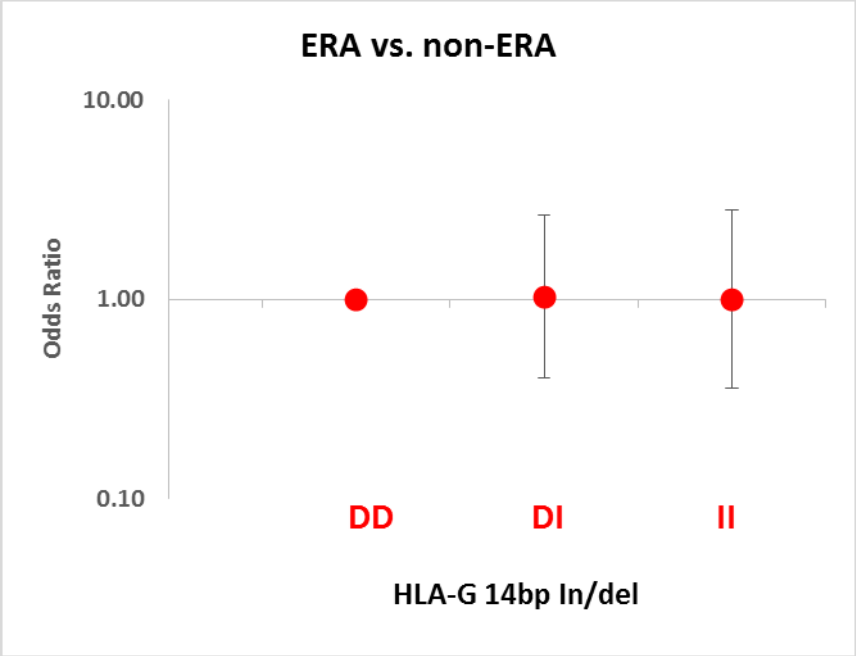

Figure 3.3. Association of HLA-G 14bp In/del varitant with ERA

Regarding HLA-DRB1 rs660895 SNP, there were no significant associations between either heterozygous or homozygous genotypes with ERA (OR=0.98, 95% CI=0.37-2.61, *p*= 0.97; OR=1.26, 95% CI=0.11-14.47, *p*= 0.86, respectively) (Figure 3.4).

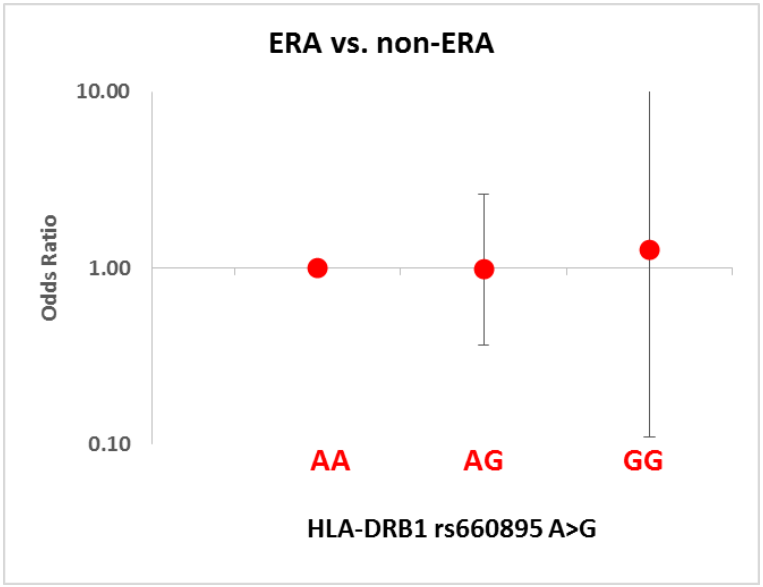

Figure 3.4. Association of HLA-DRB1 rs660895 with ERA

Regarding HLA-DRB1 rs6910071 SNP, there was no significant association between AG heterozygous genotype and ERA (OR=1.22, 95% CI=0.49-3.04, *p*= 0.68); however due to lack of GG homozygous patients, assessing association was not possible (Figure 3.5).

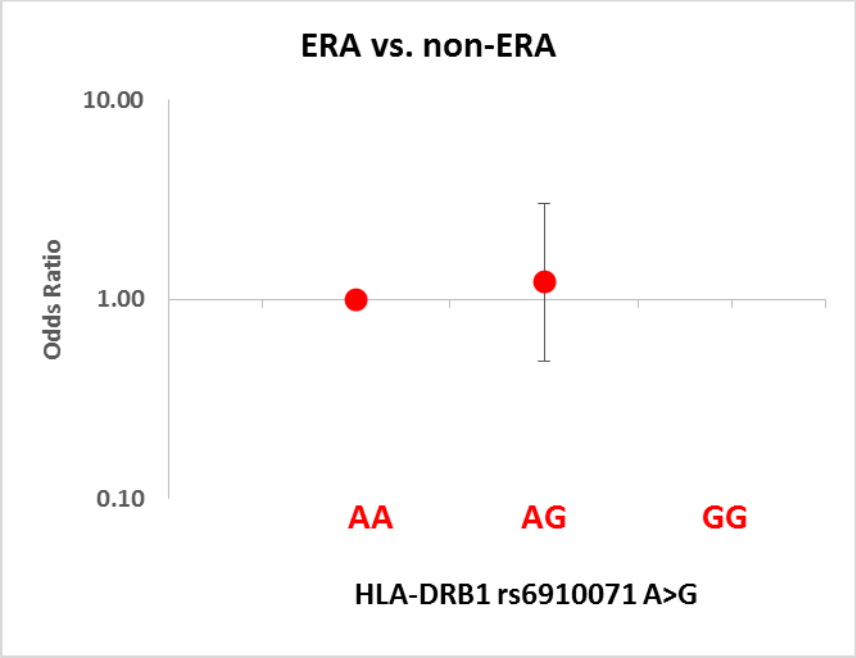

Figure 3.5. Association of HLA-DRB1 rs6910071 with ERA

As shown in figure 3.6, the association of HLA-DQA2 rs9275595 in both TC heterozygous and CC homozygous groups in ERA development was almost null (OR=1.10, 95% CI=0.46-2.66, *p*=0.83; OR=0.63, 95% CI=0.07-5.98, *p*=0.70, respectively).

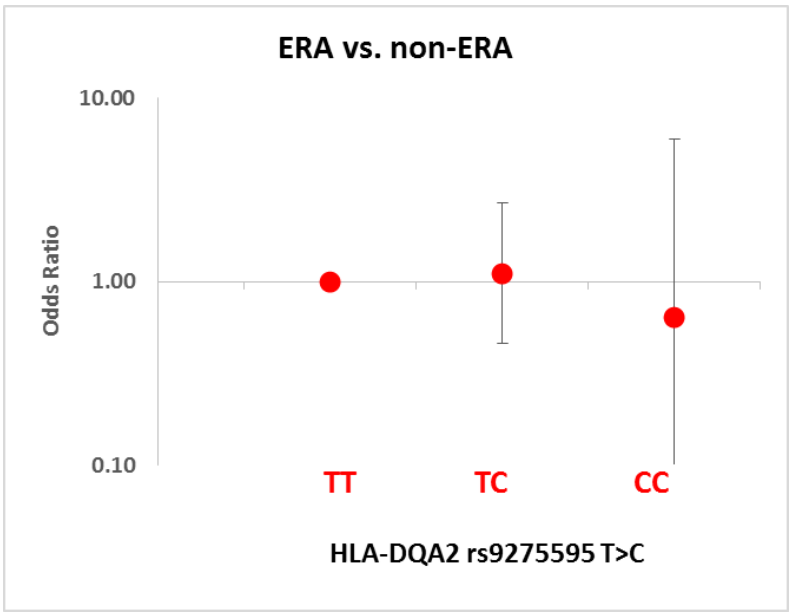

Figure 3.6. Association of HLA-DRB1 rs9275595 with ERA

The analyses showed that the association of HLA-DQB2 rs10807113 with ERA development in both CA heterozygous and AA homozygous groups were not significant (OR=1.21, 95% CI=0.52-2.82, p=0.67; OR=0.62, 95% CI=0.15-2.53, p=0.52, respectively) (Figure 3.7).

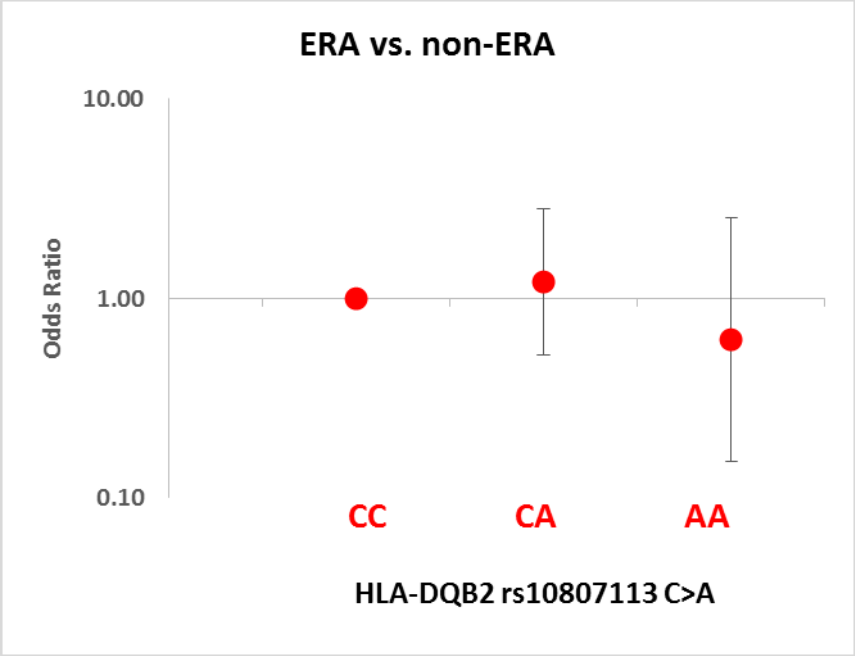

Figure 3.7. Association of HLA-DQB2 rs10807113 with ERA

Regarding PTPN22 rs246601 SNP, there was no significant association between AG heterozygous genotype and ERA (OR=1.15, 95% CI=0.33-4.00, p= 0.84); however due to lack of GG homozygous patients, assessing association was not possible (Figure 3.8).

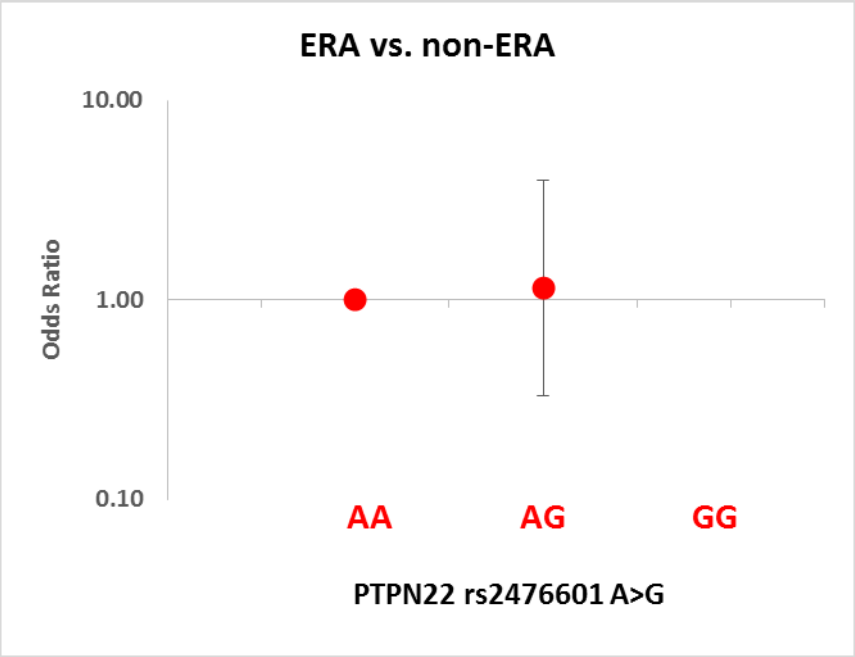

Figure 3.8. Association of PTPN22 rs2476601 with ERA

As shown in figure 3.9, the association of PADI4 rs2240340 in both TC heterozygous and CC homozygous groups in ERA development was not significant (OR=1.49, 95% CI=0.58-3.81, *p*=0.41; OR=1.35, 95% CI=0.36-5.02, *p*=0.66, respectively).

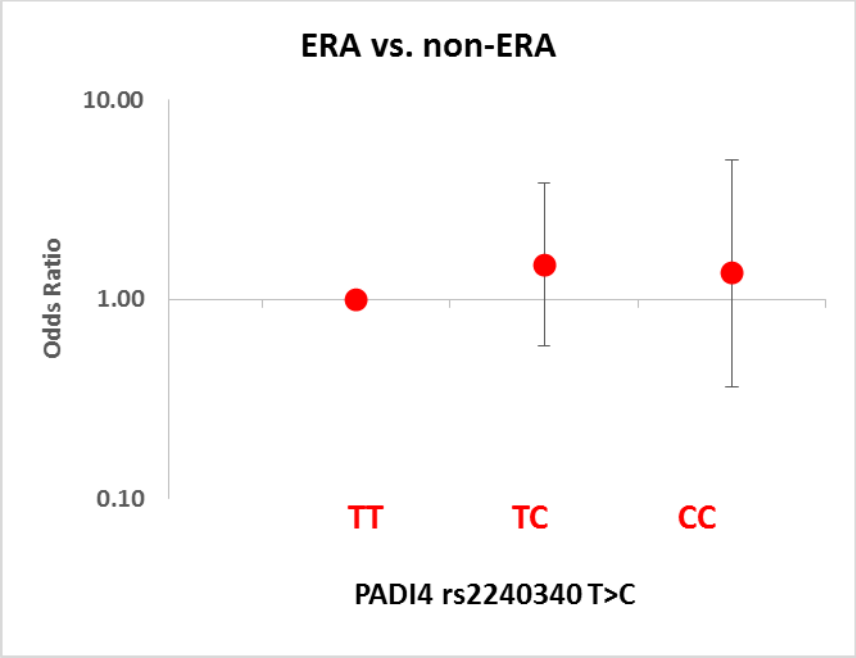

Figure 3.9. Association of PADI4 rs2240340 with ERA

The analyses showed a trend of association of STAT4 rs7574865 with ERA development in both TG heterozygous and GG homozygous groups, however the observations were not significant (OR=0.69, 95% CI=0.28-1.70, p=0.43; OR=0.15, 95% CI=0.02-1.24, p=0.08, respectively) (Figure 3.10).

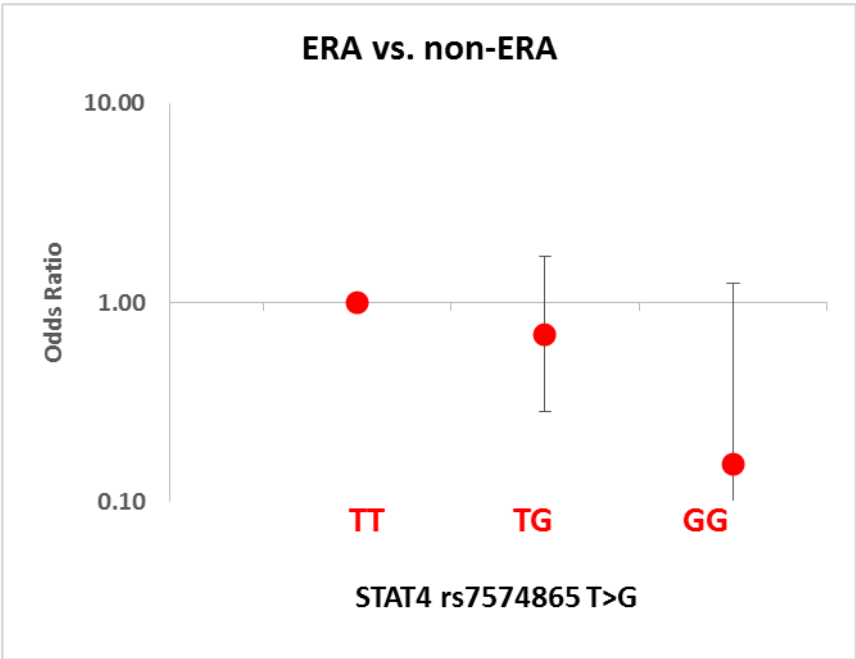

Figure 3.10. Association of STAT4 rs7574865 with ERA

Regarding CTLA4 rs231775 SNP, there were no significant associations between either heterozygous or homozygous genotypes with ERA (OR=1.23, 95% CI=0.53-2.85, p= 0.63; OR=1.25, 95% CI=0.34-4.61, p= 0.74, respectively) (Figure 3.11).

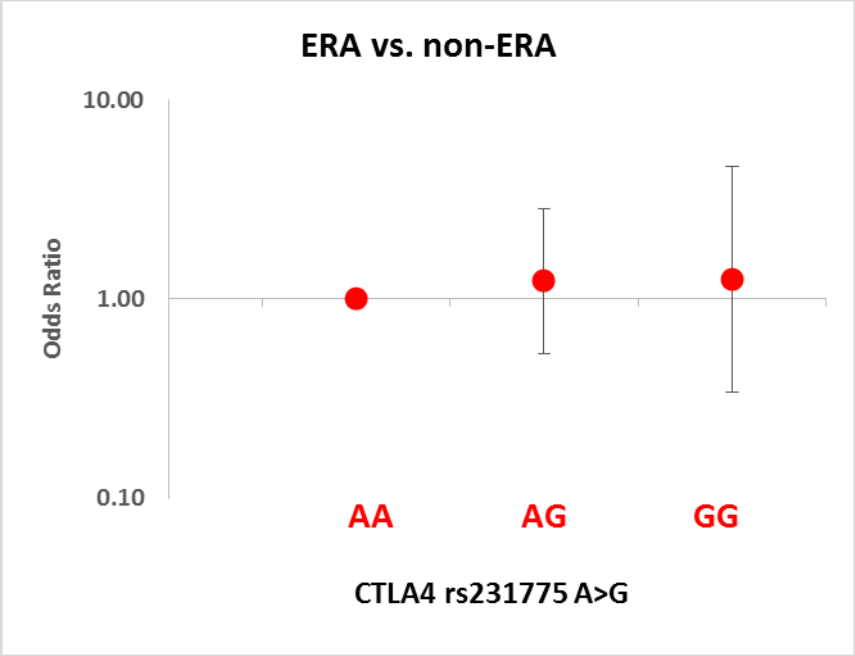

Figure 3.11. Association of CTLA4 rs231775 with ERA

As shown in figure 3.12, the reverse associations of TRAF1 rs3761847 in both GA heterozygous and AA homozygous groups in ERA development were not significant (OR=0.58, 95% CI=0.25-1.37, *p*=0.22; OR=0.58, 95% CI=0.17-2.04, *p*=0.40, respectively).

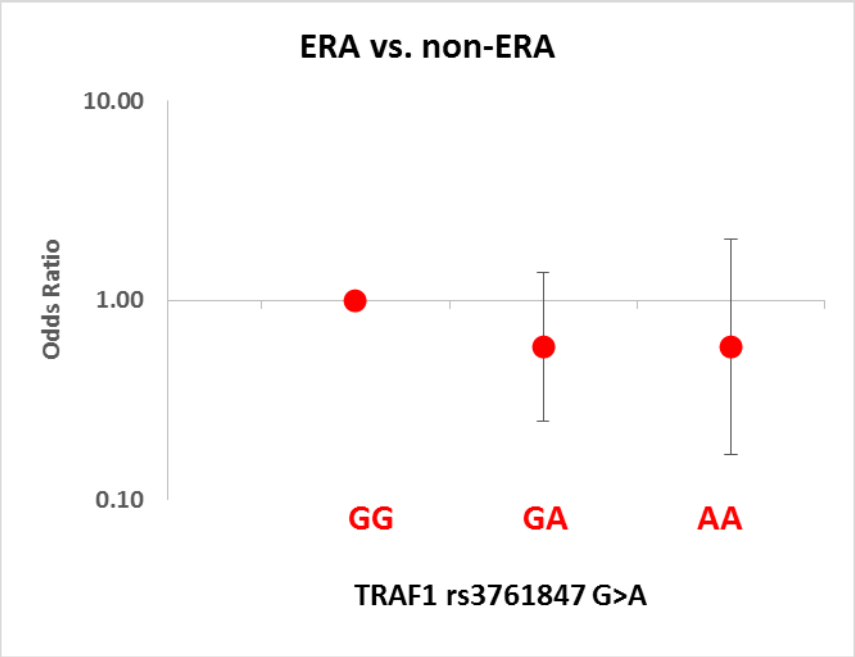

Figure 3.12. Association of TRAF1 rs3761847 with ERA

The analyses showed that there were no associations of IL-10 rs1800871 with ERA development in both AG heterozygous and GG homozygous genotypes (OR=0.64, 95% CI=0.26-1.58, p=0.34; OR=1.14, 95% CI=0.35-3.76, p=0.84, respectively) (Figure 3.13).

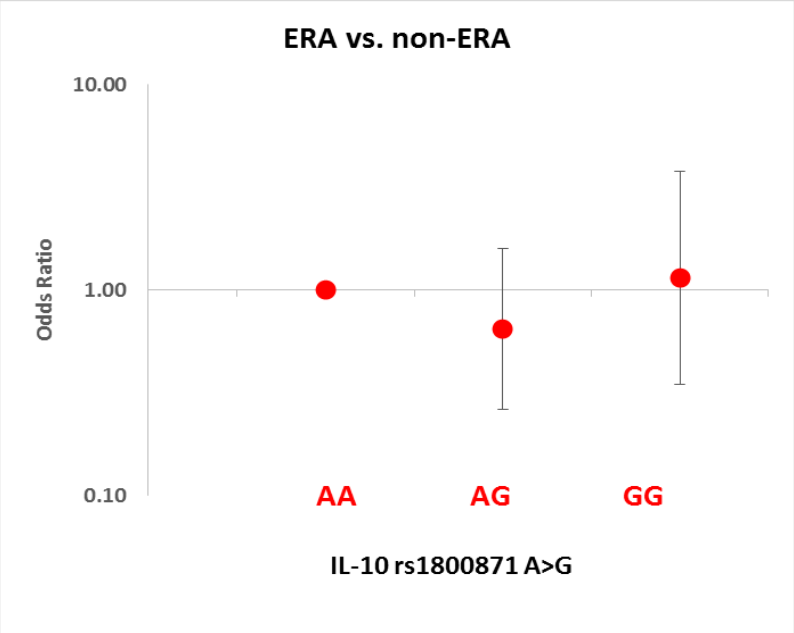

Figure 3.13. Association of IL-10 rs1800871 with ERA

The analyses showed a trend of reverse association of IL-6 rs1800795 with ERA development in both CG heterozygous and GG homozygous groups, however the associations were not significant (OR=0.87, 95% CI=0.38-2.00, p=0.75; OR=0.59, 95% CI=0.11-3.06, p=0.54, respectively) (Figure 3.14).

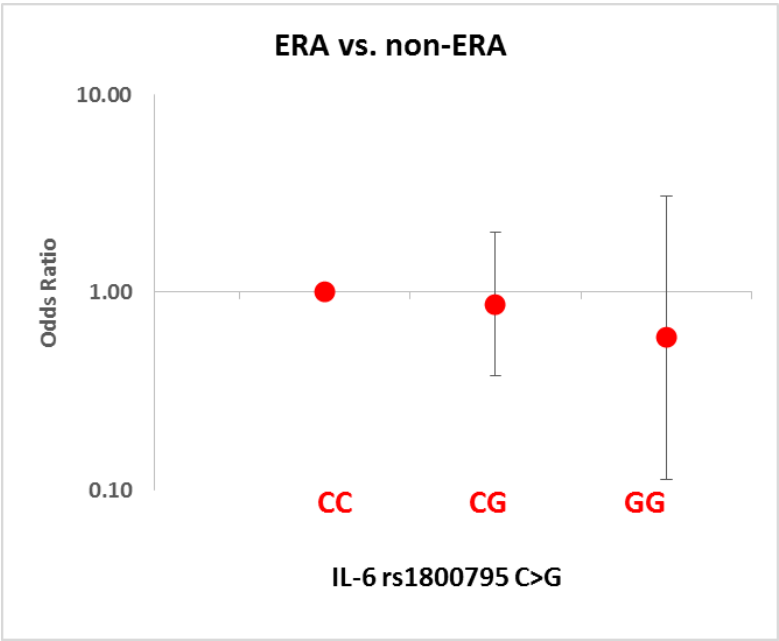

Figure 3.14. Association of IL-6 rs1800795 with ERA

Early Rheumatoid Arthritis Development-Sex

The results of genotyping for 14 candidate SNPs stratified by sex are presented in table 3.3 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	Male			Female			Male			Female		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	49	12	0	23	4	0	22	1	0	9	3	0
HLA-G rs1063320 C>G	23	28	11	12	11	4	10	8	4	3	7	2
HLA-G 14bp In/del	18	27	18	8	11	8	7	9	7	3	6	3
HLA-DRB1 rs660895 A>G	47	13	1	21	5	1	17	5	1	10	2	0
HLA-DRB1 rs6910071 A>G	45	15	2	22	4	1	19	4	0	7	5	0
HLA-DQA2 rs9275595 T>C	39	19	3	22	4	1	14	8	1	10	2	0
HLA-DQB2 rs10807113 C>A	22	27	12	13	13	1	9	12	2	4	6	1
PTPN22 rs2476601 A>G	57	5	0	23	4	0	20	3	0	11	1	0
PADI4 rs2240340 T>C	19	34	6	7	14	6	4	15	4	4	7	1
STAT4 rs7574865 T>G	34	19	9	16	7	4	17	5	1	8	4	0
CTLA4 rs231775 A>G	36	22	4	12	10	5	9	11	3	8	3	1
TRAF1 rs3761847 G>A	20	29	12	16	10	1	15	4	4	4	8	0
IL-10 rs1800871 A>G	34	21	8	14	11	2	14	5	4	7	4	1
IL-6 rs1800795 C>G	27	25	7	13	13	1	11	10	1	6	4	1

Table 3.3. Genotyping data of non-ERA and ERA patients stratified by sex. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping stratified by sex for HLA-G rs1233334 showed a reverse association of GA heterozygous with ERA development in male patients (OR=0.19, 95% CI=0.02-1.52, p=0.12) and an association in females (OR=1.92, 95% CI=0.36-10.32, p=0.46). However, none of the observed associations were significant. Due to lack of AA homozygous patients, evaluating association was not possible (Figure 3.15).

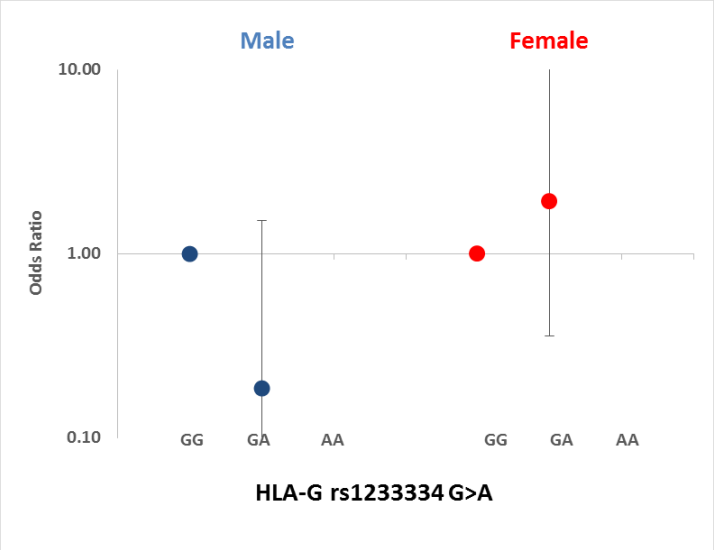

Figure 3.15. Association of HLA-G rs1233334 with ERA according to patients' sex.

The results of sex-based genotyping for HLA-G rs1063320 showed a reverse association of CG heterozygous (OR=0.66, 95% CI=0.22-1.94, p=0.45) and GG homozygous (OR=0.84, 95% CI=0.21-3.27, p=0.81) in male patients and an association of CG heterozygous (OR=2.54, 95% CI=0.52-12.37, p=0.25) and GG homozygous (OR=2.00, 95% CI=0.24-16.61, p=0.53) in females with ERA development. However, none of the observed associations were significant (Figure 3.16).

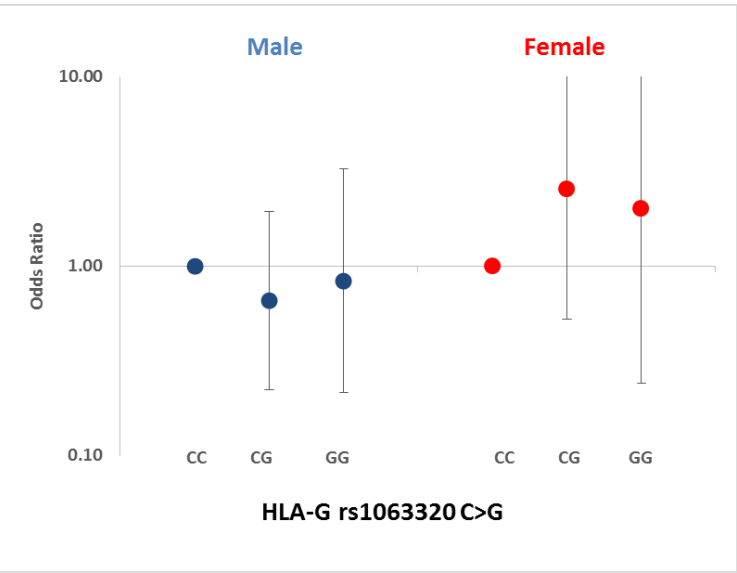

Figure 3.16. Association of HLA-G rs1063320 with ERA according to patients' sex.

The results of genotyping for HLA-G 14bp In/del showed a reverse association of DI heterozygous (OR=0.86, 95% CI=0.27-2.72, p=0.81) and lack of association of II homozygous (OR=1.00, 95% CI=0.29-3.44, p=1.00) in male patients and an association of DI heterozygous (OR=1.45, 95% CI=0.28-7.64, p=0.67) and null association of II homozygous (OR=1.00, 95% CI=0.15-6.53, p=1.00) in females with ERA development. However, none of the observed associations were significant (Figure 3.17).

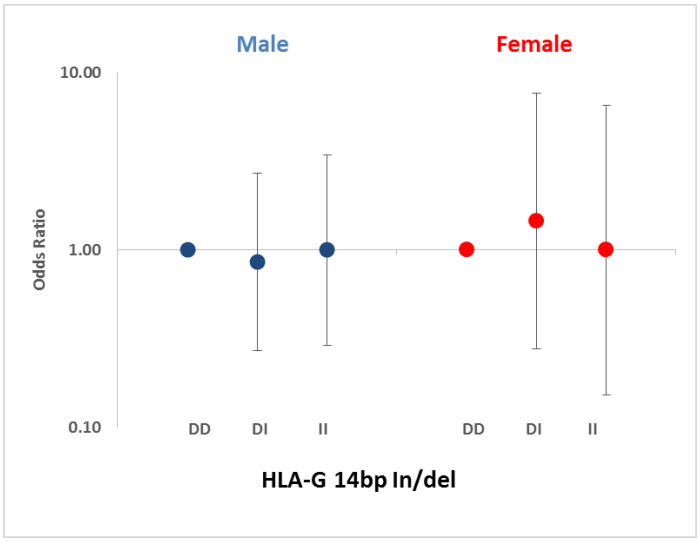

Figure 3.17. Association of HLA-G 14bp In/del with ERA according to patients' sex.

The genotyping of HLA-DRB1 rs660895 showed almost lack of association of AG heterozygous (OR=1.06, 95% CI=0.33-3.43, p=0.92) and an almost 2.80-fold association of GG homozygous (OR=2.76, 95% CI=0.16-46.70, p=0.49) in male patients and also a slight reverse association of AG heterozygous (OR=0.84, 95% CI=0.14-5.10, p=0.86) in females with ERA development.), however due to lack of GG homozygous females, assessing association was not possible. Beside, none of the observed associations were significant (Figure 3.18).

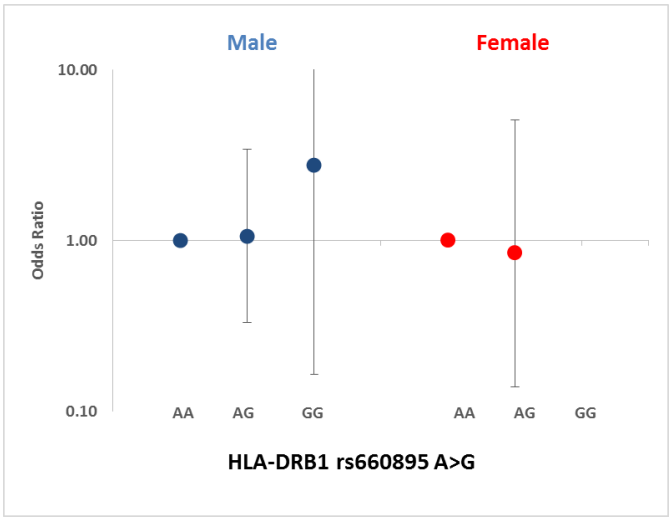

Figure 3.18. Association of HLA-DRB1 rs660895 with ERA according to patients' sex.

The results of genotyping stratified by sex for HLA-DRB1 rs6910071 showed a reverse association of AG heterozygous with ERA development in male patients (OR=0.63, 95% CI=018-2.15, p=0.47) and an association in females (OR=3.93, 95% CI=0.82-18.81, p=0.09). However, none of the observed associations were significant. Due to lack of GG homozygous patients, evaluating association was not possible (Figure 3.19).

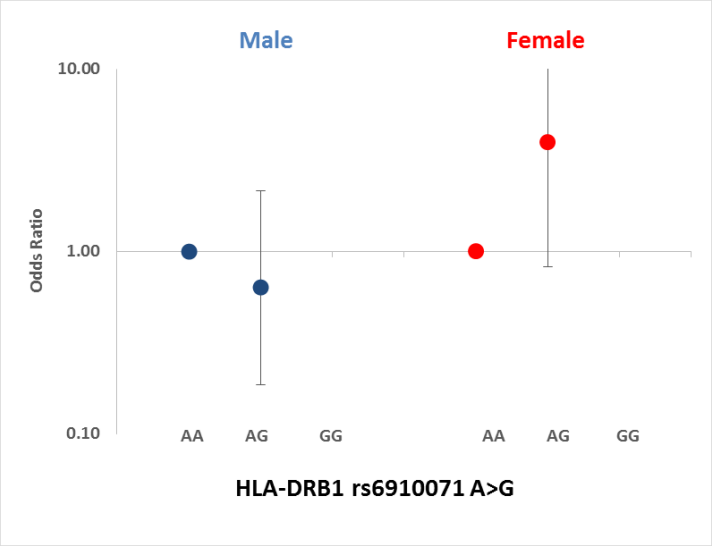

Figure 3.19. Association of HLA-DRB1 rs6910071 with ERA according to patients' sex.

The results of genotyping for HLA-DQA2 rs9275595 showed a weak association of TC heterozygous (OR=1.17, 95% CI=0.42-3.28, p=0.77) and almost lack of association of CC homozygous (OR=0.93, 95% CI=0.09-9.68, p=0.95) in male patients and almost null association of TC heterozygous (OR=1.10, 95% CI=0.17-7.03, p=0.93) in females with ERA development. Because of lacking CC homozygous patients, evaluating association was not possible However, none of the observations were statistically significant (Figure 3.20).

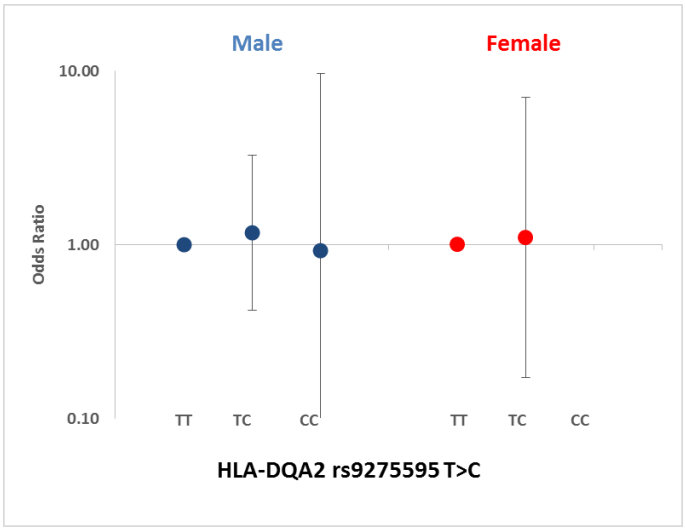

Figure 3.20. Association of HLA-DQA2 rs9275595 with ERA according to patients' sex.

The results of genotyping for HLA-DQB2 rs10807113 showed lack of association of CA heterozygous (OR=1.09, 95% CI=0.39-3.05, p=0.88) and a reverse association of AA homozygous (OR=0.41, 95% CI=0.07-2.20, p=0.30) in male patients and an association of CA heterozygous (OR=1.50, 95% CI=0.34-6.60, p=0.60) and AA homozygous (OR=3.25, 95% CI=0.16-64.62, p=0.45) in females with ERA development. Although, there was a trend of association in females, none of the observed associations were significant (Figure 3.21).

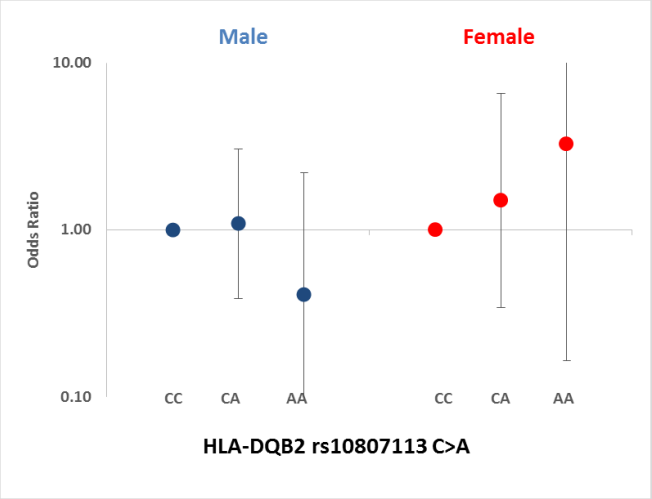

Figure 3.21. Association of HLA-DQB2 rs10807113 with ERA according to patients' sex.

The results of genotyping stratified by sex for PTPN22 rs2476601 showed an association of AG heterozygous with ERA development in male patients (OR=1.71, 95% CI=0.37-7.81, p=0.50) and a reverse association in females (OR=0.52, 95% CI=0.05-5.25, p=0.59). However, none of the observed associations were significant. Due to lack of GG homozygous patients, evaluating association was not possible (Figure 3.22).

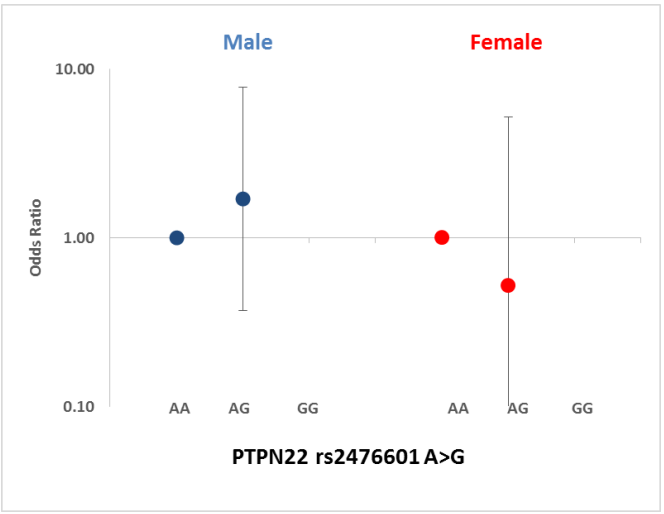

Figure 3.22. Association of PTPN22 rs2476601 with ERA according to patients' sex.

The results of genotyping for PADI4 rs2240340 showed a trend of association of TC heterozygous (OR=2.10, 95% CI=0.61-7.22, p=0.24) and CC homozygous (OR=3.62, 95% CI=0.60-16.69, p=0.17) in male patients and also a trend of reverse association of TC heterozygous (OR=0.87, 95% CI=0.19-4.03, p=0.87) and CC homozygous (OR=0.29, 95% CI=0.02-3.37, p=0.33) in females with ERA development. However, none of the observed trends were significant (Figure 3.23).

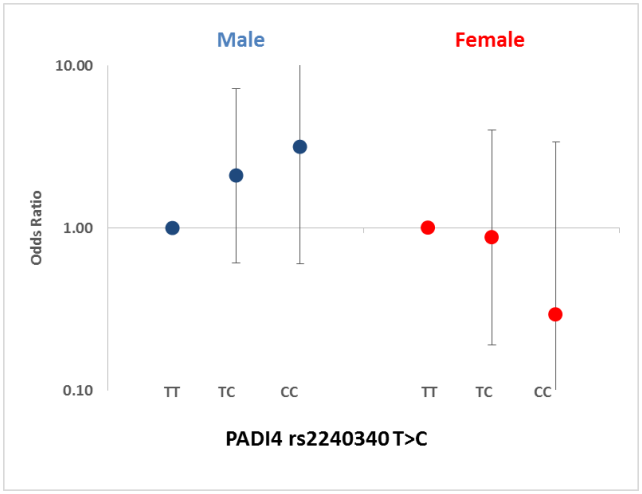

Figure 3.23. Association of PADI4 rs2240340 with ERA according to patients' sex.

As shown in figure 3.24, STAT4 rs7574865 had a reverse association of TG heterozygous (OR=0.53, 95% CI=0.17-1.65, p=0.27) and GG homozygous (OR=0.22, 95% CI=0.03-1.90, p=0.17) in male patients and almost null association of TG heterozygous (OR=1.14, 95% CI=0.26-5.09, p=0.87) in females with ERA development. Because of lacking GG homozygous female patients, evaluating association was not possible. However, none of the observations were statistically significant.

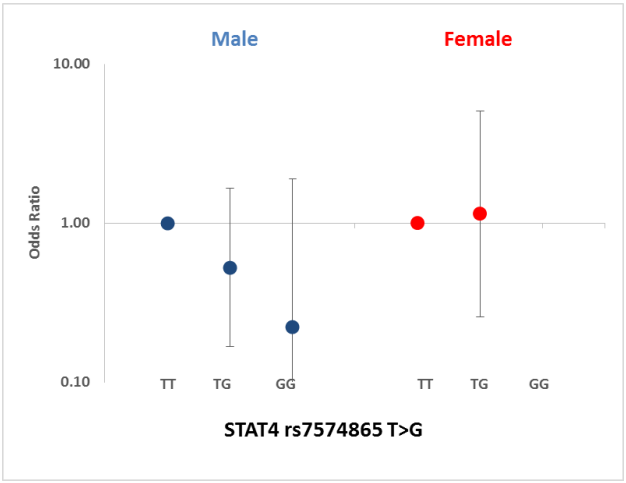

Figure 3.24. Association of STAT4 rs7574865 with ERA according to patients' sex.

The results of genotyping for CTLA4 rs231775 showed a trend of association of AG heterozygous (OR=2.00, 95% CI=0.71-5.60, p=0.19) and GG homozygous (OR=3.00, 95% CI=0.57-15.87, p=0.20) in male patients and also a trend of reverse association of AG heterozygous (OR=0.45, 95% CI=0.09-2.16, p=0.32) and GG homozygous (OR=0.30, 95% CI=0.03-3.07, p=0.31) in females with ERA development. However, none of the observed trends were significant (Figure 3.25).

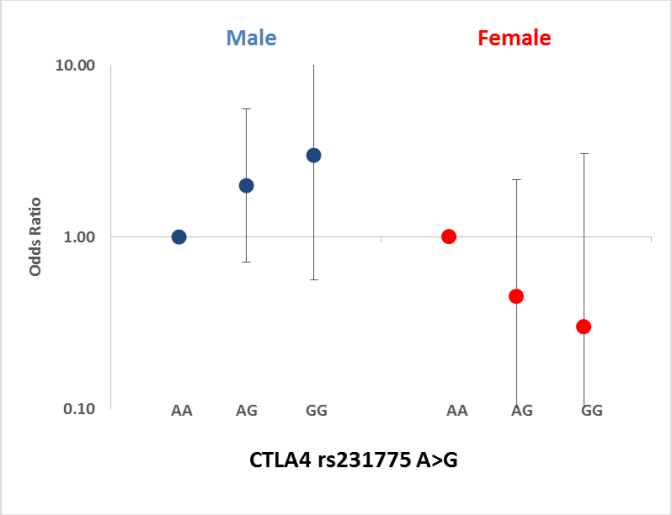

Figure 3.25. Association of CTLA4 rs231775 with ERA according to patients' sex.

As shown in figure 3.26, TRAF1 rs3761847 had a reverse significant association of GA heterozygous (OR=0.18, 95% CI=0.05-0.64, p=0.008) and not significant association of AA homozygous (OR=0.44, 95% CI=0.12-1.65, p=0.23) in male patients and an association of GA heterozygous (OR=3.20, 95% CI=0.76-13.46, p=0.11) in females with ERA development. Because of lacking AA homozygous female patients, evaluating association was not possible. The observed association in males was under dominant model (p=0.0068).

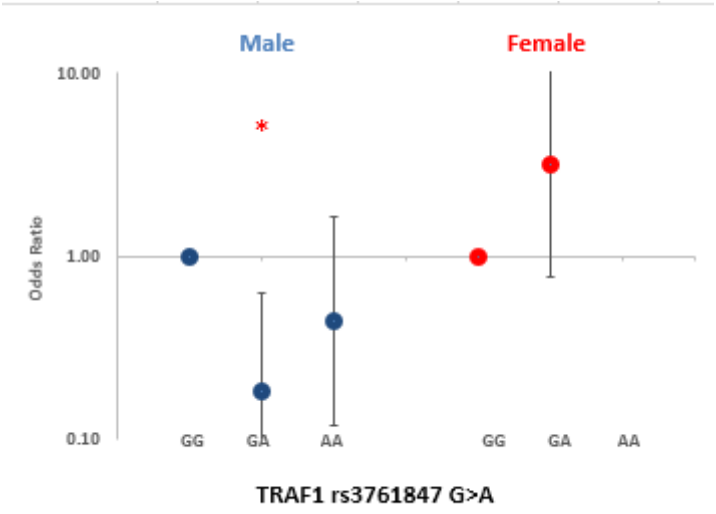

Figure 3.26. Association of TRAF1 rs3761847 with ERA according to patients' sex.

The results of genotyping for IL-10 rs1800871 showed a reverse association of AG heterozygous (OR=0.58, 95% CI=0.18-1.84, p=0.36) and almost lack of association of GG homozygous (OR=1.21, 95% CI=0.31-4.69, p=0.79) in male patients and also a reverse association of AG heterozygous (OR=0.73, 95% CI=0.17-3.13, p=0.68) and a lack of association of GG homozygous (OR=1.00, 95% CI=0.08-13.02, p=1.00) in females with ERA development. However, none of the observations were statistically significant (Figure 3.27).

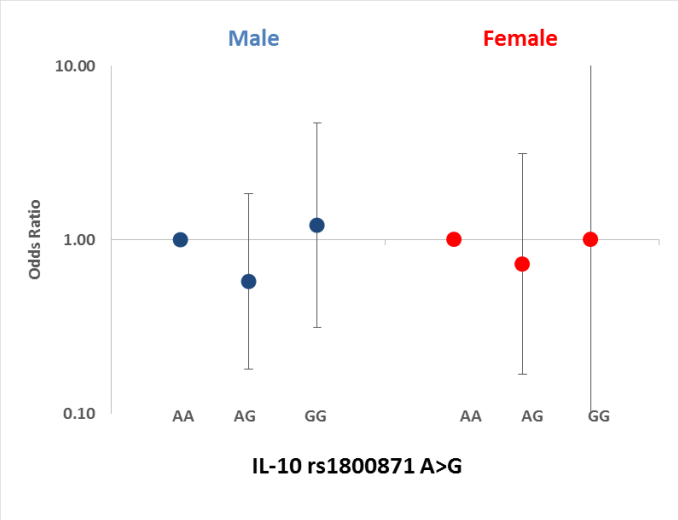

Figure 3.27. Association of IL-10 rs1800871 with ERA according to patients' sex.

As shown in figure 3.28, IL-6 rs1800795 had a null association of CG heterozygous (OR=0.98, 95% CI=0.36-2.71, p=0.97) and a reverse association of GG homozygous (OR=0.35, 95% CI=0.04-3.19, p=0.36) in male patients and also a reverse association of CG heterozygous (OR=0.67, 95% CI=0.15-2.93, p=0.60) and a 2-told association in GG homozygous (OR=2.17, 95% CI=0.11-40.81, p=0.62) in females with ERA development. However, none of the observations were statistically significant.

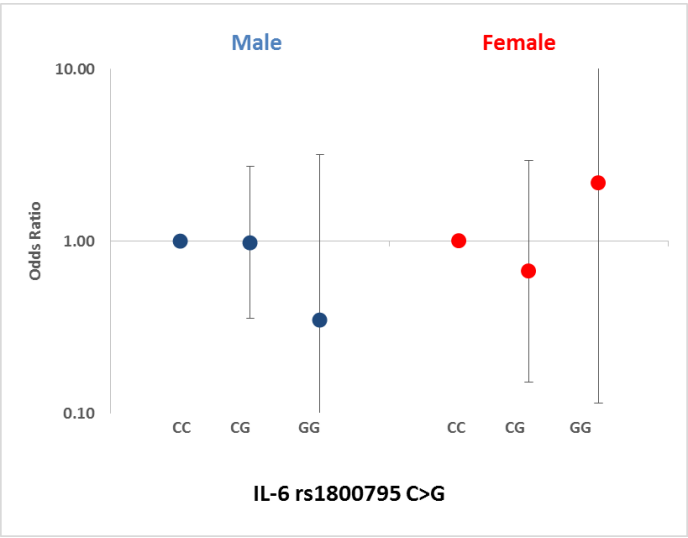

Figure 3.28. Association of IL-6 rs1800795 with ERA according to patients' sex.

Early Rheumatoid Arthritis Development-Age

The results of genotyping for 14 candidate SNPs stratified by age (<50 and >50) are presented in table 3.4 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	Age < 50 y			Age > 50 y			Age < 50 y			Age > 50 y		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	25	4	0	47	12	0	12	2	0	19	2	0
HLA-G rs1063320 C>G	12	12	5	23	27	10	5	5	4	8	10	2
HLA-G 14bp In/del	7	13	9	19	25	17	5	6	3	5	9	7
HLA-DRB1 rs660895 A>G	19	8	1	49	10	1	10	3	1	17	4	0
HLA-DRB1 rs6910071 A>G	25	3	1	42	16	2	11	3	0	15	6	0
HLA-DQA2 rs9275595 T>C	20	6	2	41	17	2	9	5	0	15	5	1
HLA-DQB2 rs10807113 C>A	10	15	3	25	25	10	4	9	1	9	9	2
PTPN22 rs2476601 A>G	23	5	0	57	4	0	13	1	0	18	3	0
PADI4 rs2240340 T>C	9	14	5	17	34	7	2	10	2	6	12	3
STAT4 rs7574865 T>G	11	11	6	39	15	7	8	6	0	17	3	1
CTLA4 rs231775 A>G	15	9	4	33	23	5	8	4	2	9	10	2
TRAF1 rs3761847 G>A	12	13	3	24	26	10	10	2	2	9	10	2
IL-10 rs1800871 A>G	15	9	5	33	23	5	10	2	2	11	7	3
IL-6 rs1800795 C>G	14	11	3	26	27	5	7	7	0	10	7	2

Table 3.4. Genotyping data of non-ERA and ERA patients stratified by age. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping stratified by age for HLA-G rs1233334 showed no association of GA heterozygous with ERA development in older patients (OR=1.04, 95% CI=0.17-6.50, p=0.97) and a reverse association in younger patients (OR=0.41, 95% CI=0.84-2.02, p=0.28). However, none of the observed associations were significant. Due to lack of AA homozygous patients, evaluating association was not possible (Figure 3.29).

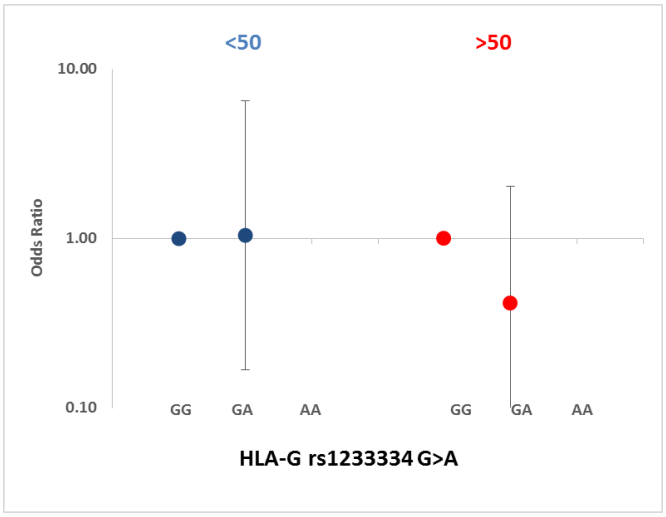

Figure 3.29. Association of HLA-G rs1233334 with ERA according to patients' age.

The results of genotyping for HLA-G rs1063320 showed lack of association of CG heterozygous (OR=1.00, 95% CI=0.23-4.37, p=1.00) and an association GG homozygous (OR=1.92, 95% CI=0.36-10.29, p=0.45) in patients below 50 years old and also null association of CG heterozygous (OR=1.06, 95% CI=0.36-3.15, p=0.92) and a reverse association GG homozygous (OR=0.57, 95% CI=0.10-3.20, p=0.53) in patients above 50 years old with ERA development. However, none of the observed associations were significant (Figure 3.30).

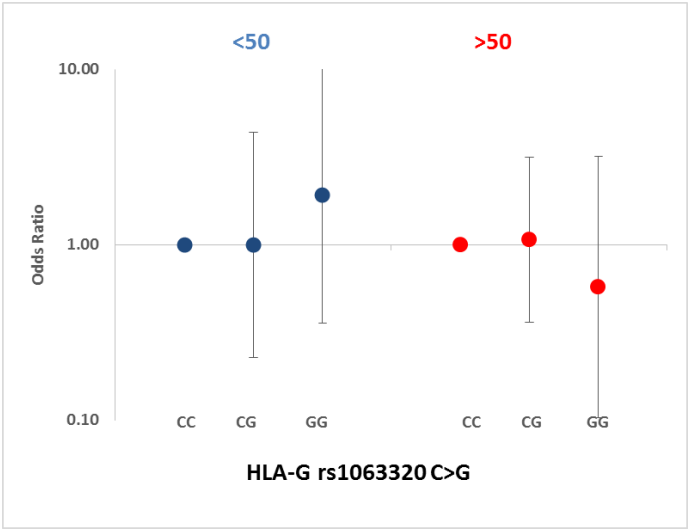

Figure 3.30. Association of HLA-G rs1063320 with ERA according to patients' age.

As presented in figure 3.31, HLA-G 14bp In/del had a trend of reverse association for DI heterozygous (OR=0.65, 95% CI=0.14-2.90, p=0.58) and II homozygous (OR=0.47, 95% CI=0.08-2.66, p=0.40) in patients below 50 years old and also a trend of association of DI heterozygous (OR=1.37, 95% CI=0.39-4.75, p=0.63) and II homozygous (OR=1.56, 95% CI=0.42-5.86, p=0.52) in patients above 50 years old with ERA development. However, none of the observed associations were significant.

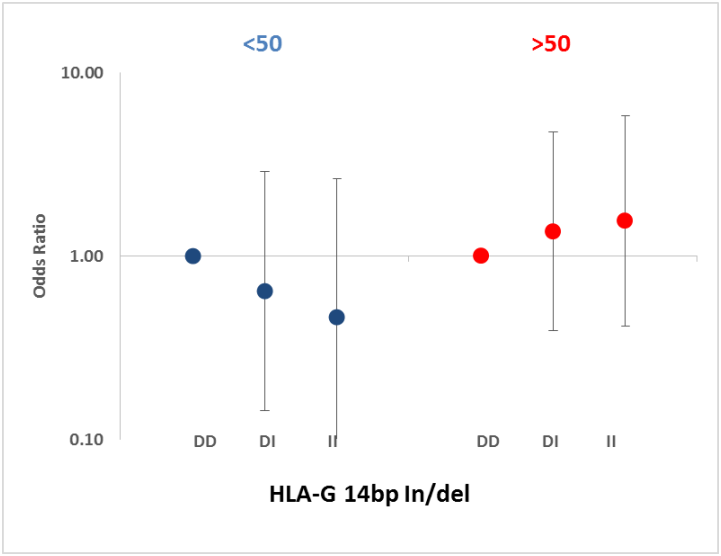

Figure 3.31. Association of HLA-G 14bp In/del with ERA according to patients' age.

The results of genotyping for HLA-DRB1 rs660895 showed a reverse association of AG heterozygous (OR=0.71, 95% CI=0.15-3.30, p=0.68) and an association for GG homozygous (OR=1.90, 95% CI=0.11-33.70, p=0.67) in patients below 50 years old and almost null association of AG heterozygous (OR=1.15, 95% CI=0.32-4.16, p=0.84) in patients above 50 years old with ERA development. However, none of the observed associations were significant (Figure 3.32).

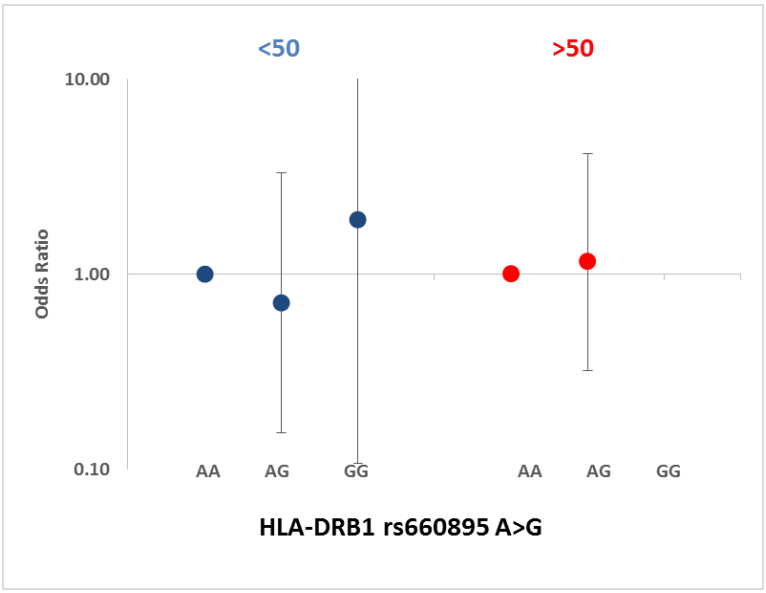

Figure 3.32. Association of HLA-DRB1 rs660895 with ERA according to patients' age.

As shown in figure 3.33, HLA-DRB1 rs6910071 had an association for AG heterozygous (OR=2.27, 95% CI=0.39-13.08, p=0.36) in patients below 50 years old and also a null association of AG heterozygous (OR=1.05, 95% CI=0.35-3.18, p=0.94) in patients above 50 years old with ERA development. Due to lack of GG homozygous patients, evaluating associations were not possible. Beside, none of the observed associations were significant.

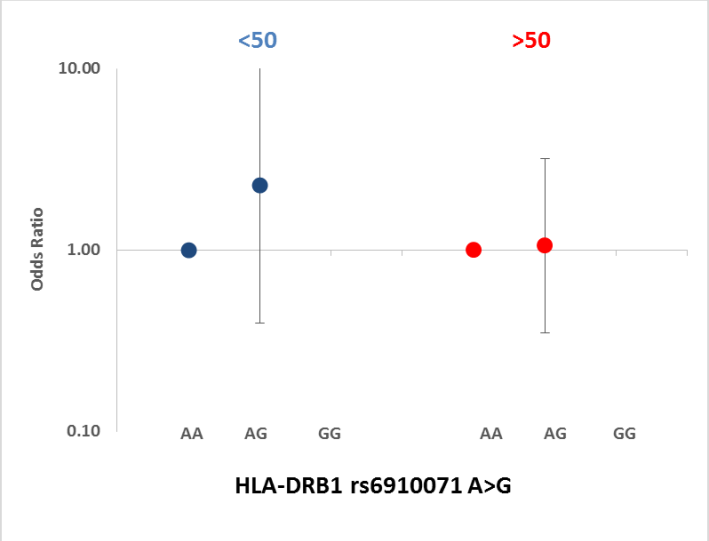

Figure 3.33. Association of HLA-DRB1 rs6910071 with ERA according to patients' age.

Regarding the genotyping for HLA-DQA2 rs9275595, there was an association of TC heterozygous (OR=1.85, 95% CI=0.45-7.69, p=0.40) in patients below 50 years old and a slight reverse association of TC heterozygous (OR=0.80, 95% CI=0.25-2.56, p=0.72) and weak association of CC homozygous (OR=1.37, 95% CI=0.11-16.19, p=0.82) in patients above 50 years old with ERA development. However, none of the observed associations were significant (Figure 3.34).

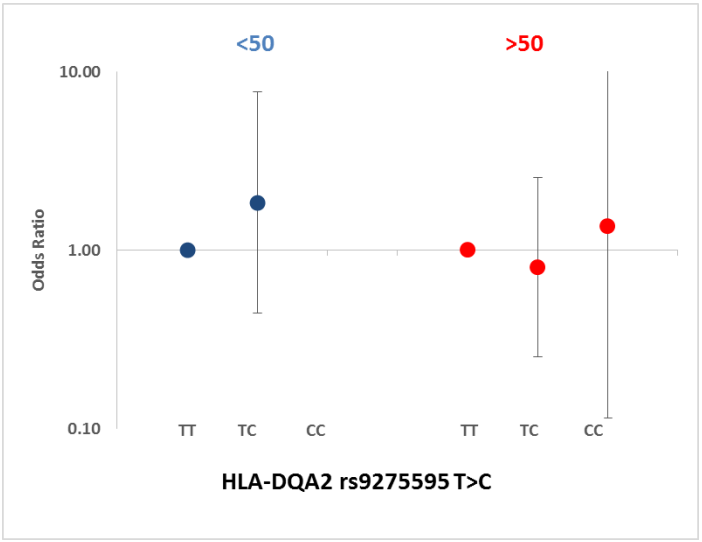

Figure 3.34. Association of HLA-DQA2 rs9275595 with ERA according to patients' age.

As shown in figure 3.35, HLA-DQB2 rs10807113 demonstrated an association for CA heterozygous (OR=1.50, 95% CI=0.36-6.23, p=0.59) and also an almost null association of AA homozygous (OR=0.83, 95% CI=0.07-10.60, p=0.90) in patients below 50 years old. There were a null association in CA heterozygous (OR=1.00, 95% CI=0.34-2.94, p=0.59) and a reverse association in AA homozygous (OR=0.56, 95% CI=0.10-3.04, p=0.51) in patients above 50 years old with ERA development. However, none of the observed associations were significant.

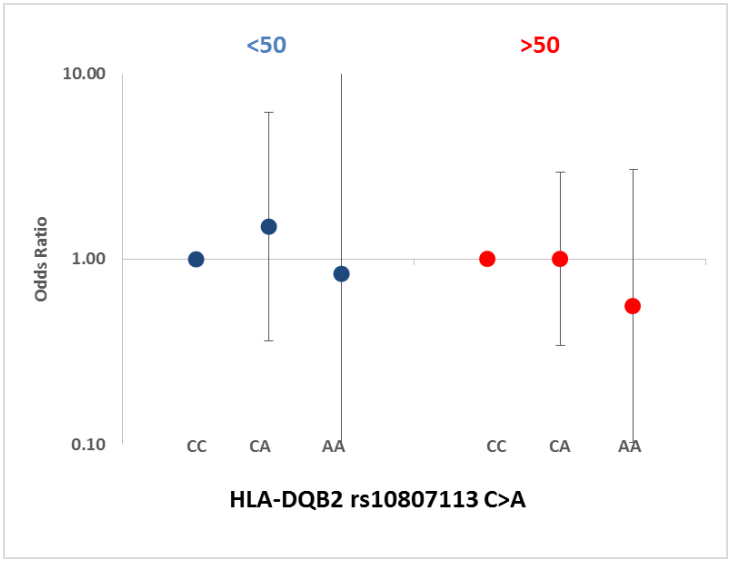

Figure 3.35. Association of HLA-DQB2 rs10807113 with ERA according to patients' age.

The results of genotyping for PTPN22 rs2476601 showed a reverse association of AG heterozygous (OR=0.35, 95% CI=0.04-3.36, p=0.37) in patients below 50 years old and an association of AG heterozygous (OR=2.37, 95% CI=0.48-11.62, p=0.29) in patients above 50 years old with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. However, none of the observed associations were significant (Figure 3.36).

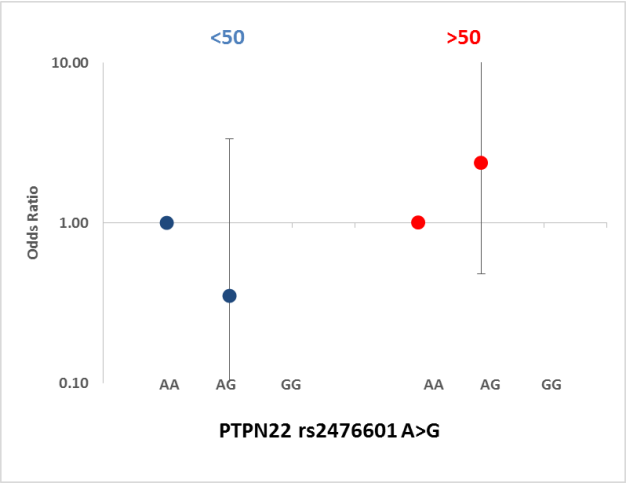

Figure 3.36. Association of PTPN22 rs2476601 with ERA according to patients' age.

As presented in figure 3.37, PADI4 rs2240340 had associations for TC heterozygous (OR=3.21, 95% CI=0.57-18.20, p=0.19) and CC homozygous (OR=1.80, 95% CI=0.19-16.98, p=0.62) in patients below 50 years old and lack of association for TC heterozygous (OR=1.00, 95% CI=0.32-3.13, p=1.00) and an association for CC homozygous (OR=1.21, 95% CI=0.23-6.27, p=0.83) in patients above 50 years old with ERA development. However, none of the observed associations were significant.

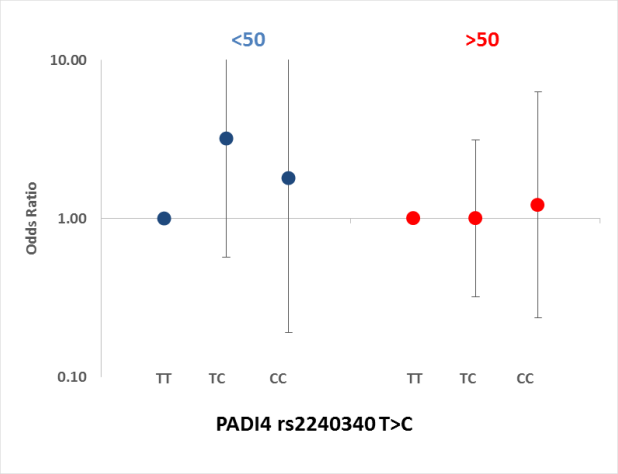

Figure 3.37. Association of PADI4 rs2240340 with ERA according to patients' age.

Regarding the genotyping for STAT4 rs7574865, there was a reverse association of TG heterozygous (OR=0.75, 95% CI=0.19-2.89, p=0.69) in patients below 50 years old and a trend of reverse association for TG heterozygous (OR=0.46, 95% CI=0.12-1.79, p=0.27) and GG homozygous (OR=0.33, 95% CI=0.04-2.87, p=0.32) in patients above 50 years old with ERA development. Due to lack of GG homozygous among younger patients, assessing associations were not possible. However, none of the observed associations were significant (Figure 3.38).

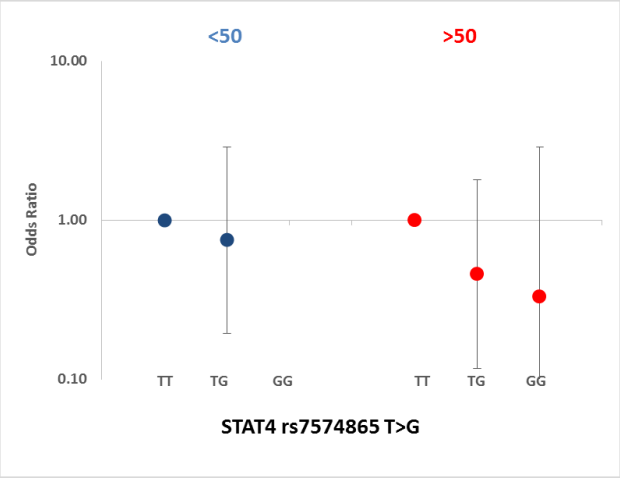

Figure 3.38. Association of STAT4 rs7574865 with ERA according to patients' age.

The results of genotyping for CTLA4 rs231775 showed almost null association of AG heterozygous (OR=0.83, 95% CI=0.19-3.58, p=0.82) and GG homozygous (OR=0.94, 95% CI=0.14-6.28, p=0.95) in patients below 50 years old and associations of AG heterozygous (OR=1.59, 95% CI=0.56-4.54, p=0.39) and GG homozygous (OR=1.47, 95% CI=0.24-8.85, p=0.69) in patients above 50 years old with ERA development. However, none of the observed associations were significant (Figure 3.39).

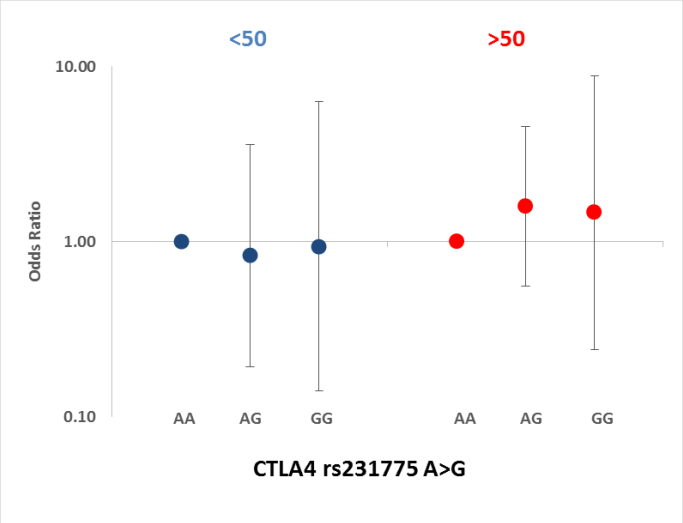

Figure 3.39. Association of CTLA4 rs231775 with ERA according to patients' age.

As shown in figure 3.40, TRAF1 rs3761847 demonstrated a reverse association for GA heterozygous (OR=0.18, 95% CI=0.03-1.02, p=0.052) and also an almost null association of AA homozygous (OR=0.80, 95% CI=0.11-5.77, p=0.84) in patients below 50 years old. There were a null association in GA heterozygous (OR=1.03, 95% CI=0.36-2.95, p=0.97) and a reverse association in AA homozygous (OR=0.53, 95% CI=0.10-2.92, p=0.48) in patients above 50 years old with ERA development. However, none of the observed associations were significant.

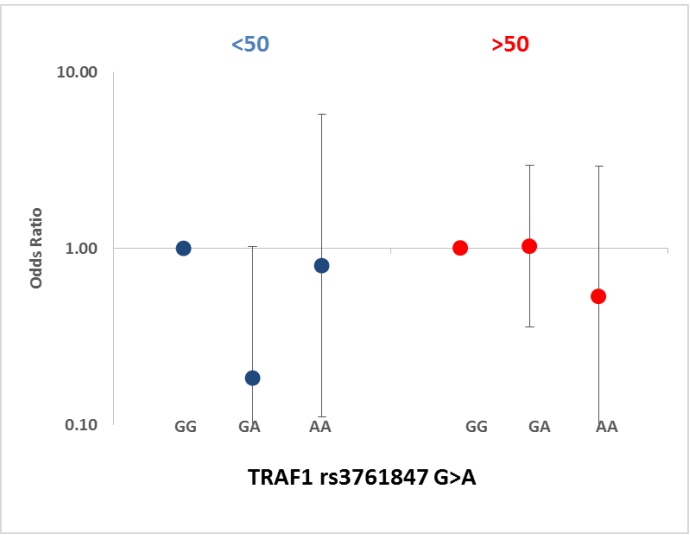

Figure 3.40. Association of TRAF1 rs3761847 with ERA according to patients' age.

Regarding the genotyping for IL-10 rs1800871, there were reverse associations of AG heterozygous (OR=0.33, 95% CI=0.06-1.88, p=0.21) and GG homozygous (OR=0.60, 95% CI=0.10-3.72, p=0.59) in patients below 50 years old and a null association for AG heterozygous (OR=0.91, 95% CI=0.31-2.71, p=0.88) and an association of GG homozygous (OR=1.80, 95% CI=0.37-8.79, p=0.48) in patients above 50 years old with ERA development. However, none of the observed associations were significant (Figure 3.41).

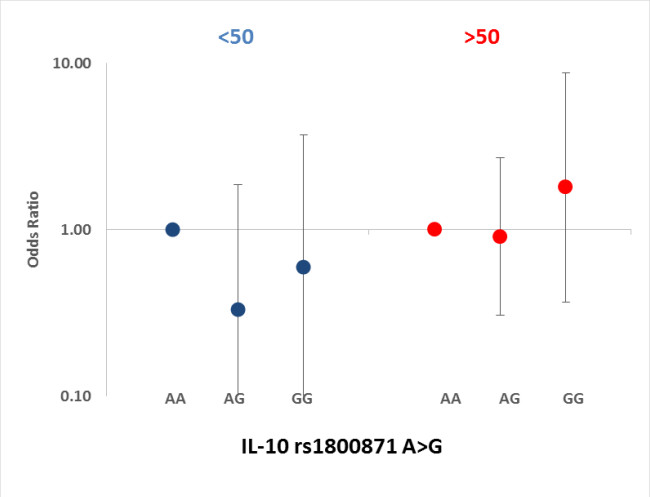

Figure 3.41. Association of IL-10 rs1800871 with ERA according to patients' age.

As presented in figure 3.42, IL-6 rs1800795 had an association for CG heterozygous (OR=1.27, 95% CI=0.34-4.73, p=0.73) in patients below 50 years old and a reverse association for CG heterozygous (OR=0.67, 95% CI=0.22-2.04, p=0.49) and a null association for GG homozygous (OR=1.04, 95% CI=0.17-6.26, p=0.97) in patients above 50 years old with ERA development. Due to lack of GG homozygous among younger patients, assessing associations were not possible. Beside, none of the observed associations were significant.

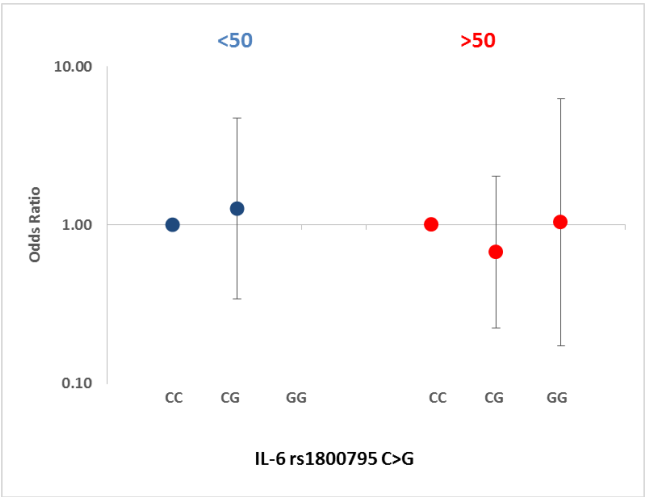

Figure 3.42. Association of IL-6 rs1800795 with ERA according to patients' age.

Early Rheumatoid Arthritis Development-Smoking

The results of genotyping for 14 candidate SNPs stratified by smoking exposure are presented in Table 3.5 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	non-Smoker			Smoker			non-Smoker			Smoker		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	50	12	0	22	4	0	18	1	0	13	3	0
HLA-G rs1063320 C>G	26	25	11	9	14	4	10	5	4	3	10	2
HLA-G 14bp In/del	18	25	20	8	13	6	6	6	7	4	9	3
HLA-DRB1 rs660895 A>G	46	14	2	16	3	0	22	4	0	11	4	1
HLA-DRB1 rs6910071 A>G	46	15	2	21	4	1	14	5	0	12	4	0
HLA-DQA2 rs9275595 T>C	42	16	4	19	7	0	13	5	1	11	5	0
HLA-DQB2 rs10807113 C>A	27	24	12	8	16	1	6	11	2	7	7	1
PTPN22 rs2476601 A>G	58	5	0	22	4	0	16	3	0	15	1	0
PADI4 rs2240340 T>C	17	36	8	9	12	4	5	12	2	3	10	3
STAT4 rs7574865 T>G	35	18	10	15	8	3	14	5	0	11	4	1
CTLA4 rs231775 A>G	35	21	7	13	11	2	8	9	2	9	5	2
TRAF1 rs3761847 G>A	25	29	8	11	10	5	9	6	4	10	6	0
IL-10 rs1800871 A>G	31	24	8	17	8	2	8	6	5	13	3	0
IL-6 rs1800795 C>G	27	27	6	13	11	2	8	10	1	9	4	1

Table 3.5. Genotyping data of non-ERA and ERA patients stratified by smoking exposure. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

As shown in figure 3.43, HLA-G rs1233334 demonstrated a reverse association for GA heterozygous (OR=0.23, 95% CI=0.03-1.91, p=0.17) in non-smokers. There was a weak association in GA heterozygous (OR=1.27, 95% CI=0.24-6.59, p=0.79) in smokers with ERA development. Due to lack of AA homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

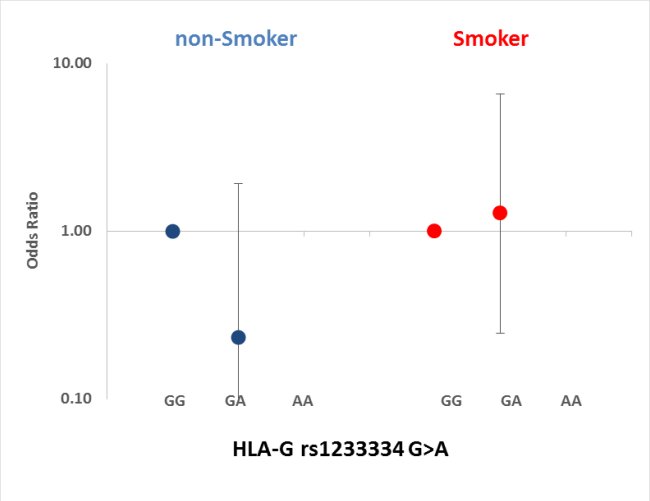

Figure 3.43. Association of HLA-G rs1233334 with ERA according to smoking exposure.

As presented in figure 3.44, HLA-G rs1063320 had a reverse association for CG heterozygous (OR=0.52, 95% CI=0.16-1.74, p=0.29) and null association of GG homozygous (OR=0.94, 95% CI=0.24-3.67, p=0.40) in non-smoker patients and also associations of CG heterozygous (OR=2.14, 95% CI=0.46-9.98, p=0.34) and GG homozygous (OR=1.50, 95% CI=0.18-12.78, p=0.72) in smokers with ERA development. However, none of the observed associations were significant.

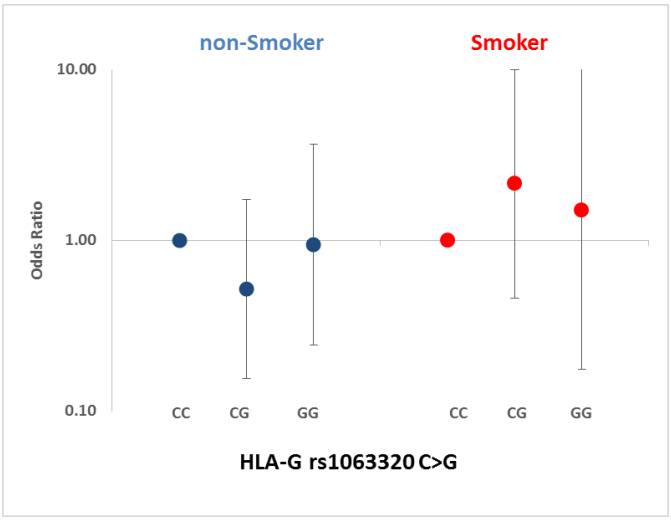

Figure 3.44. Association of HLA-G rs1063320 with ERA according to smoking exposure.

Regarding the genotyping for HLA-G 14bp In/del, there was a reverse associations of DI heterozygous (OR=0.72, 95% CI=0.20-2.60, p=0.63) and a null association of II homozygous (OR=1.05, 95% CI=0.30-3.71, p=0.94) in non-smoker patients, and an association of DI heterozygous (OR=1.38, 95% CI=0.32-6.03, p=0.68) and lack of association of II homozygous (OR=1.00, 95% CI=0.16-6.25, p=1.00) in smokers with ERA development. However, none of the observed associations were significant (Figure 3.45).

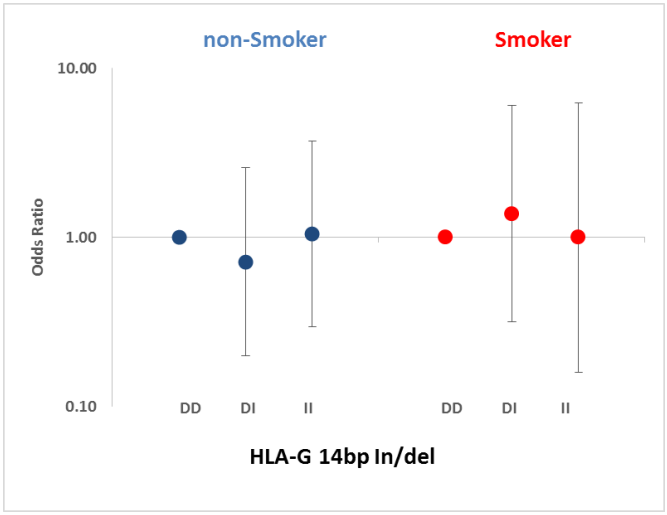

Figure 3.45. Association of HLA-G 14bp In/del with ERA according to smoking exposure.

The results of genotyping for HLA-DRB1 rs660895 showed a reverse association of AG heterozygous (OR=0.62, 95% CI=0.16-2.43, p=0.50) in non-smoker patients and an associations of AG heterozygous (OR=2.00, 95% CI=0.42-9.55, p=0.39) in smoker patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible However, none of the observed associations were significant (Figure 3.46).

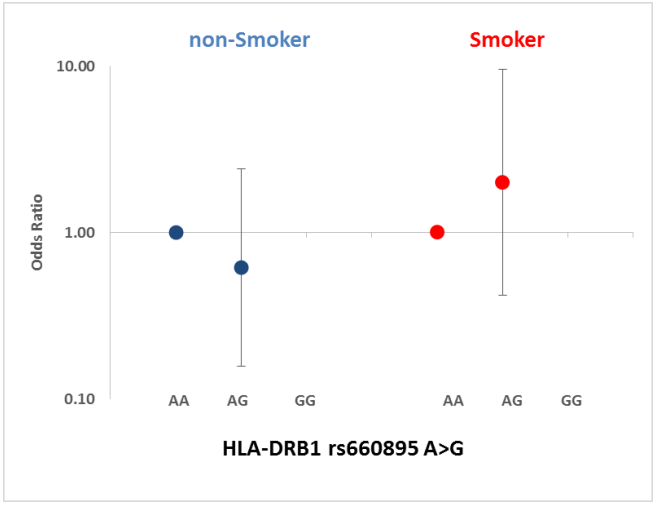

Figure 3.46. Association of HLA-DRB1 rs660895 with ERA according to smoking exposure.

As shown in figure 3.47, HLA-DRB1 rs6910071 demonstrated a null association for AG heterozygous (OR=1.09, 95% CI=0.34-3.55, p=0.89) in non-smokers. There was an association in AG heterozygous (OR=1.75, 95% CI=0.37-8.30, p=0.49) in smokers with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

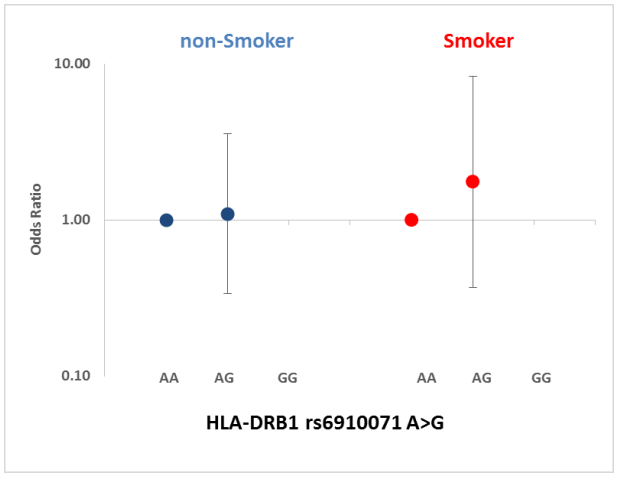

Figure 3.47. Association of HLA-DRB1 rs6910071 with ERA according to smoking exposure.

As presented in figure 3.48, HLA-DQA2 rs9275595 had a null association for TC heterozygous (OR=1.01, 95% CI=0.31-3.29, p=0.99) and a weak reverse association of CC homozygous (OR=0.81, 95% CI=0.83-7.88, p=0.86) in non-smoker patients and a slight associations of TC heterozygous (OR=1.23, 95% CI=0.31-4.84, p=0.78) in smokers with ERA development. Because of lacking of CC homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

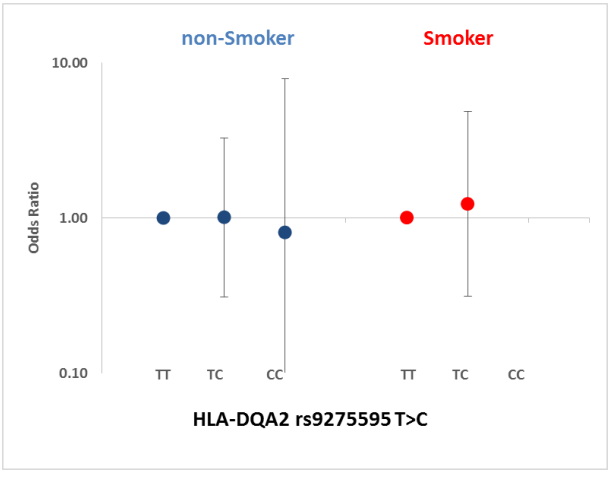

Figure 3.48. Association of HLA-DQA2 rs9275595 with ERA according to smoking exposure.

Regarding the genotyping for HLA-DQB2 rs10807113, there was a associations of CA heterozygous (OR=2.06, 95% CI=0.66-6.43, p=0.21) and a reverse association of AA homozygous (OR=0.75, 95% CI=0.13-4.27, p=0.76) in non-smoker patients, and a reverse association of CA heterozygous (OR=0.50, 95% CI=0.13-1.92, p=0.32) and almost lack of association of AA homozygous (OR=1.14, 95% CI=0.06-21.87, p=0.93) in smokers with ERA development. However, none of the observed associations were significant (Figure 3.49).

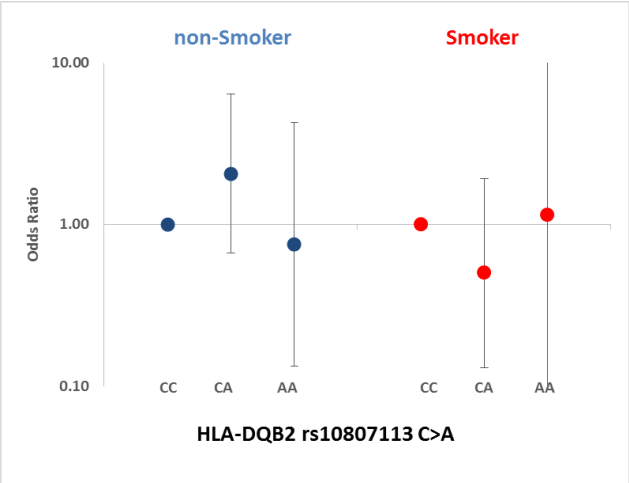

Figure 3.49. Association of HLA-DQA2 rs9275595 with ERA according to smoking exposure.

The results of genotyping for PTPN2 rs247660 showed an association of AG heterozygous (OR=2.17, 95% CI=0.47-10.10, p=0.33) in non-smoker patients and a reverse associations of AG heterozygous (OR=0.37, 95% CI=0.04-3.61, p=0.40) in smoker patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible However, none of the observed associations were significant (Figure 3.50).

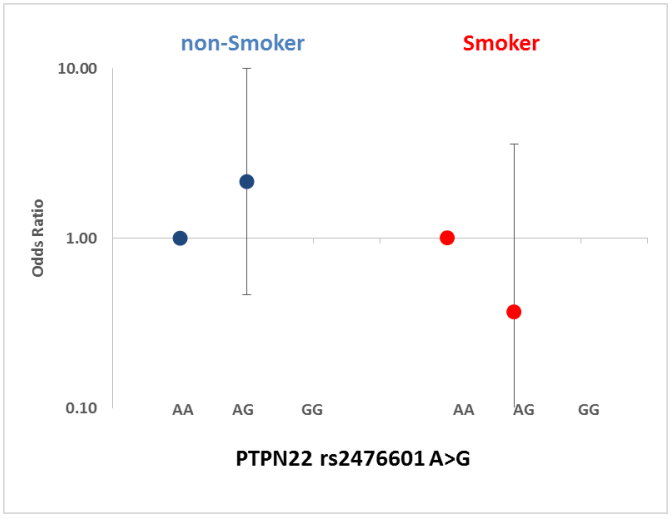

Figure 3.50. Association of PTPN22 rs247660 with ERA according to smoking exposure.

As shown in figure 3.51, PADI4 rs2240340 demonstrated an almost null associations for TC heterozygous (OR=1.13, 95% CI=0.34-3.73, p=0.85) and CC homozygous (OR=0.85, 95% CI=0.13-5.37, p=0.87) in non-smokers. There were associations of TC heterozygous (OR=2.50, 95% CI=0.53-11.81, p=0.25) and CC homozygous (OR=2.25, 95% CI=0.31-16.41, p=0.43) in smokers with ERA development. However, none of the observed associations were significant.

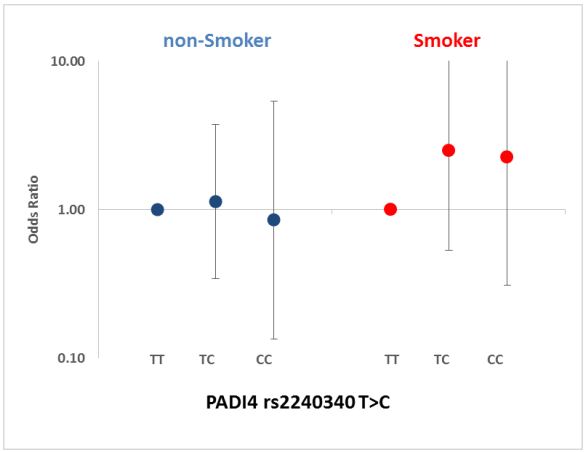

Figure 3.51. Association of PADI4 rs2240340 with ERA according to smoking exposure.

As presented in figure 3.52, STAT4 rs7574865 had a reverse association of TG heterozygous (OR=0.69, 95% CI=0.22-2.23, p=0.55) in non-smoker patients. There was a trend of reverse association of TG homozygous (OR=0.68, 95% CI=0.16-2.85, p=0.61) and GG heterozygous (OR=0.45, 95% CI=0.04-4.98, p=0.53) in smokers with ERA development. Because of lacking of GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

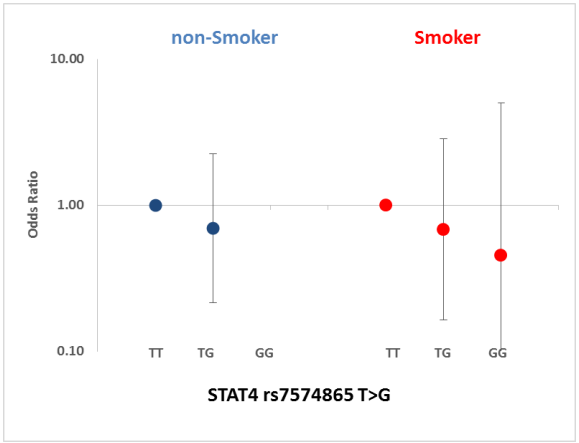

Figure 3.52. Association of STAT4 rs7574865 with ERA according to smoking exposure.

Regarding the genotyping for CTLA4 rs231775, there was an association of AG heterozygous (OR=1.87, 95% CI=0.63-5.61, p=0.26) and an almost null association of GG homozygous (OR=1.25, 95% CI=0.22-7.19, p=0.81) in non-smoker patients, and a reverse association of AG heterozygous (OR=0.66, 95% CI=0.17-2.55, p=0.55) and an association of GG homozygous (OR=1.44, 95% CI=0.17-12.32, p=0.75) in smokers with ERA development. None of the observed associations were significant (Figure 3.53).

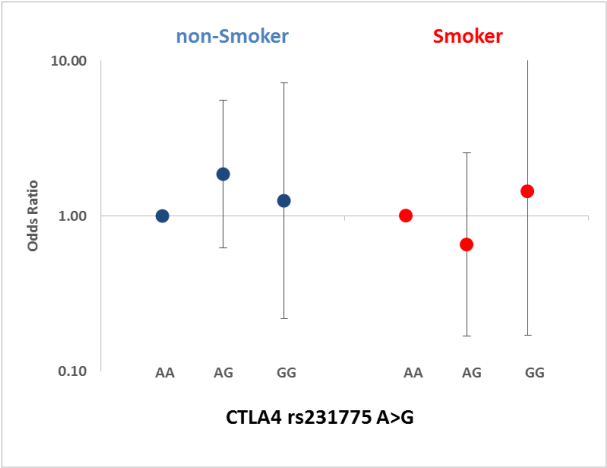

Figure 3.53. Association of CTLA4 rs231775 with ERA according to smoking exposure.

The results of genotyping for TRAF1 rs3761847 showed a reverse association of GA heterozygous (OR=0.57, 95% CI=0.18-1.84, p=0.36) and an association of AA homozygous (OR=1.39, 95% CI=0.33-5.76, p=0.66) in non-smoker patients and a reverse associations of GA heterozygous (OR=0.66, 95% CI=0.17-2.48, p=0.55) in smoker patients with ERA development. Due to lack of AA homozygous patients, assessing associations were not possible. However, none of the observed associations were significant (Figure 3.54).

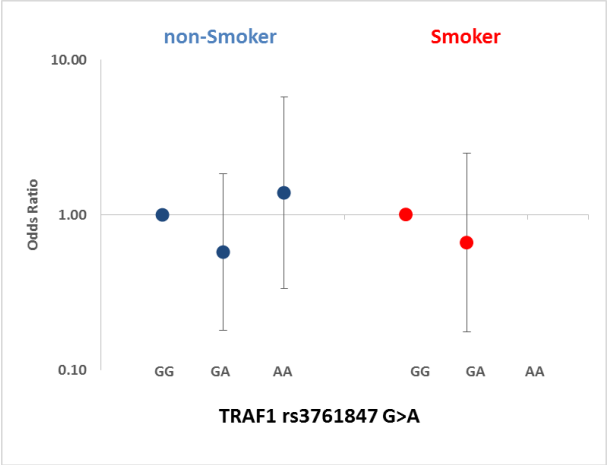

Figure 3.54. Association of TRAF1 rs3761847 with ERA according to smoking exposure.

As shown in figure 3.55, IL-10 rs1800871 demonstrated an almost null associations for AG heterozygous (OR=0.97, 95% CI=0.30-3.17, p=0.96) and an association of GG homozygous (OR=2.42, 95% CI=0.62-9.45, p=0.20) in non-smokers. There was a reverse associations of AG heterozygous (OR=0.49, 95% CI=0.11-2.22, p=0.36) in smokers with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

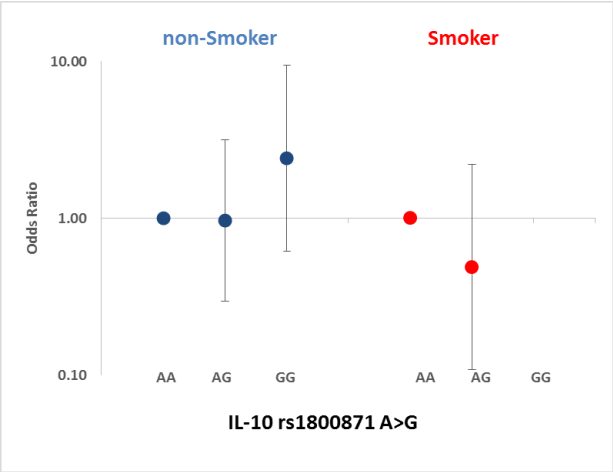

Figure 3.55. Association of IL-10 rs1800871 with ERA according to smoking exposure.

As presented in figure 3.56, IL-6 rs1800795 had an almost null association of CG heterozygous (OR=1.25, 95% CI=0.43-3.65, p=0.70) and a reverse association of GG homozygous (OR=0.56, 95% CI=0.06-5.39, p=0.63) in non-smoker patients. There were reverse associations of TG homozygous (OR=0.52, 95% CI=0.13-2.18, p=0.38) and GG heterozygous (OR=0.72, 95% CI=0.06-9.22, p=0.81) in smokers with ERA development. None of the observed associations were significant.

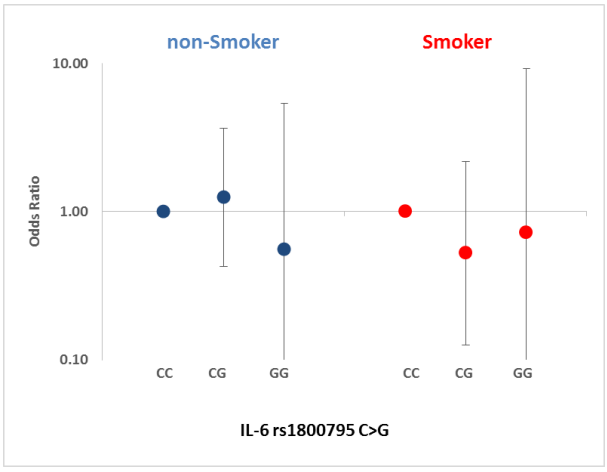

Figure 3.56. Association of IL-6 rs1800795 with ERA according to smoking exposure.

Early Rheumatoid Arthritis Development-BMI

The results of genotyping for 14 candidate SNPs stratified by BMI (<30 and >30) are presented in Table 3.6 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	BMI< 30			BMI> 30			BMI< 30			BMI> 30		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	61	14	0	11	2	0	24	3	0	7	1	0
HLA-G rs1063320 C>G	29	36	11	6	3	4	12	9	6	1	6	0
HLA-G 14bp In/del	21	36	20	5	2	6	8	11	8	2	4	2
HLA-DRB1 rs660895 A>G	58	16	1	10	2	1	22	4	1	5	3	0
HLA-DRB1 rs6910071 A>G	59	15	2	8	4	1	19	8	0	7	1	0
HLA-DQA2 rs9275595 T>C	52	20	3	9	3	1	19	7	1	5	3	0
HLA-DQB2 rs10807113 C>A	28	36	11	7	4	2	7	17	2	6	1	1
PTPN22 rs2476601 A>G	67	9	0	13	0	0	25	2	0	6	2	0
PADI4 rs2240340 T>C	21	40	12	5	8	0	6	17	4	2	5	1
STAT4 rs7574865 T>G	44	22	10	6	4	3	19	8	0	6	1	1
CTLA4 rs231775 A>G	42	26	8	6	6	1	14	11	2	3	3	2
TRAF1 rs3761847 G>A	32	33	10	4	6	3	15	9	3	4	3	1
IL-10 rs1800871 A>G	42	28	7	6	4	3	14	8	5	7	1	0
IL-6 rs1800795 C>G	36	30	7	4	8	1	15	9	2	2	5	0

Table 3.6. Genotyping data of non-ERA and ERA patients stratified by BMI. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

As shown in figure 3.57, HLA-G rs1233334 demonstrated a reverse association for GA heterozygous (OR=0.54, 95% CI=0.14-2.07, p=0.38) in patients with BMI below 30 kg/m^2. There was a weak association in GA heterozygous (OR=0.79, 95% CI=0.06-10.38, p=0.86) in patients with BMI above 30 kg/m^2 with ERA development. Due to lack of AA homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

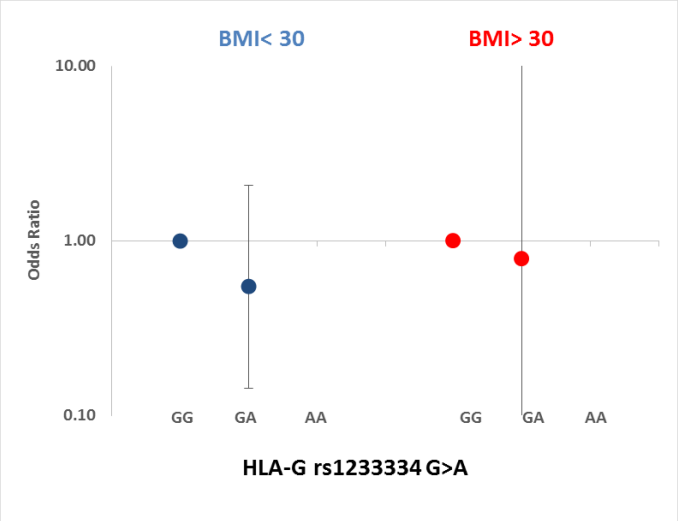

Figure 3.57. Association of HLA-G rs1233334 with ERA according to patients' BMI.

As presented in figure 3.58, HLA-G rs1063320 had a reverse association for CG heterozygous (OR=0.60, 95% CI=0.22-1.63, p=0.32) and almost null association of GG homozygous (OR=1.32, 95% CI=0.40-4.38, p=0.66) in patients with BMI below 30 kg/m^2 and 12-fold association of CG heterozygous (OR=12.00, 95% CI=0.96-150.69, p=0.05) in patients with BMI above 30 kg/m^2 with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

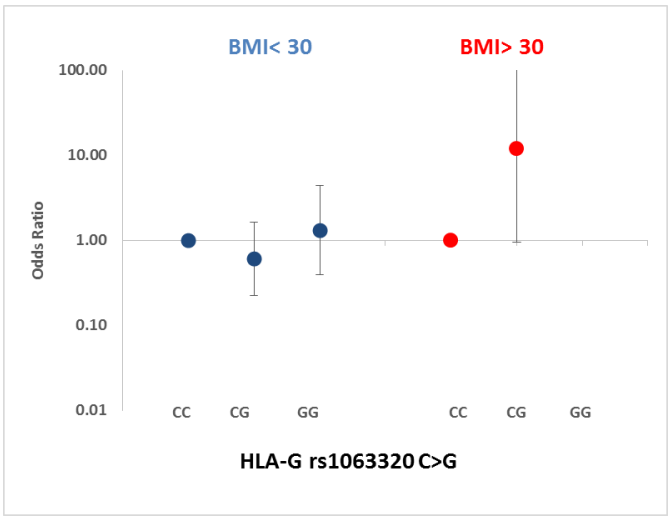

Figure 3.58. Association of HLA-G rs1063320 with ERA according to patients' BMI.

Regarding the genotyping for HLA-G 14bp In/del, there was a slight reverse associations of DI heterozygous (OR=0.80, 95% CI=0.28-2.31, p=0.70) and a null association of II homozygous (OR=1.05, 95% CI=0.33-3.33, p=0.94) in patients with BMI below 30 kg/m^2, and 5-fold association of DI heterozygous (OR=5.00, 95% CI=0.47-52.96, p=0.18) and slight reverse association of II homozygous (OR=0.83, 95% CI=0.08-8.24, p=0.88) in patients with BMI above 30 kg/m^2 with ERA development. However, none of the observed associations were significant (Figure 3.59).

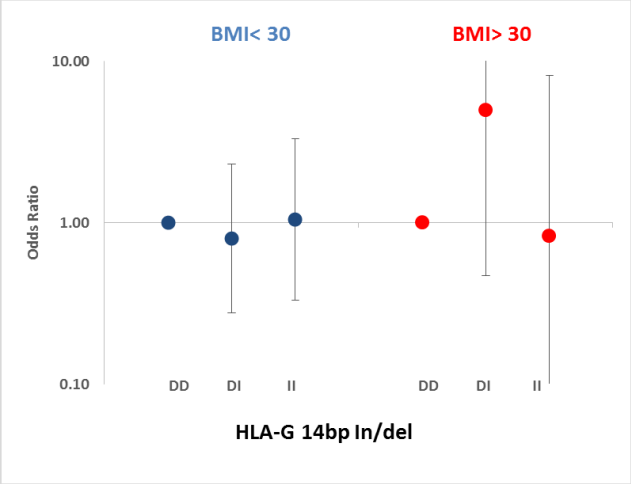

Figure 3.59. Association of HLA-G 14bp In/del with ERA according to patients' BMI.

The results of genotyping for HLA-DRB1 rs660895 showed a reverse association of AG heterozygous (OR=0.66, 95% CI=0.20-2.19, p=0.50) and an association of GG homozygous (OR=2.64, 95% CI=0.16-44.01, p=0.51) in patients with BMI below 30 kg/m^2, and an associations of AG heterozygous (OR=3.00, 95% CI=0.37-24.17, p=0.31) in patients with BMI above 30 kg/m^2, with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible However, none of the observed associations were significant (Figure 3.60).

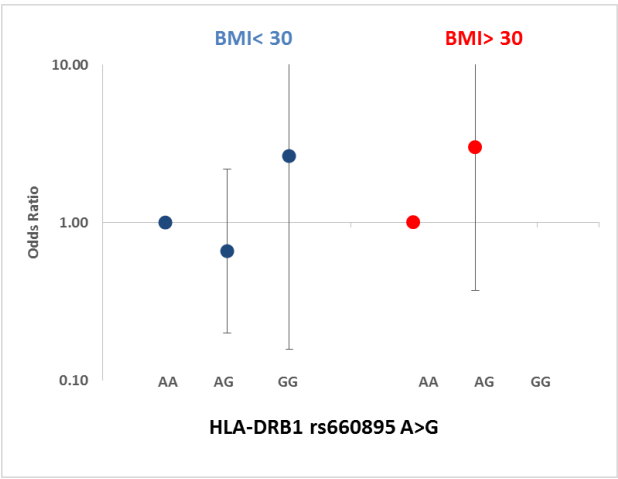

Figure 3.60. Association of HLA-DRB1 rs660895 with ERA according to patients' BMI.

As shown in figure 3.61, HLA-DRB1 rs6910071 demonstrated an association for AG heterozygous (OR=1.66, 95% CI=0.61-4.51, p=0.33) in patients with BMI below 30 kg/m^2. There was a reverse association in AG heterozygous (OR=0.29, 95% CI=0.03-3.20, p=0.31) in patients with BMI above 30 kg/m^2 with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant.

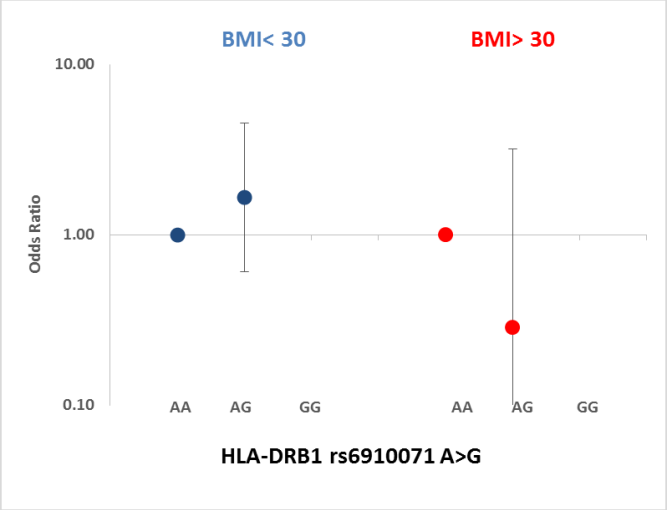

Figure 3.61. Association of HLA-DRB1 rs6910071 with ERA according to patients' BMI.

As presented in figure 3.62, HLA-DQA2 rs9275595 had almost null associations for TC heterozygous (OR=0.96, 95% CI=0.35-2.63, p=0.94) and CC homozygous (OR=0.91, 95% CI=0.90-9.31, p=0.94) in patients with BMI below 30 kg/m^2, and an association of TC heterozygous (OR=1.80, 95% CI=0.26-12.50, p=0.56) in patients with BMI above 30 kg/m^2 with ERA development. Because of lacking CC homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

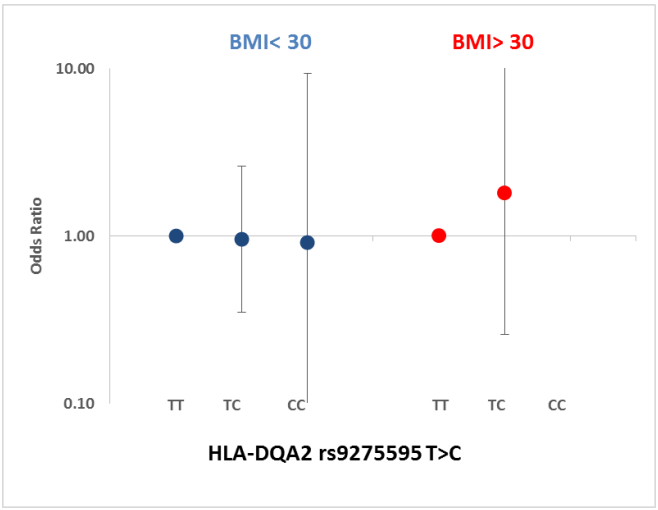

Figure 3.62. Association of HLA-DQA2 rs9275595 with ERA according to patients' BMI.

Regarding the genotyping for HLA-DQB2 rs10807113, there was an association of CA heterozygous (OR=1.89, 95% CI=0.69-5.18, p=0.22) and a reverse association of AA homozygous (OR=0.73, 95% CI=0.13-4.06, p=0.73) in patients with BMI below 30 kg/m^2, and reverse associations of CA heterozygous (OR=0.29, 95% CI=0.02-3.37, p=0.33) and AA homozygous (OR=0.58, 95% CI=0.04-8.15, p=0.70) in patients with BMI above 30 kg/m^2 with ERA development. However, none of the observed associations were significant (Figure 3.63).

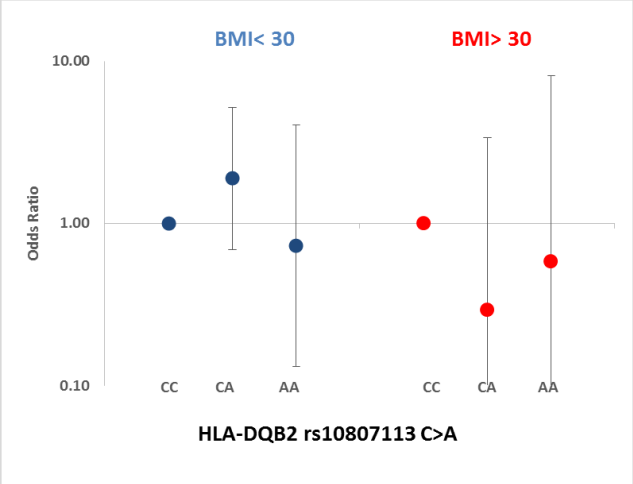

Figure 3.63. Association of HLA-DQB2 rs10807113 with ERA according to patients' BMI.

The results of genotyping for PTPN2 rs247660 showed a reverse association of AG heterozygous (OR=0.60, 95% CI=0.12-2.95, p=0.54) in patients with BMI below 30 kg/m^2 with ERA development. As shown in figure 3.64, due to lack of other genotypes, assessing associations were not possible. The observed association was not significant.

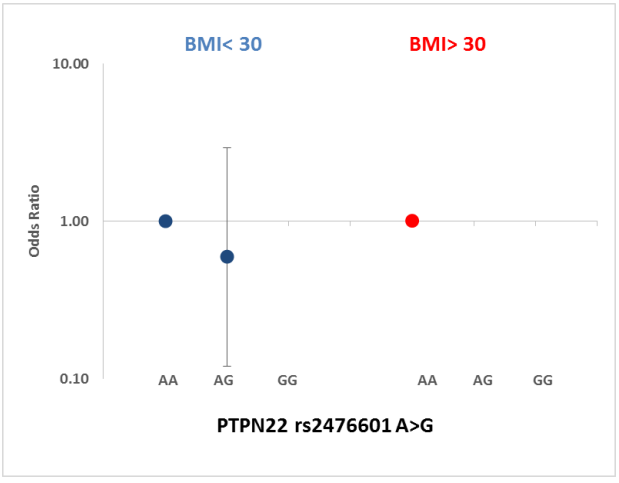

Figure 3.64. Association of PTPN22 rs2476601 with ERA according to patients' BMI.

As shown in figure 3.65, PADI4 rs2240340 demonstrated an association for TC heterozygous (OR=1.49, 95% CI=0.51-4.34, p=0.48) and almost null association of CC homozygous (OR=1.17, 95% CI=0.27-4.98, p=0.85) in patients with BMI below 30 kg/m^2. There was association of TC heterozygous (OR=1.56, 95% CI=0.21-11.37, p=0.67) in patients with BMI above 30 kg/m^2 with ERA development. Because of lacking CC homozygous assessing association was not possible. None of the observed associations were significant.

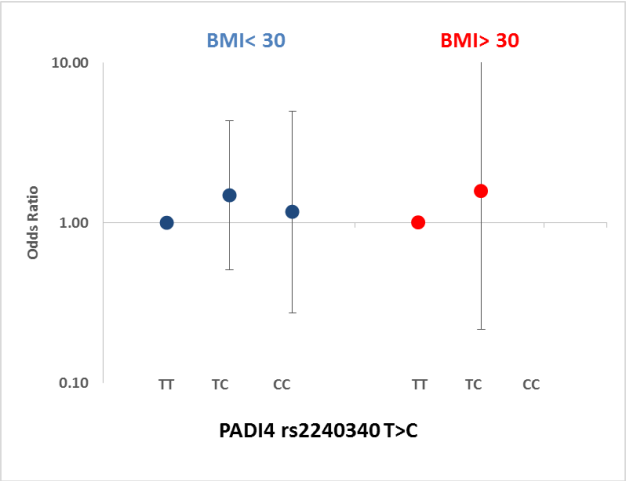

Figure 3.65. Association of PADI4 rs2240340 with ERA according to patients' BMI.

As presented in figure 3.66, STAT4 rs7574865 had an almost null association of TG heterozygous (OR=0.84, 95% CI=0.32-2.22, p=0.74) in patients with BMI below 30 kg/m^2. Because of lacking of GG homozygous patients, assessing association was not possible. There were reverse associations of TG homozygous (OR=0.25, 95% CI=0.02-2.94, p=0.27) and GG heterozygous (OR=0.33, 95% CI=0.03-4.19, p=0.40) in patients with BMI above 30 kg/m^2 with ERA development. None of the observed associations were significant.

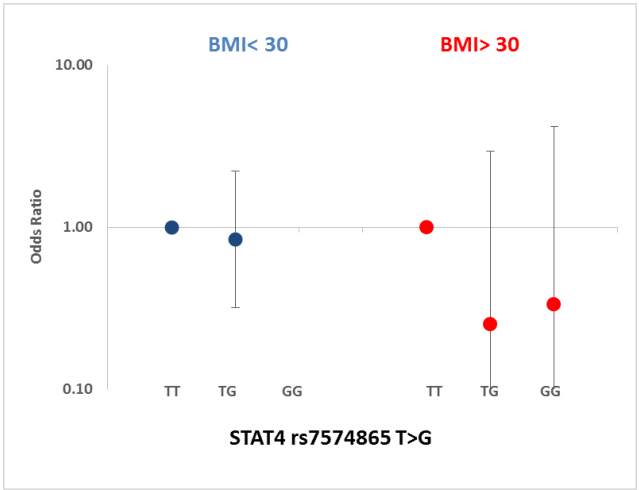

Figure 3.66. Association of STAT4 rs7574865 with ERA according to patients' BMI.

Regarding the genotyping for CTLA4 rs231775, there were almost null associations of AG heterozygous (OR=1.27, 95% CI=0.50-3.21, p=0.63) and GG homozygous (OR=0.75, 95% CI=0.14-3.96, p=0.75) in patients with BMI below 30 kg/m^2, and also null association of AG heterozygous (OR=1.00, 95% CI=0.14-7.10, p=1.00) and a 4-fold association of GG homozygous (OR=4.00, 95% CI=0.25-63.95, p=0.33) in patients with BMI above 30 kg/m^2 with ERA development. None of the observed associations were significant (Figure 3.67).

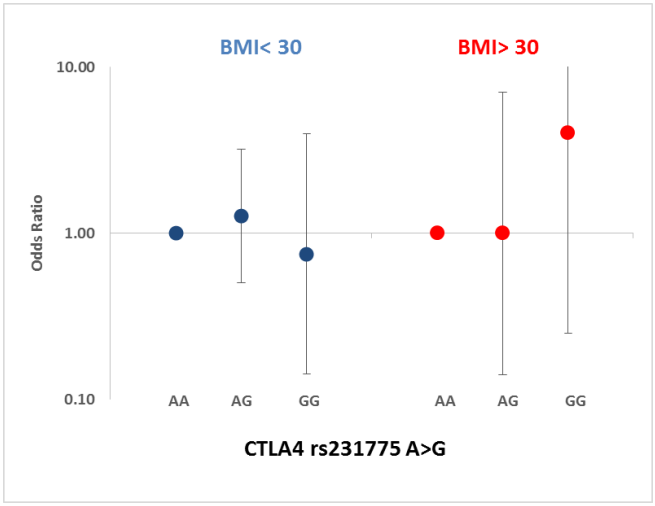

Figure 3.67. Association of CTLA4 rs231775 with ERA according to patients' BMI.

The results of genotyping for TRAF1 rs3761847 showed reverse associations of GA heterozygous (OR=0.58, 95% CI=0.22-1.52, p=0.27) and AA homozygous (OR=0.64, 95% CI=0.15-2.67, p=0.55) in patients with BMI below 30 kg/m^2 and a trend of reverse associations of GA heterozygous (OR=0.50, 95% CI=0.07-3.55, p=0.50) and AA homozygous (OR=0.33, 95% CI=0.02-4.74, p=0.42) in patients with BMI above 30 kg/m^2 with ERA development. However, none of the observed associations were significant (Figure 3.68).

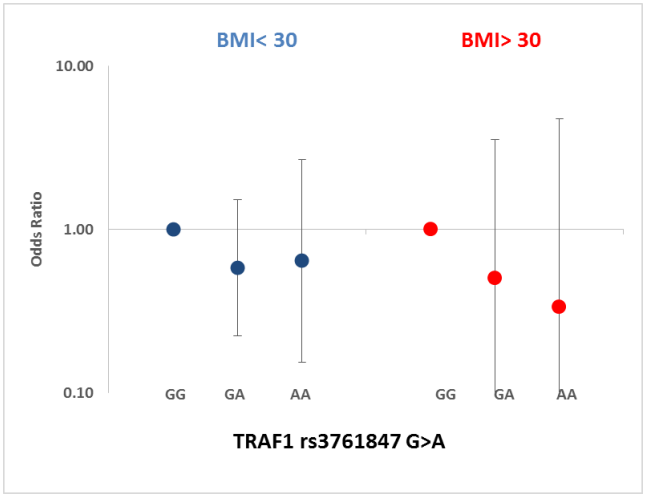

Figure 3.68. Association of TRAF1 rs3761847 with ERA according to patients' BMI.

As shown in figure 3.69, IL-10 rs1800871 demonstrated an almost null associations for AG heterozygous (OR=0.86, 95% CI=0.32-2.31, p=0.77) and an association of GG homozygous (OR=2.14, 95% CI=0.59-7.84, p=0.25) in patients with BMI below 30 kg/m^2. There was a reverse associations of AG heterozygous (OR=0.21, 95% CI=0.02-2.48, p=0.22) in patients with BMI above 30 kg/m^2 with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant.

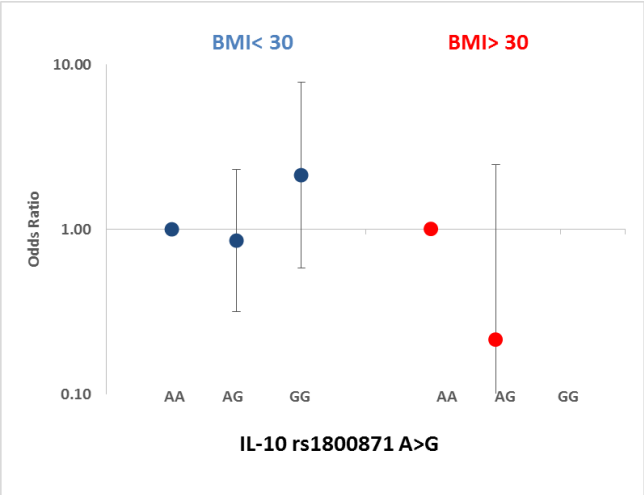

Figure 3.69. Association of IL-10 rs1800871 with ERA according to patients' BMI.

As presented in figure 3.70, IL-6 rs1800795 had reverse associations of CG heterozygous (OR=0.72, 95% CI=0.28-1.88, p=0.51) and GG homozygous (OR=0.69, 95% CI=0.13-3.69, p=0.67) in patients with BMI below 30 kg/m^2. There was a slight association of TG homozygous (OR=1.25, 95% CI=0.16-9.54, p=0.84) in patients with BMI above 30 kg/m^2 with ERA development. Because of lacking of GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

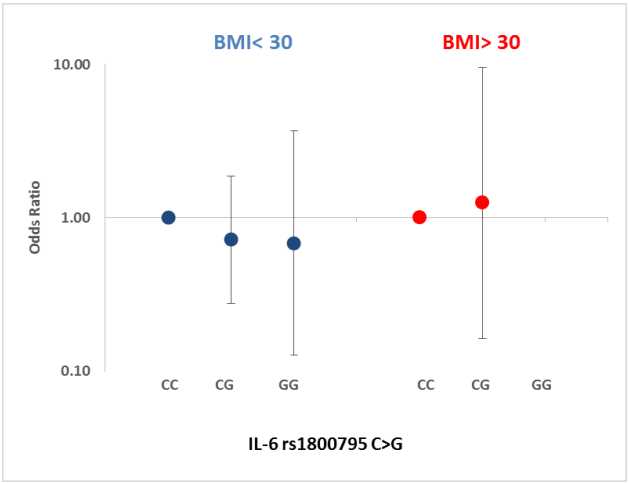

Figure 3.70. Association of IL-6 rs1800795 with ERA according to patients' BMI.

Early Rheumatoid Arthritis Development-RF

The results of genotyping for 14 candidate SNPs stratified by presence or absence of rheumatoid factor are presented in Table 3.7 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	RF-			RF+			RF-			RF+		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	53	11	0	15	5	0	21	3	0	10	1	0
HLA-G rs1063320 C>G	26	29	10	7	8	5	7	11	5	6	4	1
HLA-G 14bp In/del	19	28	19	6	8	6	9	10	5	1	5	5
HLA-DRB1 rs660895 A>G	54	9	2	12	7	0	20	3	1	7	4	0
HLA-DRB1 rs6910071 A>G	51	11	3	12	8	0	20	4	0	6	5	0
HLA-DQA2 rs9275595 T>C	46	15	4	11	8	0	18	6	0	6	4	1
HLA-DQB2 rs10807113 C>A	27	26	12	6	12	1	9	13	1	4	5	2
PTPN22 rs2476601 A>G	57	9	0	19	0	0	21	3	0	10	1	0
PADI4 rs2240340 T>C	18	38	8	8	8	2	4	16	4	4	6	1
STAT4 rs7574865 T>G	36	23	7	13	2	4	18	5	1	7	4	0
CTLA4 rs231775 A>G	36	25	5	10	6	3	13	10	1	4	4	3
TRAF1 rs3761847 G>A	26	30	9	7	8	4	12	9	3	7	3	1
IL-10 rs1800871 A>G	33	25	8	12	6	2	16	5	3	5	4	2
IL-6 rs1800795 C>G	27	30	5	10	8	2	14	6	2	3	8	0

Table 3.7. Genotyping data of non-ERA and ERA patients stratified by Rheumatoid Factor (RF). **Genotype 1**: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

As shown in figure 3.71, HLA-G rs1233334 demonstrated a reverse association for GA heterozygous (OR=0.69, 95% CI=0.17-2.72, p=0.61) in RF negative patients. There was also a reverse association in GA heterozygous (OR=0.30, 95% CI=0.03-2.97, p=0.31) in RF positive patients with ERA development. Due to lack of AA homozygous patients, assessing associations were not possible. However, none of the observed associations were significant.

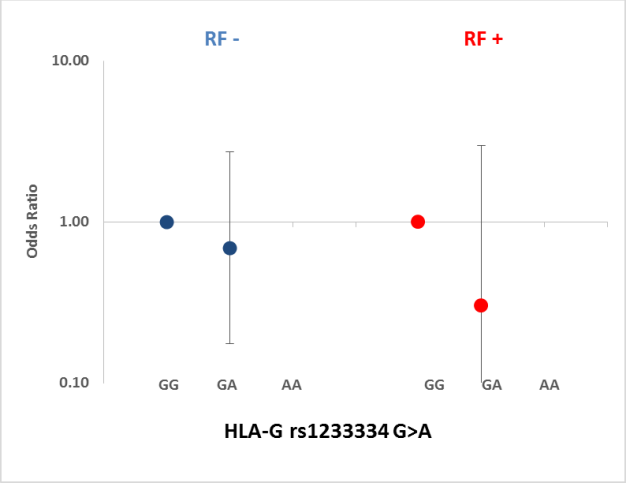

Figure 3.71. Association of HLA-G rs1233334 with ERA according to rheumatoid factor status.

As presented in figure 3.72, HLA-G rs1063320 had a trend of association for CG heterozygous (OR=1.41, 95% CI=0.48-4.17, p=0.55) and GG homozygous (OR=1.86, 95% CI=0.48-7.23, p=0.38) in RF negative patients and a trend of reverse association of CG heterozygous (OR=0.58, 95% CI=0.11-2.95, p=0.24) and GG homozygous (OR=0.23, 95% CI=0.02-2.59, p=0.24) in RF positive patients with ERA development. However, none of the observed associations were significant.

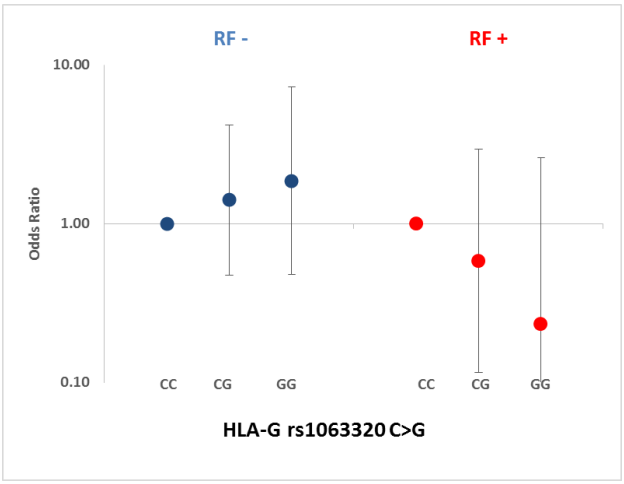

Figure 3.72. Association of HLA-G rs1063320 with ERA according to rheumatoid factor status.

Regarding the genotyping for HLA-G 14bp In/del, there was a trend of reverse associations of DI heterozygous (OR=0.75, 95% CI=0.26-2.20, p=0.62) and II homozygous (OR=0.56, 95% CI=0.16-1.97, p=0.37) in RF negative patients, and a trend of association of DI heterozygous (OR=3.75, 95% CI=0.34-41.08, p=0.28) and II homozygous (OR=5.00, 95% CI=0.44-56.63, p=0.19) in RF positive patients with ERA development. However, none of the observed associations were significant (Figure 3.73).

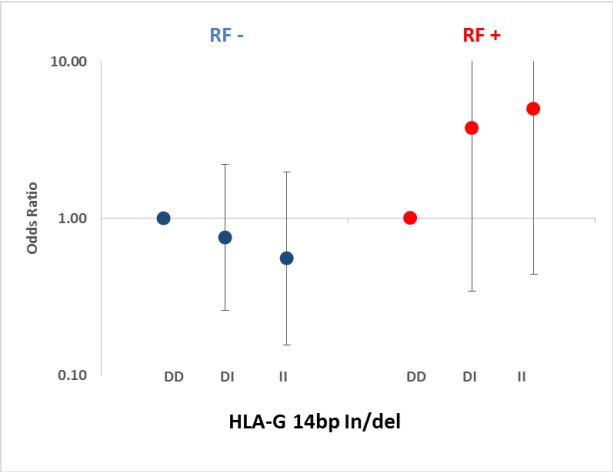

Figure 3.73. Association of HLA-G 14bp In/del with ERA according to rheumatoid factor status.

The results of genotyping for HLA-DRB1 rs660895 showed an almost null association of AG heterozygous (OR=0.90, 95% CI=0.22-3.66, p=0.89) and a slight association of GG homozygous (OR=1.35, 95% CI=0.12-15.72, p=0.82) in RF negative patients and also a null associations of AG heterozygous (OR=0.98, 95% CI=0.21-4.58, p=0.98) in RF positive patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant (Figure 3.74).

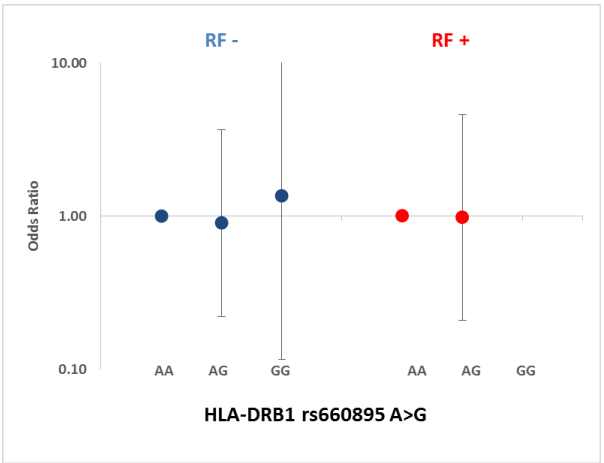

Figure 3.74. Association of HLA-DRB1 rs660895 with ERA according to rheumatoid factor status.

As shown in figure 3.75, HLA-DRB1 rs6910071 demonstrated a lack of association for AG heterozygous (OR=0.93, 95% CI=0.26-3.25, p=0.91) in RF negative patients. There was a slight association in AG heterozygous (OR=1.25, 95% CI=0.28-5.53, p=0.78) in RF positive patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant.

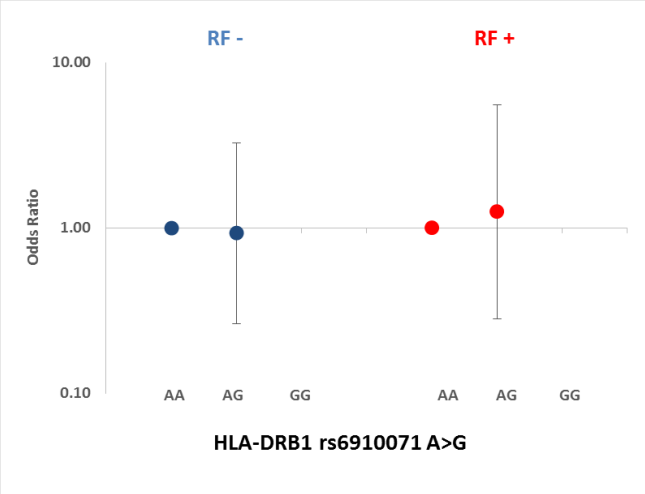

Figure 3.75. Association of HLA-DRB1 rs6910071 with ERA according to rheumatoid factor status.

As presented in figure 3.76, HLA-DQA2 rs9275595 had almost null associations for TC heterozygous (OR=1.02, 95% CI=0.34-3.05, p=0.97) in RF negative patients, and also an almost null association of TC heterozygous (OR=0.92, 95% CI=0.19-4.36, p=0.92) in RF positive patients with ERA development. Because of lacking CC homozygous patients, assessing associations were not possible. None of the observed associations were significant.

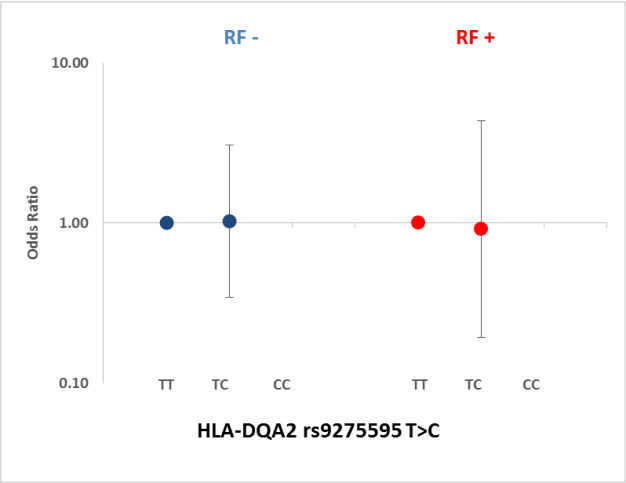

Figure 3.76. Association of HLA-DQA2 rs9275595 with ERA according to rheumatoid factor status.

Regarding the genotyping for HLA-DQB2 rs10807113, there was an association of CA heterozygous (OR=1.50, 95% CI=0.55-4.10, p=0.44) and a reverse association of AA homozygous (OR=0.25, 95% CI=0.03-2.20, p=0.21) in RF negative patients, and reverse associations of CA heterozygous (OR=0.62, 95% CI=0.12-3.22, p=0.59) and a 3-fold association of AA homozygous (OR=3.00, 95% CI=0.20-45.25, p=0.44) in RF positive patients with ERA development. However, none of the observed associations were significant (Figure 3.77).

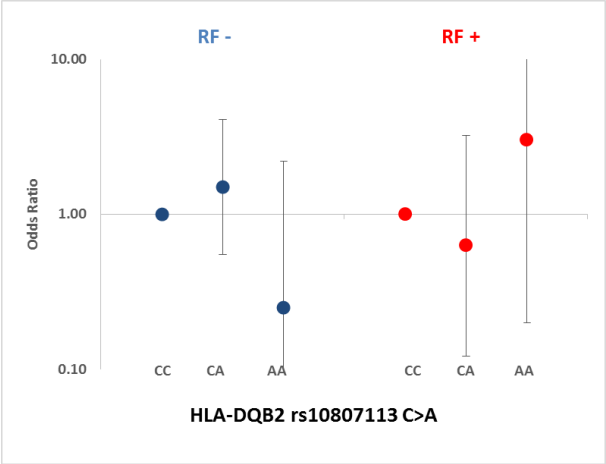

Figure 3.77. Association of HLA-DQB2 rs10807113 with ERA according to rheumatoid factor status.

The results of genotyping for PTPN2 rs247660 showed an almost null association of AG heterozygous (OR=0.90, 95% CI=0.22-3.67, p=0.90) in RF negative patients with ERA development. As shown in figure 3.78, due to lack of other genotypes, assessing associations were not possible. The observed association was not significant.

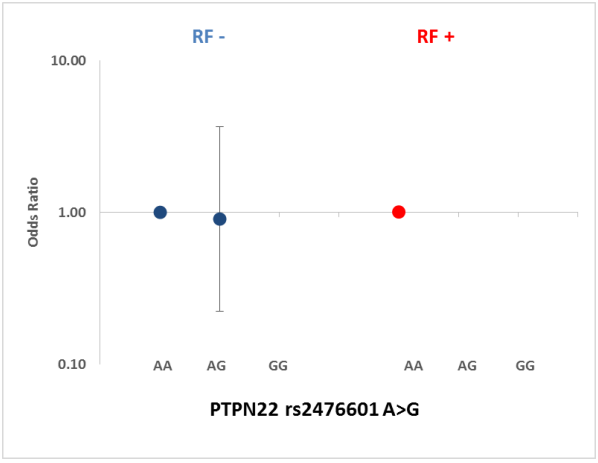

Figure 3.78. Association of PTPN22 rs2476601 with ERA according to rheumatoid factor status.

As shown in figure 3.79, PADI4 rs2240340 demonstrated a trend of associations for TC heterozygous (OR=1.89, 95% CI=0.55-6.49, p=0.31) and CC homozygous (OR=2.25, 95% CI=0.45-11.33, p=0.33) in RF negative patients. There was an association of TC heterozygous (OR=1.50, 95% CI=0.30-7.43, p=0.63) and no association for CC homozygous (OR=1.00, 95% CI=0.07-14.64, p=1.00) in RF positive patients with ERA development. None of the observed associations were significant.

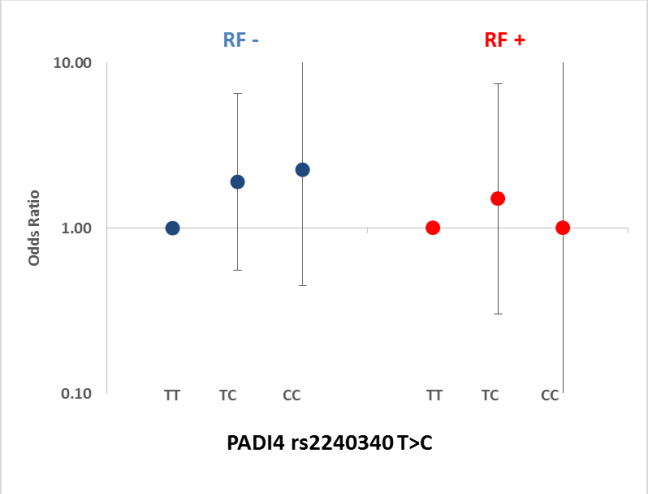

Figure 3.79. Association of PADI4 rs2240340 with ERA according to rheumatoid factor status.

As presented in figure 3.80, STAT4 rs7574865 had a trend of reverse association of TG heterozygous (OR=0.43, 95% CI=0.14-1.33, p=0.14) and GG homozygous (OR=0.29, 95% CI=0.03-2.50, p=0.26) in RF negative patients. There was an association of TG homozygous (OR=3.71, 95% CI=0.54-25.59, p=0.18) in RF positive patients with ERA development. Because of lacking of GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

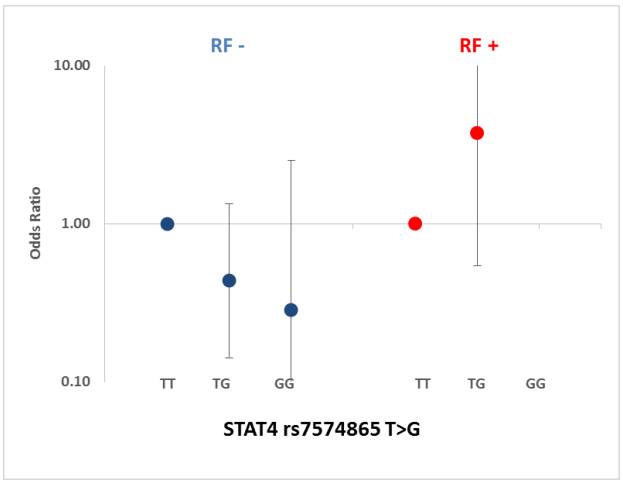

Figure 3.80. Association of STAT4 rs7574865 with ERA according to rheumatoid factor status.

Regarding the genotyping for CTLA4 rs231775, there were almost null associations of AG heterozygous (OR=1.11, 95% CI=0.42-2.92, p=0.85) and a reverse association of GG homozygous (OR=0.55, 95% CI=0.06-5.20, p=0.62) in RF negative patients, and also a trend of association of AG heterozygous (OR=1.67, 95% CI=0.30-9.27, p=0.57) and GG homozygous (OR=2.50, 95% CI=0.35-18.04, p=0.37) in RF positive patients with ERA development. None of the observed associations were significant (Figure 3.81).

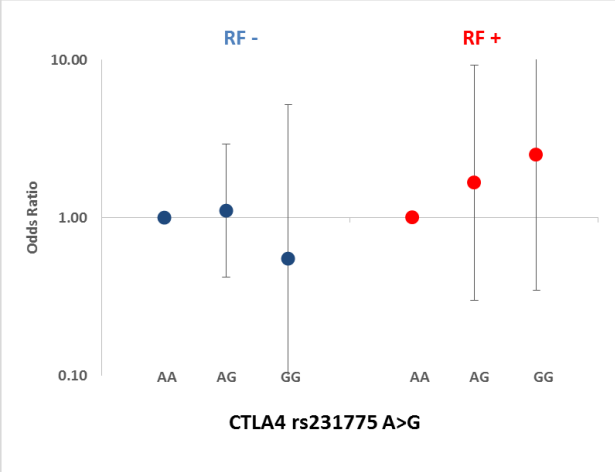

Figure 3.81. Association of CTLA4 rs231775 with ERA according to rheumatoid factor status.

The results of genotyping for TRAF1 rs3761847 showed reverse associations of GA heterozygous (OR=0.65, 95% CI=0.24-1.79, p=0.41) and AA homozygous (OR=0.72, 95% CI=0.16-3.16, p=0.68) in RF negative patients and a trend of reverse associations of GA heterozygous (OR=0.37, 95% CI=0.07-2.03, p=0.27) and AA homozygous (OR=0.25, 95% CI=0.02-2.84, p=0.27) in RF positive patients with ERA development. However, none of the observed associations were significant (Figure 3.82).

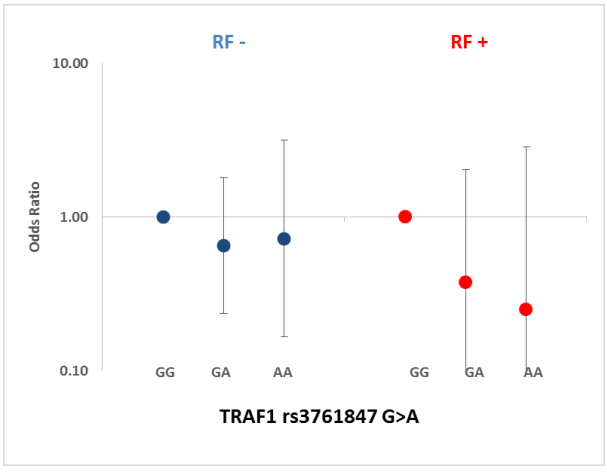

Figure 3.82. Association of TRAF1 rs3761847 with ERA according to rheumatoid factor status.

As shown in figure 3.83, IL-10 rs1800871 demonstrated a reverse associations for AG heterozygous (OR=0.41, 95% CI=0.13-1.28, p=0.12) and GG homozygous (OR=0.77, 95% CI=0.18-3.31, p=0.74) in RF negative patients. There was a trend of association of AG heterozygous (OR=1.60, 95% CI=0.31-8.25, p=0.59) and GG homozygous (OR=2.40, 95% CI=0.26-22.11, p=0.45) in RF positive patients with ERA development. None of the observed associations were significant.

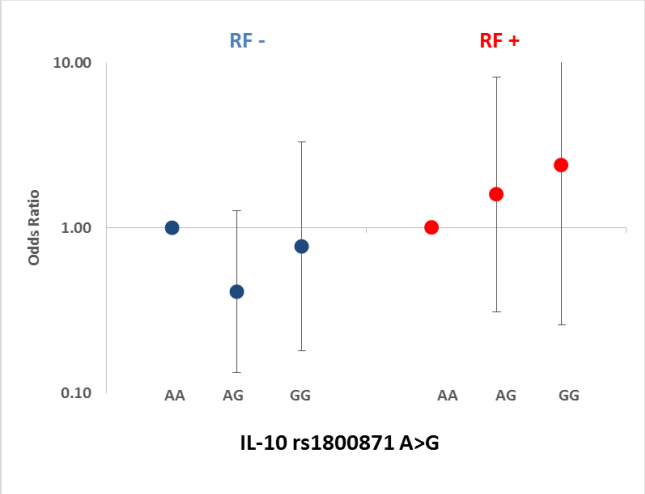

Figure 3.83. Association of IL-10 rs1800871 with ERA according to rheumatoid factor status.

As presented in figure 3.84, IL-6 rs1800795 had reverse associations of CG heterozygous (OR=0.39, 95% CI=0.13-1.15, p=0.09) and GG homozygous (OR=0.77, 95% CI=0.13-4.49, p=0.78) in RF negative patients. There was an association of TG homozygous (OR=3.33, 95% CI=0.66-16.85, p=0.15) in RF positive patients with ERA development. Because of lacking of GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

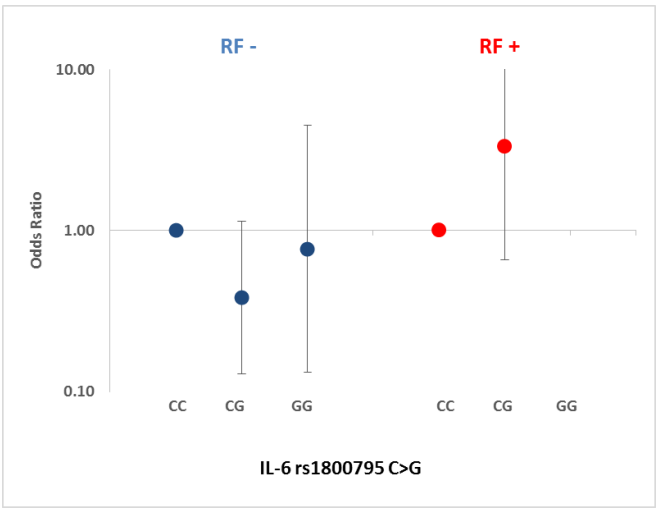

Figure 3.84. Association of IL-6 rs1800795 with ERA according to rheumatoid factor status.

Early Rheumatoid Arthritis Development-ACPA

The results of genotyping for 14 candidate SNPs stratified by presence or absence of anti-citrllinated Protein Antibody are presented in Table 3.8 and subsequently followed by the association graphs.

Gene variant	non-ERA						ERA					
	ACPA-			ACPA+			ACPA-			ACPA+		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
HLA-G rs1233334 G>A	56	14	0	12	2	0	23	3	0	7	1	0
HLA-G rs1063320 C>G	27	32	12	6	5	3	9	11	5	3	4	1
HLA-G 14bp In/del	21	30	21	4	6	4	9	11	6	1	4	3
HLA-DRB1 rs660895 A>G	58	11	2	8	5	0	23	3	0	3	4	1
HLA-DRB1 rs6910071 A>G	51	17	3	12	2	0	20	6	0	5	3	0
HLA-DQA2 rs9275595 T>C	50	17	4	7	6	0	19	7	0	5	3	0
HLA-DQB2 rs10807113 C>A	30	28	13	3	10	0	9	14	2	4	3	1
PTPN22 rs2476601 A>G	64	8	0	12	1	0	23	3	0	7	1	0
PADI4 rs2240340 T>C	20	40	9	6	6	1	5	17	4	2	5	1
STAT4 rs7574865 T>G	41	23	8	8	2	3	18	7	1	6	2	0
CTLA4 rs231775 A>G	39	28	5	7	3	3	16	9	1	1	4	3
TRAF1 rs3761847 G>A	29	30	12	4	8	1	14	9	3	4	3	1
IL-10 rs1800871 A>G	33	30	9	12	1	1	16	6	4	5	2	1
IL-6 rs1800795 C>G	31	30	7	6	8	0	14	8	2	3	5	0

Table 3.7. Genotyping data of non-ERA and ERA patients stratified by anti-Citrllinated Protein Antidoby (ACPA). Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

As shown in figure 3.85, HLA-G rs1233334 demonstrated a reverse association for GA heterozygous (OR=5.22, 95% CI=0.14-1.99, p=0.35) in ACPA negative patients. There was also a slight reverse association in GA heterozygous (OR=0.86, 95% CI=0.06-11.26, p=0.91) in RF positive patients with ERA development. Due to lack of AA homozygous patients, assessing associations were not possible. None of the observed associations were significant.

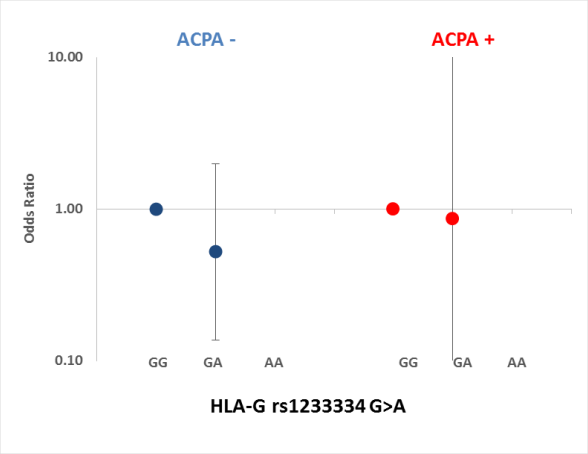

Figure 3.85. Association of HLA-G rs1233334 with ERA according to ACPA status.

As presented in figure 3.86, HLA-G rs1063320 had almost null associations for CG heterozygous (OR=1.03, 95% CI=0.37-2.86, p=0.96) and GG homozygous (OR=1.25, 95% CI=0.34-4.53, p=0.75) in ACPA negative patients and an association of CG heterozygous (OR=1.60, 95% CI=0.24-10.81, p=0.64) and a reverse association of GG homozygous (OR=0.67, 95% CI=0.05-9.47, p=0.78) in ACPA positive patients with ERA development. However, none of the observed associations were significant.

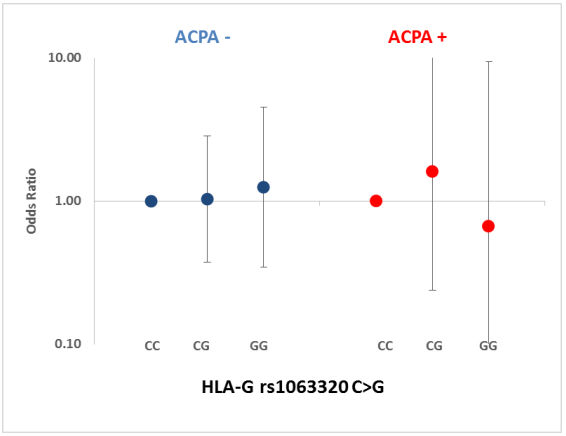

Figure 3.86. Association of HLA-G rs1063320 with ERA according to ACPA status.

Regarding the genotyping for HLA-G 14bp In/del, there was a trend of reverse associations of DI heterozygous (OR=0.86, 95% CI=0.30-2.43, p=0.78) and II homozygous (OR=0.67, 95% CI=0.20-2.20, p=0.52) in ACPA negative patients, and a trend of association of DI heterozygous (OR=2.67, 95% CI=0.21-33.49, p=0.46) and II homozygous (OR=3.00, 95% CI=0.21-42.63, p=0.42) in ACPA positive patients with ERA development. However, none of the observed associations were significant (Figure 3.87).

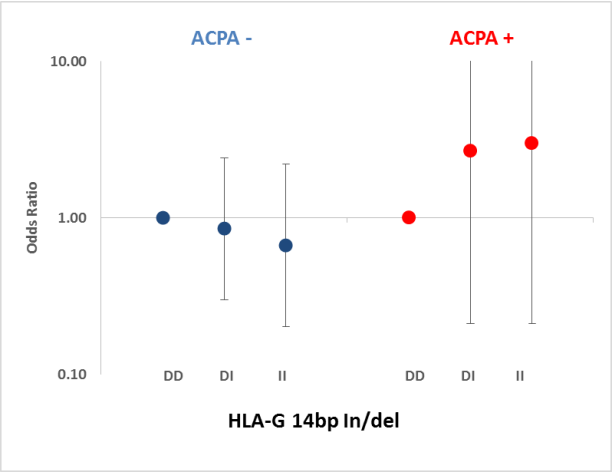

Figure 3.87. Association of HLA-G 14bp In/del with ERA according to ACPA status.

The results of genotyping for HLA-DRB1 rs660895 showed a reverse association of AG heterozygous (OR=0.69, 95% CI=0.18-2.69, p=0.60) in ACPA negative patients and also an association of AG heterozygous (OR=2.13, 95% CI=0.33-13.81, p=0.43) in ACPA positive patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant (Figure 3.88).

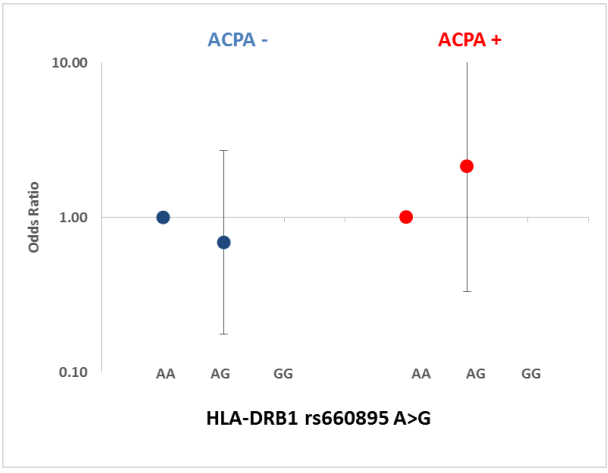

Figure 3.88. Association of HLA-DRB1 rs660895 with ERA according to ACPA status.

As shown in figure 3.89, HLA-DRB1 rs6910071 demonstrated a lack of association for AG heterozygous (OR=0.90, 95% CI=0.31-2.61, p=0.86) in ACPA negative patients. There was an association in AG heterozygous (OR=3.60, 95% CI=0.45-28.56, p=0.23) in ACPA positive patients with ERA development. Due to lack of GG homozygous patients, assessing associations were not possible. None of the observed associations were significant.

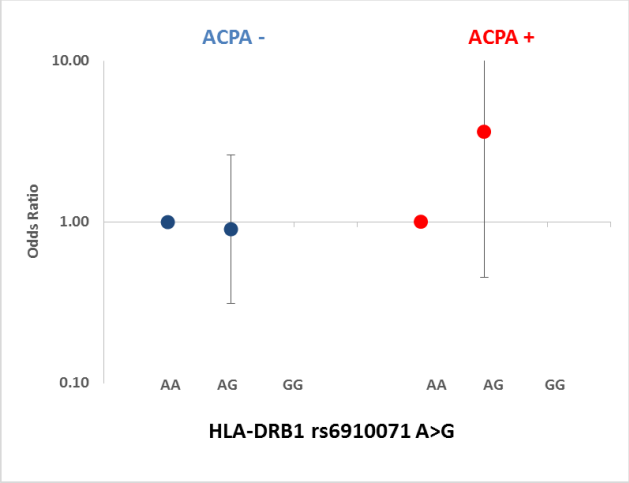

Figure 3.89. Association of HLA-DRB1 rs6910071 with ERA according to ACPA status.

As presented in figure 3.90, HLA-DQA2 rs9275595 had almost null association for TC heterozygous (OR=1.08, 95% CI=0.39-3.02, p=0.87) in ACPA negative patients, and also a reverse association of TC heterozygous (OR=0.70, 95% CI=0.12-4.23, p=0.71) in ACPA positive patients with ERA development. Because of lacking CC homozygous patients, assessing associations were not possible. None of the observed associations were significant.

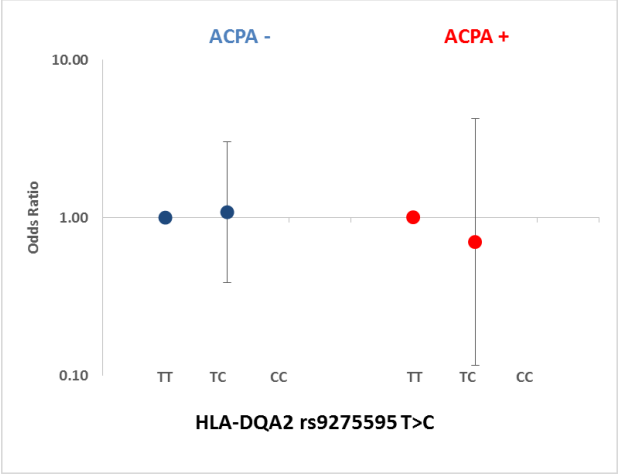

Figure 3.90. Association of HLA-DQA2 rs9275595 with ERA according to ACPA status.

Regarding the genotyping for HLA-DQB2 rs10807113, there was an association of CA heterozygous (OR=1.67, 95% CI=0.62-4.45, p=0.31) and a reverse association of AA homozygous (OR=0.51, 95% CI=0.08-2.71, p=0.44) in ACPA negative patients, and a reverse association of CA heterozygous (OR=0.22, 95% CI=0.03-1.62, p=0.14) in ACPA positive patients with ERA development. However, none of the observed associations were significant (Figure 3.91).

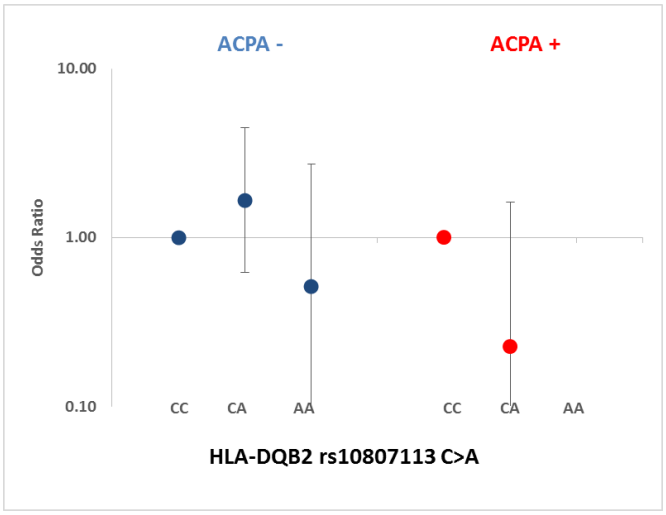

Figure 3.91. Association of HLA-DQA2 rs9275595 with ERA according to ACPA status.

The results of genotyping for PTPN2 rs247660 showed an almost null association of AG heterozygous (OR=1.04, 95% CI=0.25-4.27, p=0.96) in ACPA negative patients with ERA development, and an association of CA heterozygous (OR=1.71, 95% CI=0.09-31.92, p=0.73) in ACPA positive patients with ERA development. As shown in figure 3.92, due to lack of other genotypes, assessing associations were not possible. The observed association was not significant.

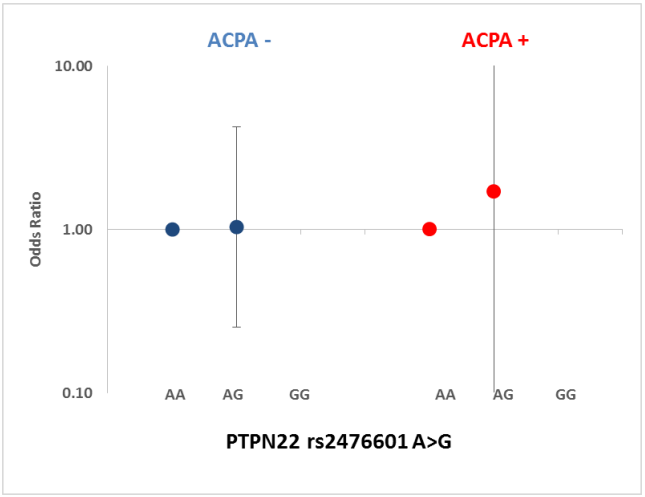

Figure 3.92. Association of PTPN22 rs2476601 with ERA according to ACPA status.

As shown in figure 3.93, PADI4 rs2240340 demonstrated a trend of associations for TC heterozygous (OR=1.70, 95% CI=0.55-5.27, p=0.36) and CC homozygous (OR=1.78, 95% CI=0.38-8.23, p=0.47) in ACPA negative patients. There was a trend of association of TC heterozygous (OR=2.50, 95% CI=0.34-18.33, p=0.37) and CC homozygous (OR=3.00, 95% CI=0.12-73.65, p=0.51) in ACPA positive patients with ERA development. None of the observed associations were significant.

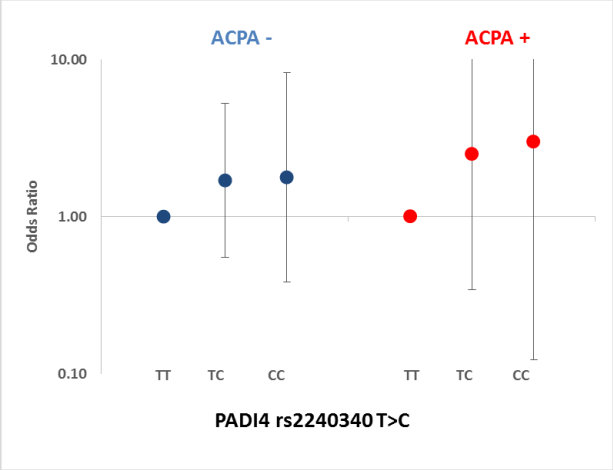

Figure 3.93. Association of PADI4 rs2240340 with ERA according to ACPA status.

As presented in figure 3.94, STAT4 rs7574865 had a trend of reverse association of TG heterozygous (OR=0.69, 95% CI=0.25-1.91, p=0.49) and GG homozygous (OR=0.28, 95% CI=0.03-2.45, p=0.25) in ACPA negative patients. There was an association of TG homozygous (OR=1.33, 95% CI=0.14-12.37, p=0.81) in ACPA positive patients with ERA development. Because of lacking GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

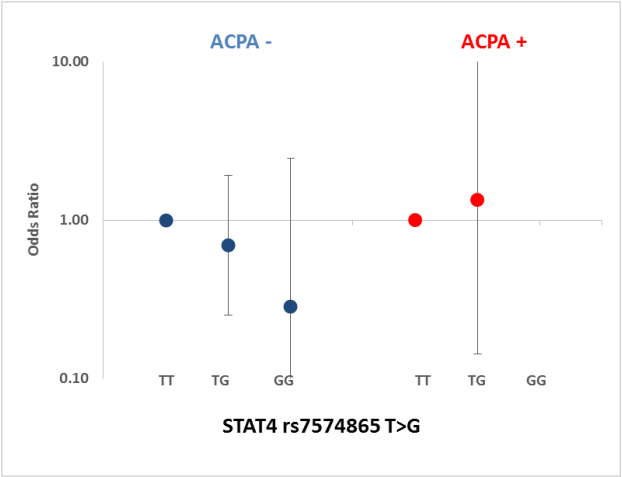

Figure 3.94. Association of STAT4 rs7574865 with ERA according to ACPA status.

Regarding the genotyping for CTLA4 rs231775, there were almost null associations of AG heterozygous (OR=0.78, 95% CI=0.30-2.03, p=0.63) and a reverse association of GG homozygous (OR=0.49, 95% CI=0.05-4.51, p=0.54) in ACPA negative patients, and also association of AG heterozygous (OR=9.33, 95% CI=0.70-122.58, p=0.09) and GG homozygous (OR=7.00, 95% CI=0.50-97.76, p=0.15) in ACPA positive patients with ERA development. None of the observed associations were significant (Figure 3.95).

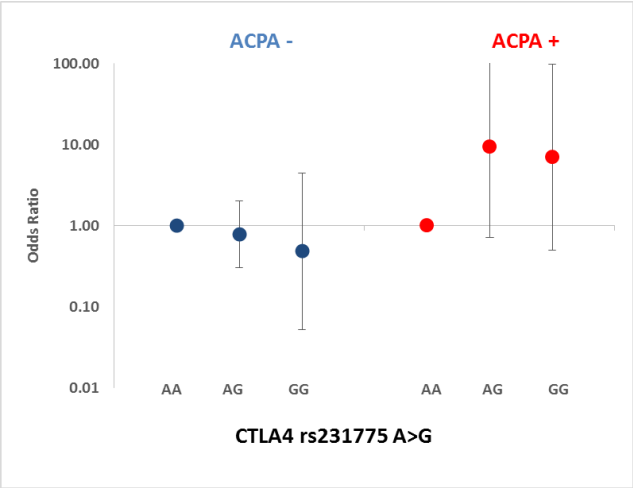

Figure 3.95. Association of CTLA4 rs231775 with ERA according to ACPA status.

The results of genotyping for TRAF1 rs3761847 showed reverse associations of GA heterozygous (OR=0.62, 95% CI=0.23-1.66, p=0.35) and AA homozygous (OR=0.52, 95% CI=0.13-2.14, p=0.37) in ACPA negative patients and a reverse association of GA heterozygous (OR=0.37, 95% CI=0.05-2.55, p=0.32) and lack of association of AA homozygous (OR=1.00, 95% CI=0.04-22.18, p=1.00) in ACPA positive patients with ERA development. However, none of the observed associations were significant (Figure 3.96).

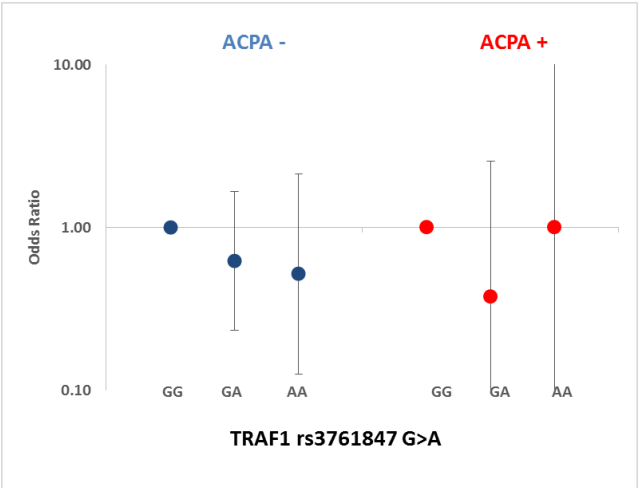

Figure 3.96. Association of TRAF1 rs3761847 with ERA according to ACPA status.

As shown in figure 3.97, IL-10 rs1800871 demonstrated a reverse association for AG heterozygous (OR=0.41, 95% CI=0.14-1.19, p=0.10) and an almost null association of GG homozygous (OR=0.92, 95% CI=0.24-4.43, p=0.90) in ACPA negative patients. There were associations of AG heterozygous (OR=4.80, 95% CI=0.35-65.76, p=0.24) and GG homozygous (OR=2.40, 95% CI=0.12-46.39, p=0.57) in ACPA positive patients with ERA development. None of the observed associations were significant.

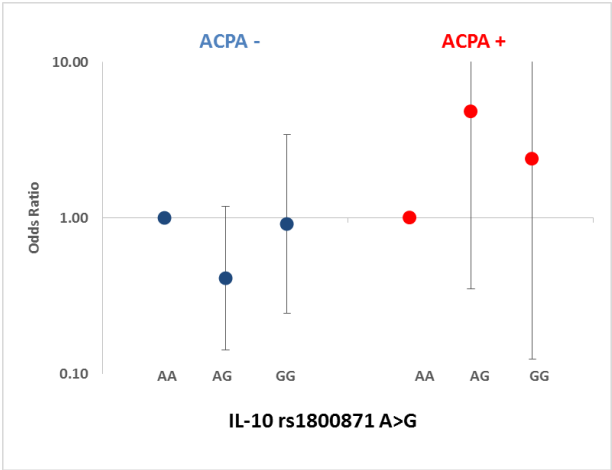

Figure 3.97. Association of IL-10 rs1800871 with ERA according to ACPA status.

As presented in figure 3.98, IL-6 rs1800795 had reverse associations of CG heterozygous (OR=0.59, 95% CI=0.22-1.61, p=0.31) and GG homozygous (OR=0.63, 95% CI=0.12-3.44, p=0.61) in ACPA negative patients. There was an association of TG homozygous (OR=1.25, 95% CI=0.21-7.41, p=0.82) in ACPA positive patients with ERA development. Because of lacking of GG homozygous patients, assessing association was not possible. None of the observed associations were significant.

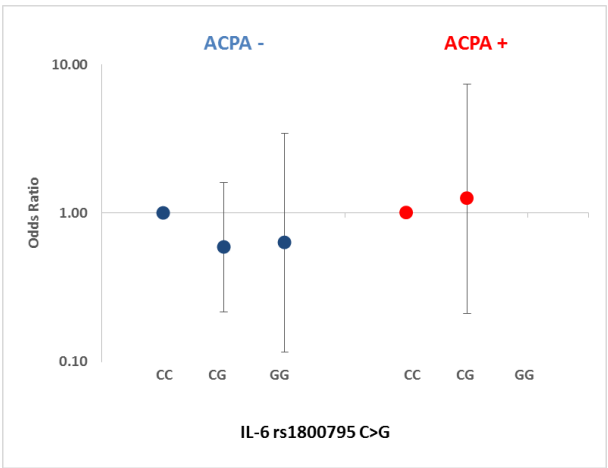

Figure 3.98. Association of IL-6 rs1800795 with ERA according to ACPA status.

MDR Analyses

MDR analysis identified that smoking and APCA interaction play a role in ERA development (figure 3.99). As comparing ACPA negative non-smokers to ACPA negative smokers, the probability of remaining in non-ERA condition was significantly 3 times higher than developing ERA (OR=3.14, 95% CI=1.41-7.00, p=5.28E-03). However, the ratio of ERA patients among non-smokers and smokers was identical. On the other hand, among ACPA positive patients, the probability of remaining in non-ERA condition in smokers was 2 times more than non-smoker patients (Figure 3.100).

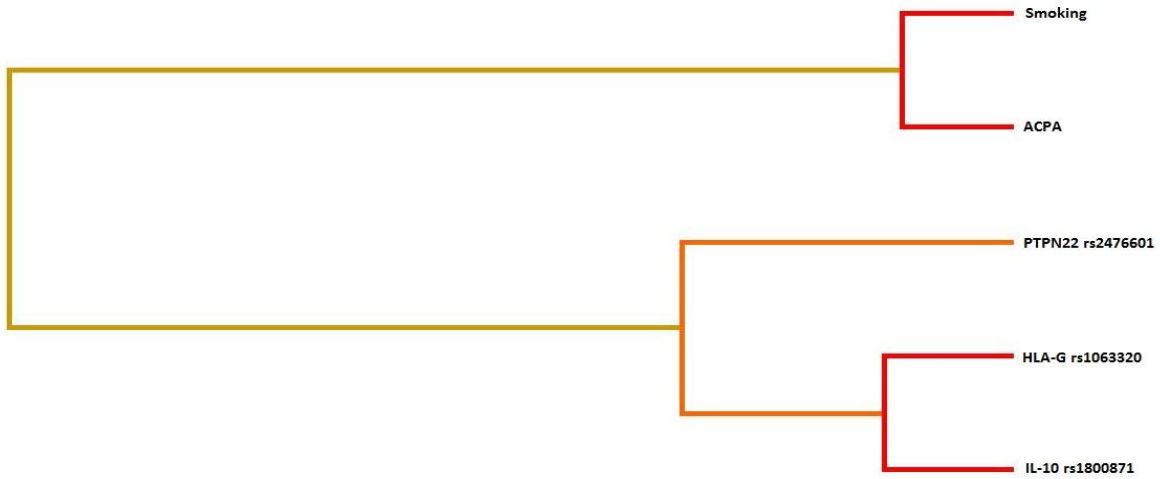

Figure 3.99. MDR analysis. Dendrogram of interaction of smoking and ACPA status in ERA development.

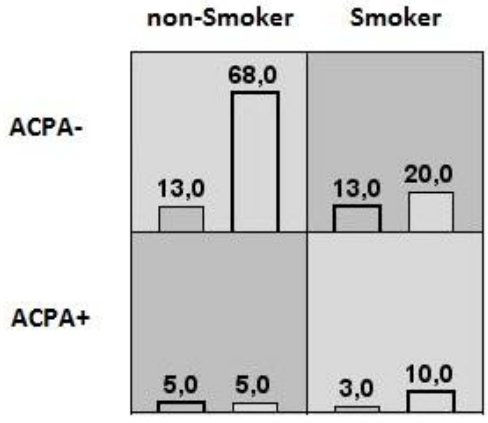

Figure 3.100. MDR analysis. Interaction of Smoking and ACPA status in ERA development.

Dark gray bar: ERA patients, Light gray bar: non-ERA patients.

As shown in figure 3.101, MDR analysis revealed an interaction of TRAF1 rs3761847 and sex in ERA development. As such, among GG homozygous patients, the risk of developing ERA was higher among females (36.58%) compared to males (18.18%) that considering the rate of ERA development in AA heterozygous patients, the MDR interaction analysis demonstrates the protective role of G allele in male patients (Figure 3.102). The probability of maintaining non-ERA condition in GG homozygous male or an A allele carrier female was significantly 2 times more comparing to being GG homozygous female or an A allele carrier male (OR=2.14, 95% CI=1.01-4.51, p=4.53E-02).

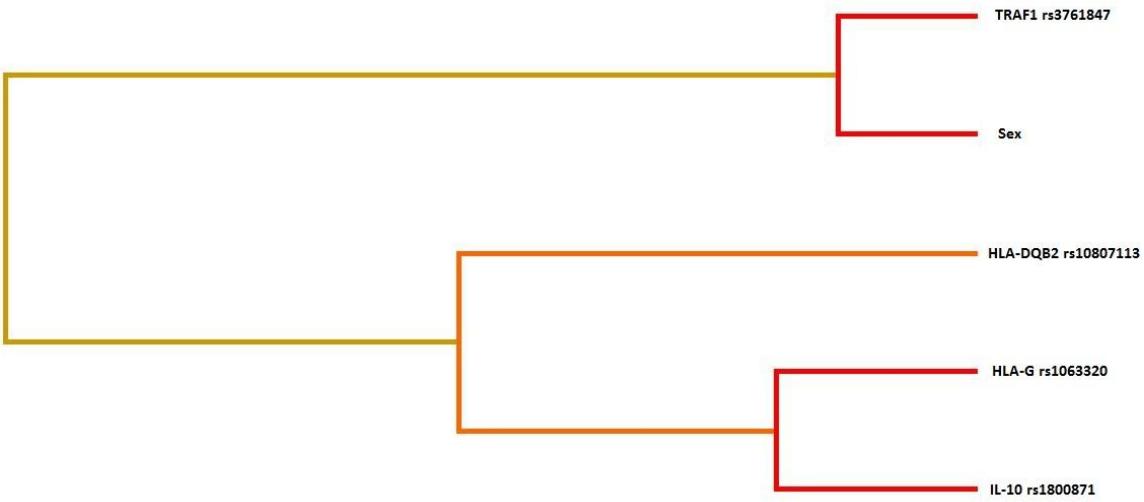

Figure 3.101. MDR analysis. Dendrogram of interaction of TRAF1 rs3761847 and sex in ERA development.

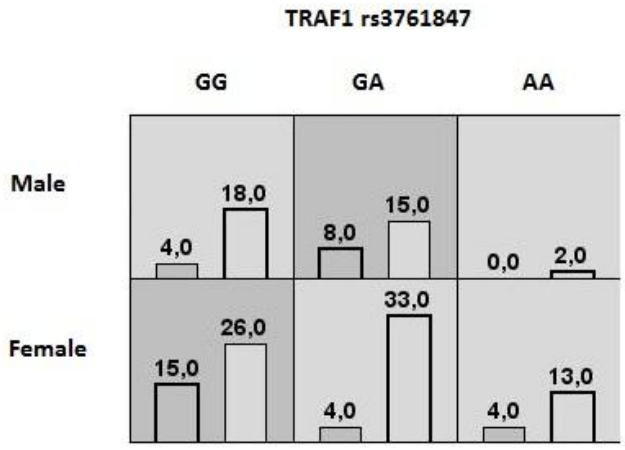

Figure 3.102. MDR analysis. Interaction of TRAF1 rs3761847 and sex in ERA development.

Dark gray bar: ERA patients, Light gray bar: non-ERA patients.

Pharmacogenetic of Early Rheumatoid Arthritis

The Second part of the present study focused on genetics of response to the therapy in patients who developed early rheumatoid arthritis. 379 patients had been recruited that after 6 months follow up according to ACR/EULAR criteria, 55 of them identified as non-responders, and 324 as responders. The mean ± standard deviation (SD) regarding the age, body mass index (BMI), and diseasy activity indexes and percentage of female sex, smokers, rheumatoeid factor positive and anti-citrullinated protein antibody positive of non-ERA and ERA patients were presented in Table 3.8. The difference between BMI, RF, TJC/28, SJC/28, SJC/44, GH, and CDAI between non-responder and responder patients were significant.

Parameter	non-Responder (55)	Responder (324)	*p*
Age	58±14	60±16	0.28
BMI Kg/m^2	62	73	0.01
Female (%)	26.56±4.87	24.58±7.6	0.11
Smokers (%)	49	38	0.14
RF+ (%)	55	40	0.049
ACPA+ (%)	44	37	0.33
TJC/28	4.52±5.06	6.69±5.39	5.00E-03
TJC/44	5.24±7.05	7.23±8.12	0.06
SJC/28	2.69±3.28	5.59±4.87	1.93E-07
SJC/44	3.04±4.41	5.99±7.09	6.73E-05
GH	42.36±26.92	55.06±26.37	2.00E-03
HAQ	0.84±0.75	1.70±11.74	0.19
CDAI	15.75±11.52	22.47±12.30	1.69E-04
SDAI	16.84±11.96	23.64±13.26	2.51E-04
VAS	45.16±29.09	55.46±27.76	1.70E-02
DAS28	3.75±1.39	4.93±1.24	1.11E-07

Table 3.8. Characteristics of drug responder and non-responder patients. BMI: Body Mass Index; **RF**: Rheumatoid Factor; **ACPA**: Anti-Citrullinated Protein Antibody; **TJC28/44**: Tender 28/44 Joints Count; **SJC 28/44**: Swollen 28/44 Joints Count; **GH**: General Health Score; **HAQ**: Healthy Assessment Questionnaire; **CDAI**: Clinical Disease Activity Index; **SDAI**: Simplified Disease Activity Index; **VAS**: Visual Analogue Scale; **DAS28**: Diseasy Activity Score on 28 Joints.

Six single nucleotide polymorphisms belonging to folate genes had been studied in order to invesitgate the association of SNP between first line drug responders and non-responders. The number of successful genotyping, Hardy Weinberg Equilibrium and rare allele frequency for each SNP had been presented in Table 3.9. All SNPs except ATIC rs2372536 (p=0.01) were in Hardy Weinberg Equilibrium.

Gene variant	Genotype Count						HW (p)	Minor allele Frequency	
	non-Responder			Responder					
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3		non-Responder	Responder
ATIC rs2372536 C>G	132	160	27	27	37	3	0.01	33.50%	32.00%
MTRR rs1801394 A>G	99	149	71	20	34	13	0.69	45.60%	44.80%
SHMT1 rs1979277 C>T	190	105	19	36	27	4	0.27	22.80%	26.10%
SLC19A1 rs1051266 C>T	94	154	62	19	34	13	0.98	44.80%	45.50%
MTHFR rs1801133 A>G	87	165	57	21	32	13	0.49	45.10%	43.90%
MTHFR rs1801131 T>G	158	124	28	26	34	6	1.00	29.00%	34.80%

Table 3.8. Genotyping data, Hardy Weinberg Equilibrium and rare allele frequency of drug responder and non-responder patients. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous; **HW**: Hardy Weinberg Equilibrium; ***p:*** p-Value.

Regarding ATIC rs2372536 SNP, there was no significant association between heterozygous genotype (OR=0.88, 95% CI=0.51-1.53, *p*= 0.67), GG homozygous (OR=1.84, 95% CI=0.52-6.51, *p*= 0.35) and drug response (Figure 3.103).

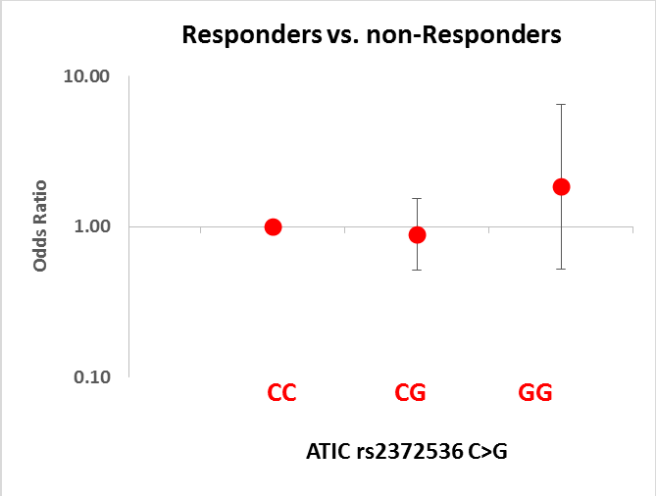

Figure 3.103. Association of ATIC rs2372536 SNP with drug response.

As shown in figure 3.104, regarding MTRR rs1801394 SNP, the association of both AG heterozygous and GG homozygous patients in response to therapy was almost null (OR=0.88, 95% CI=0.48-1.63, *p*=0.71; OR=1.10, 95% CI=0.51-2.36, *p*=0.81, respectively).

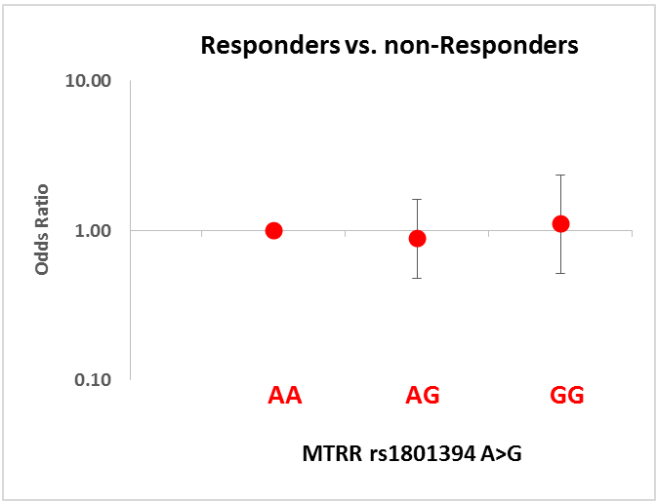

Figure 3.104. Association of MTRR rs1801394 SNP with drug response.

As shown in figure 3.105, the association of SHMT1 rs1979277 in both CT heterozygous and TT homozygous groups in response to therapy was almost null, likewise (OR=0.74, 95% CI=0.42-1.28, *p*=0.28; OR=0.90, 95% CI=0.29-2.80, *p*=0.87, respectively).

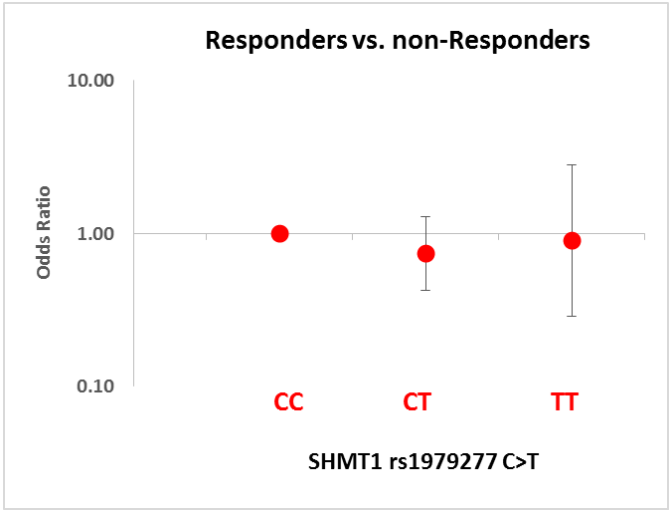

Figure 3.105. Association of SHMT1 rs1979277 SNP with drug response.

Regarding SLC19A1 SNP, there were no significant associations between either heterozygous or homozygous genotypes with drug response (OR=0.92, 95% CI=0.49-1.70, *p*= 0.79; OR=0.96, 95% CI=0.44-2.09, *p*= 0.93, respectively) (Figure 3.106).

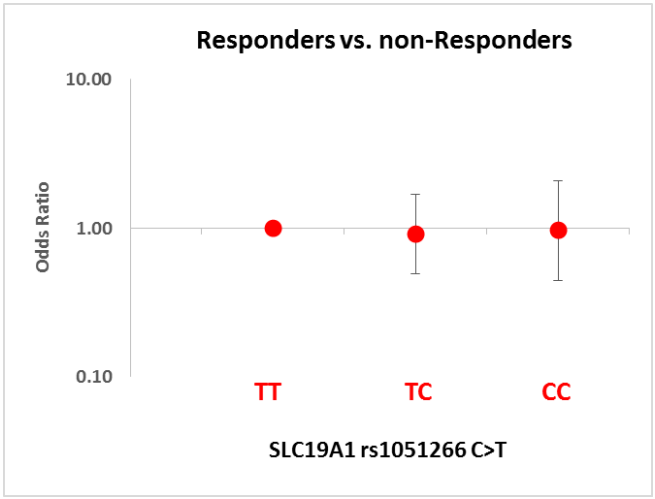

Figure 3.106. Association of SLC19A1 rs1051266 SNP with drug response.

Regarding MTHFR rs1801133 SNP, there was no significant association between GA heterozygous (OR=1.24, 95% CI=0.67-2.29, *p*= 0.49) and AA homozygous (OR=1.06, 95% CI=0.49-2.28, *p*= 0.89) with response to therapy (Figure 3.107).

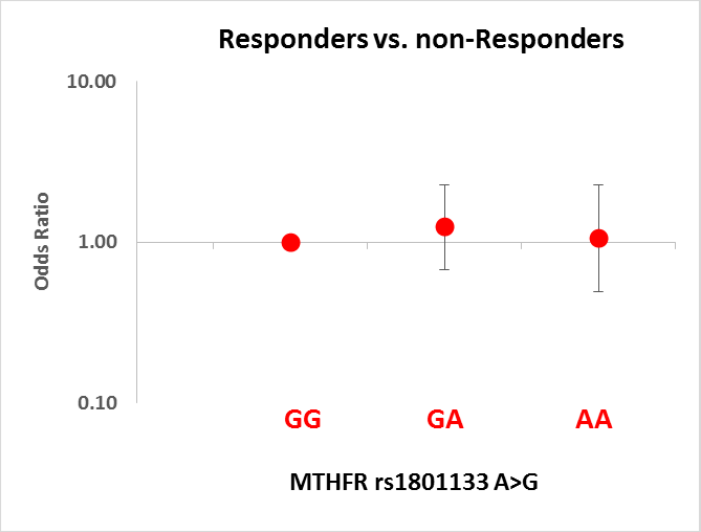

Figure 3.107. Association of MTHFE rs1801133 SNP with drug response.

As shown in figure 3.108, the association of MTHFR rs1801131 in both TG heterozygous and GG homozygous groups with response to therapy were not significant (OR=0.60, 95% CI=0.34-1.05, *p*=0.07; OR=0.77, 95% CI=0.29-2.03, *p*=0.61, respectively).

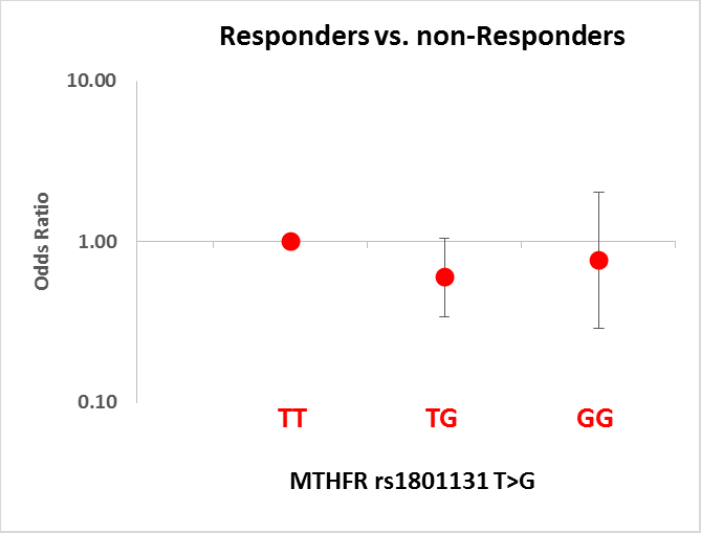

Figure 3.108. Association of MTHFE rs1801131 SNP with drug response.

Pharmacogenetic of Early Rheumatoid Arthritis-Sex

The results of genotyping for 6 candidate SNPs stratified by sex are presented in Table 3.9 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	Male			Female			Male			Female		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	101	113	19	31	47	8	16	25	2	11	12	1
MTRR rs1801394 A>G	75	106	52	24	43	19	13	21	9	7	13	4
SHMT1 rs1979277 C>T	137	80	12	53	25	7	25	16	2	11	11	2
SLC19A1 rs1051266 C>T	65	115	46	29	39	16	14	20	9	5	14	4
MTHFR rs1801133 A>G	64	123	38	23	42	19	16	18	9	5	14	4
MTHFR rs1801131 T>G	108	96	22	50	28	6	16	22	5	10	12	1

Table 3.9. Genotyping data of non-responder and responder patients to the therapy stratified by sex. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed a reverse association of CG heterozygous (OR=0.72, 95% CI=0.36-1.42, p=0.34) and an association of GG homozygous (OR=1.50, 95% CI=0.32-7.09, p=0.62) in male patients and also a trend of association of CG heterozygous (OR=1.39, 95% CI=0.54-3.54, p=0.50) and GG homozygous (OR=2.84, 95% CI=0.32-25.36, p=0.36) in females with drug response. However, none of the observed results were significant (Figure 3.109).

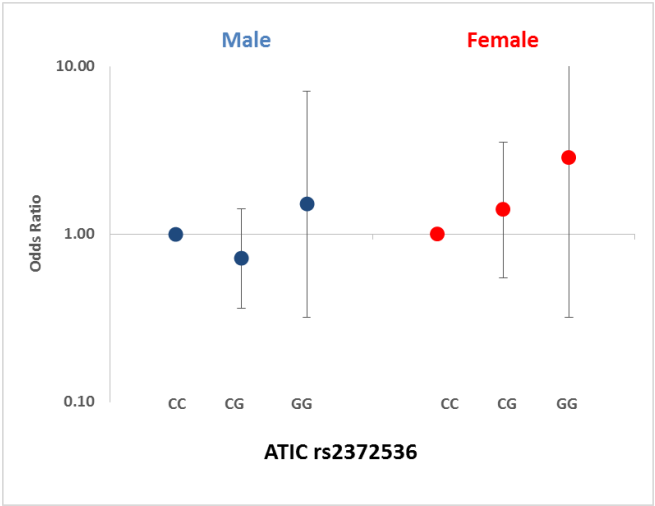

Figure 3.109. Association of ATIC rs2372536 SNP with drug response according to patients' sex.

The results of genotyping for MTRR rs1891394 showed almost lack of association of AG heterozygous (OR=0.87, 95% CI=0.41-1.86, p=0.74) and GG homozygous (OR=1.00, 95% CI=0.40-2.51, p=1.00) in male patients and also lack of association of AG heterozygous (OR=0.96, 95% CI=0.34-2.74, p=0.95) but an association of GG homozygous (OR=1.38, 95% CI=0.35-5.44, p=0.65) in females with response to the therapy. Although, none of the observed associations were significant (Figure 3.110).

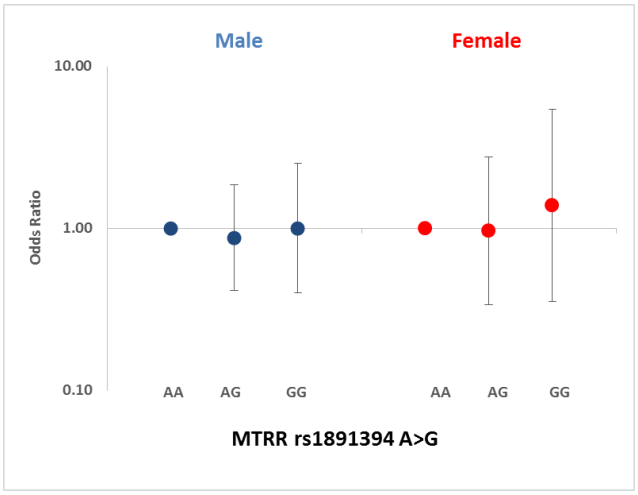

Figure 3.110. Association of MTRR rs1891394 SNP with drug response according to patients' sex.

As shown in figure 3.111, SHMT1 rs1979277 had an almost null association of CT heterozygous (OR=0.91, 95% CI=0.46-1.81, p=0.80) and TT homozygous (OR=1.09, 95% CI=0.23-5.19, p=0.92) in male patients and reverse associations of CT heterozygous (OR=0.47, 95% CI=0.18-1.23, p=0.13) and TT homozygous (OR=0.73, 95% CI=0.13-3.98, p=0.73) in females with response to the therapy. However, none of the observations were statistically significant.

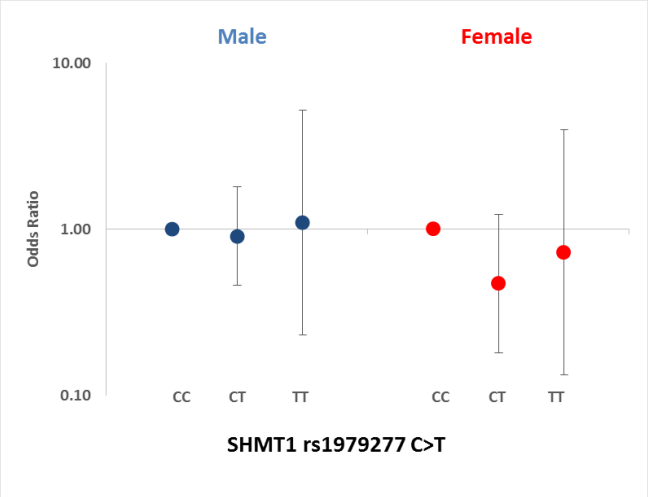

Figure 3.111. Association of SHMT1 rs1979277 SNP with drug response according to patients' sex.

The results of genotyping for SLC19A1 rs1051266 showed an almost null associations of TC heterozygous (OR=1.24, 95% CI=0.59-2.62, p=0.59) and CC homozygous (OR=1.10, 95% CI=0.44-2.79, p=0.85) in male patients and also reverse associations of TC heterozygous (OR=0.48, 95% CI=0.15-1.48, p=0.20) and CC homozygous (OR=0.69, 95% CI=0.16-2.94, p=0.63) in females with response to the therapy. However, none of the observed trends were significant (Figure 3.112).

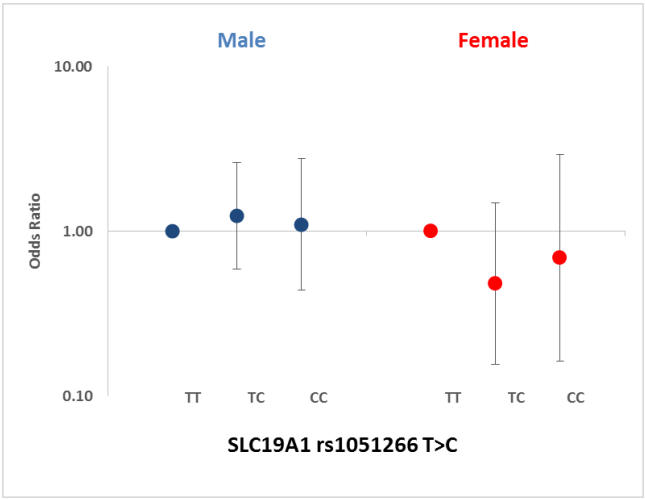

Figure 3.112. Association of SLC19A1 rs1051266 SNP with drug response according to patients' sex.

The results of genotyping stratified by sex for MTHFR rs1801133 showed an association of GA heterozygous (OR=1.71, 95% CI=0.82-3.57, p=0.16) and lack of association of AA homozygous (OR=1.06, 95% CI=0.42-2.62, p=0.91) with response to the therapy in male patients and a reverse association of GA heterozygous (OR=0.65, 95% CI=0.21-2.04, p=0.47) and also null association of AA homozygous (OR=1.03, 95% CI=0.24-4.39, p=0.97) in females. However, none of the observed associations were significant (Figure 3.113).

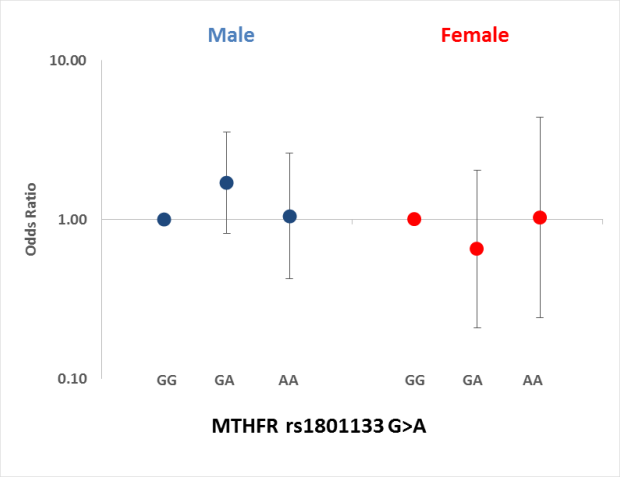

Figure 3.113. Association of MTHFR rs1801133 SNP with drug response according to patients' sex.

As shown in figure 3.114, MTHFR rs1801131 had reverse associations of TG heterozygous (OR=0.65, 95% CI=0.32-1.30, p=0.22) and GG homozygous (OR=0.65, 95% CI=0.22-1.97, p=0.46) in male patients and reverse association of TG heterozygous (OR=0.47, 95% CI=0.18-1.22, p=0.12) and GG homozygous (OR=1.20, 95% CI=0.13-11.08, p=0.88) in females with response to the therapy. However, none of the observations were statistically significant.

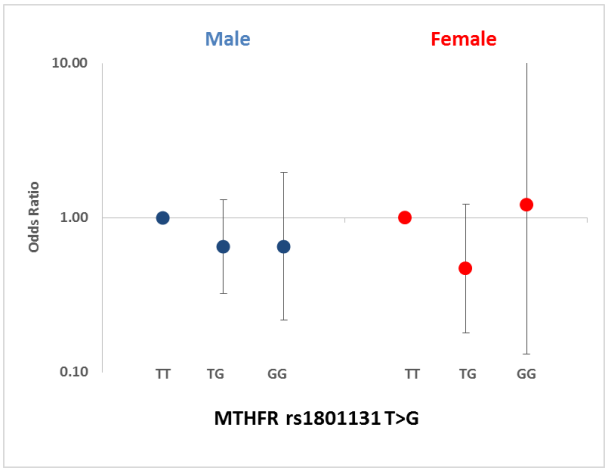

Figure 3.114. Association of MTHFR rs1801131 SNP with drug response according to patients' sex.

Pharmacogenetic of Early Rheumatoid Arthritis-Age

The results of genotyping for 6 candidate SNPs stratified by age are presented in Table 3.10 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	Age < 50 y			Age > 50 y			Age < 50 y			Age > 50 y		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	40	42	4	92	118	23	10	11	0	17	26	3
MTRR rs1801394 A>G	29	40	17	70	109	54	5	10	6	15	24	7
SHMT1 rs1979277 C>T	48	34	3	142	71	16	11	9	1	25	18	3
SLC19A1 rs1051266 C>T	28	42	13	66	112	49	8	9	4	11	25	9
MTHFR rs1801133 A>G	29	42	11	58	123	46	6	8	7	15	24	6
MTHFR rs1801131 T>G	40	39	4	118	85	24	10	10	1	16	24	5

Table 3.10. Genotyping data of non-responder and responder patients to the therapy stratified by age. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed a null association of CG heterozygous (OR=0.95, 95% CI=0.37-2.49, p=0.93) in younger patients and also an opposite association of CG heterozygous (OR=0.84, 95% CI=0.43-1.64, p=0.62) and GG homozygous (OR=1.42, 95% CI=0.38-5.25, p=0.61) in older patients with drug response. Due to lack of GG homozygous in younger group evaluation was not possible. However, none of the observed results were significant (Figure 3.115).

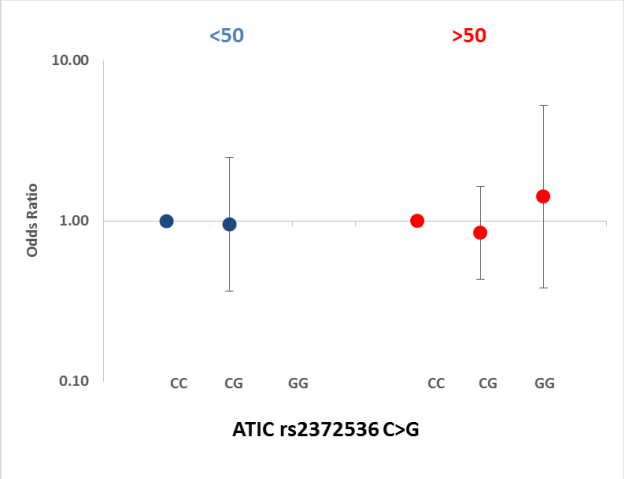

Figure 3.115. Association of ATIC rs2372536 SNP with drug response according to patients' age.

The results of genotyping for MTRR rs1891394 showed a trend of reverse association of AG heterozygous (OR=0.69, 95% CI=0.21-2.23, p=0.55) and GG homozygous (OR=0.49, 95% CI=0.13-1.85, p=0.29) in patients below 50 years old, and also lack of association of AG heterozygous (OR=0.97, 95% CI=0.48-1.98, p=0.95) but an association of GG homozygous (OR=1.65, 95% CI=0.63-4.34, p=0.31) in patients above 50 years old with response to the therapy. Although, none of the observed associations were significant (Figure 3.116).

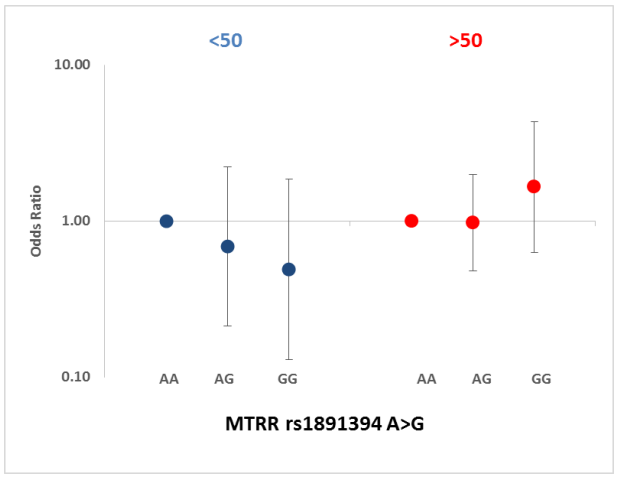

Figure 3.116. Association of MTRR rs1891394 SNP with drug response according to patients' age.

As shown in figure 3.117, SHMT1 rs1979277 had an almost null association of CT heterozygous (OR=0.87, 95% CI=0.32-2.32, p=0.79) and a reverse association of TT homozygous (OR=0.69, 95% CI=0.06-7.25, p=0.77) in younger patients and reverse association of CT heterozygous (OR=0.69, 95% CI=0.36-1.36, p=0.29) and almost null association of TT homozygous (OR=0.94, 95% CI=0.25-3.46, p=0.93) in older patients with response to the therapy. However, none of the observations were statistically significant.

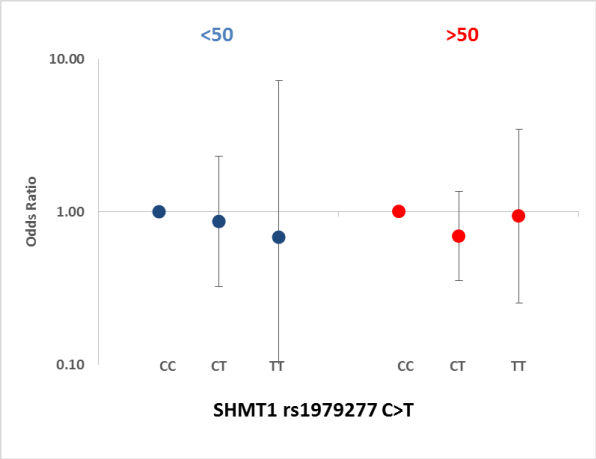

Figure 3.117. Association of SHMT1 rs1979277 SNP with drug response according to patients' age.

The results of genotyping for SLC19A1 rs1051266 showed an almost null associations of TC heterozygous (OR=1.33, 95% CI=0.46-3.87, p=0.61) and CC homozygous (OR=0.93, 95% CI=0.24-3.65, p=0.92) in patients below 50 years old and also reverse association of TC heterozygous (OR=0.75, 95% CI=0.34-1.61, p=0.47) and null association of CC homozygous (OR=0.91, 95% CI=0.35-2.36, p=0.85) in patients above 50 years old with response to the therapy. However, none of the observed trends were significant (Figure 3.118).

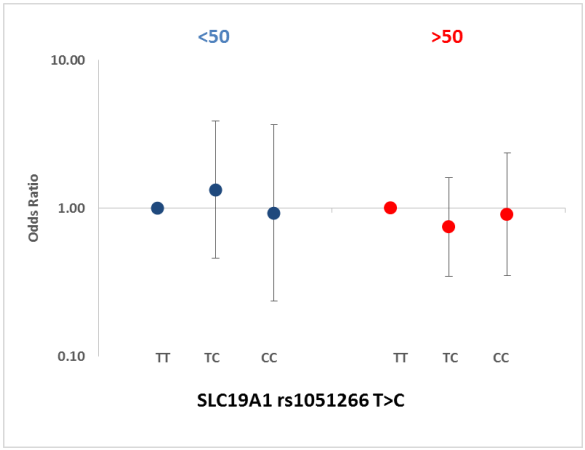

Figure 3.118. Association of SLC19A1 rs1051266 with drug response according to patients' age.

The results of genotyping stratified by age for MTHFR rs1801133 showed lack of association of GA heterozygous (OR=1.09, 95% CI=0.34-3.46, p=0.90) and reverse association of AA homozygous (OR=0.32, 95% CI=0.09-1.18, p=0.09) with response to the therapy in younger patients and a trend of association of GA heterozygous (OR=1.32, 95% CI=0.65-2.71, p=0.45) and AA homozygous (OR=1.98, 95% CI=0.71-5.51, p=0.19) in older patients. However, none of the observed associations were significant (Figure 3.119).

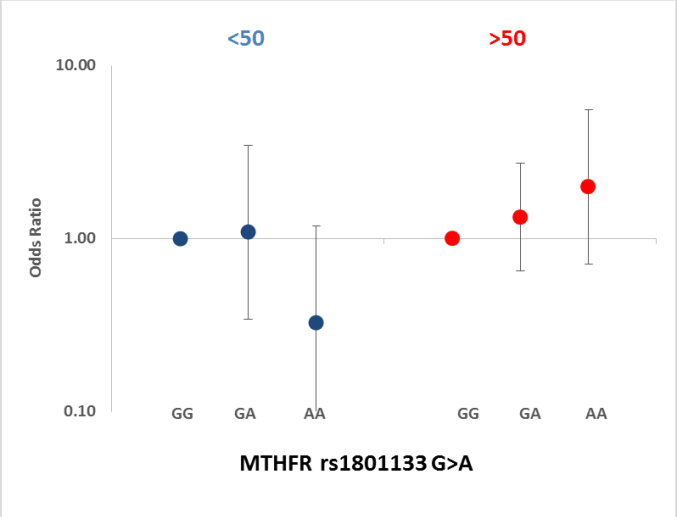

Figure 3.119. Association of MTHFR rs1801133 SNP with drug response according to patients' age.

As shown in figure 3.120, MTHFR rs1801131 had null associations of TG heterozygous (OR=1.09, 95% CI=0.34-3.46, p=0.90) and GG homozygous (OR=0.32, 95% CI=0.09-1.18, p=0.09) in patients below 50 years old and reverse associations of TG heterozygous (OR=1.32, 95% CI=0.65-2.71, p=0.45) and GG homozygous (OR=1.98, 95% CI=0.71-5.51, p=0.19) in patients above 50 years old with response to the therapy. However, none of the observations were statistically significant.

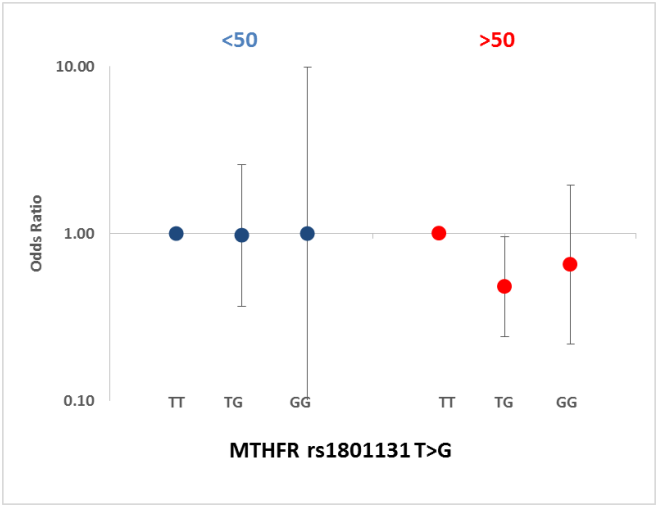

Figure 3.120. Association of MTHFR rs1801131 SNP with drug response according to patients' age.

Pharmacogenetic of Early Rheumatoid Arthritis-Smoking

The results of genotyping for 6 candidate SNPs stratified by smoking state are presented in Table 3.11 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	non-Smoker			Smoker			non-Smoker			Smoker		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	80	101	16	52	59	11	10	23	1	17	14	2
MTRR rs1801394 A>G	58	97	42	41	52	29	3	22	9	17	12	4
SHMT1 rs1979277 C>T	116	70	8	74	35	11	18	13	3	18	14	1
SLC19A1 rs1051266 C>T	61	94	35	33	60	27	13	18	3	6	16	10
MTHFR rs1801133 A>G	54	104	32	33	61	25	11	17	6	10	15	7
MTHFR rs1801131 T>G	89	85	16	69	39	12	12	18	4	14	16	2

Table 3.11. Genotyping data of non-responder and responder patients to the therapy stratified by smoking state. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed a reverse association of CG heterozygous (OR=0.55, 95% CI=0.25-1.22, p=0.14) and an association of GG homozygous (OR=2.00, 95% CI=0.24-16.74, p=0.53) in non-smoker patients and also a trend of association of CG heterozygous (OR=1.38, 95% CI=0.62-3.06, p=0.44) and GG homozygous (OR=1.80, 95% CI=0.36-8.93, p=0.48) in smokers with drug response. However, none of the observed results were significant (Figure 3.121).

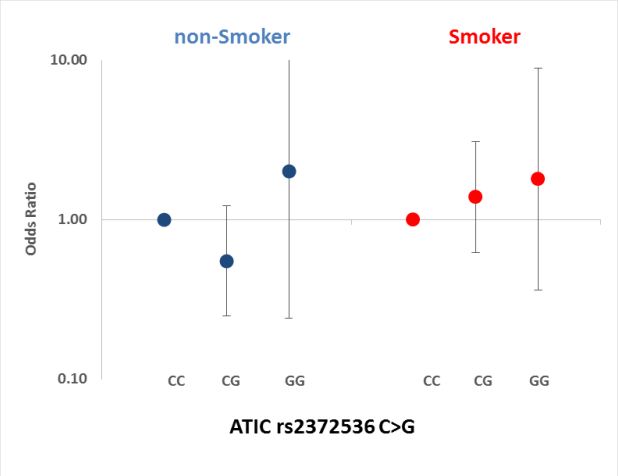

Figure 3.121. Association of ATIC rs2372536 SNP with drug response according to patients' smoking status.

The results of genotyping for MTRR rs1891394 showed a significant reverse associations of AG heterozygous (OR=0.23, 95% CI=0.06-0.79, p=0.02) and GG homozygous (OR=0.24, 95% CI=0.06-0.95, p=0.04) in non-smokers, and also a trend of association of AG heterozygous (OR=1.80, 95% CI=0.77-4.18, p=0.17) and GG homozygous (OR=3.01, 95% CI=0.92-9.87, p=0.07) in smoker patients with response to the therapy (Figure 3.122).

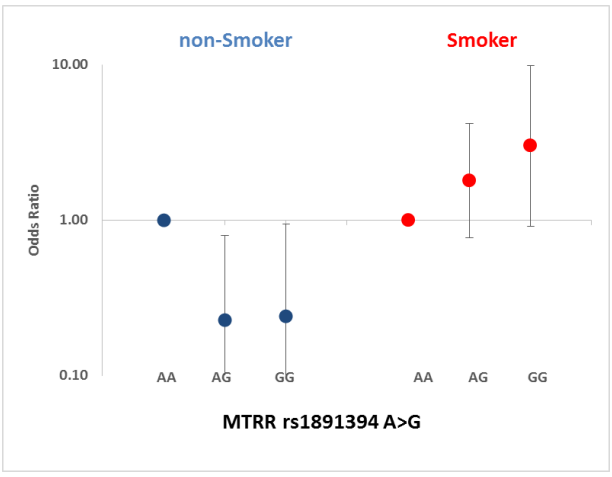

Figure 3.122. Association of MTRR rs1891394 SNP with drug response according to patients' smoking status.

As shown in figure 3.123, SHMT1 rs1979277 had a reverse association of CT heterozygous (OR=0.84, 95% CI=0.39-1.81, p=0.66) and TT homozygous (OR=0.41, 95% CI=0.10-1.71, p=0.22) in non-smoker patients and reverse association of CT heterozygous (OR=0.61, 95% CI=0.27-1.36, p=0.23) and an association of TT homozygous (OR=2.68, 95% CI=0.32-22.09, p=0.37) in smokers with response to the therapy. However, none of the observations were statistically significant.

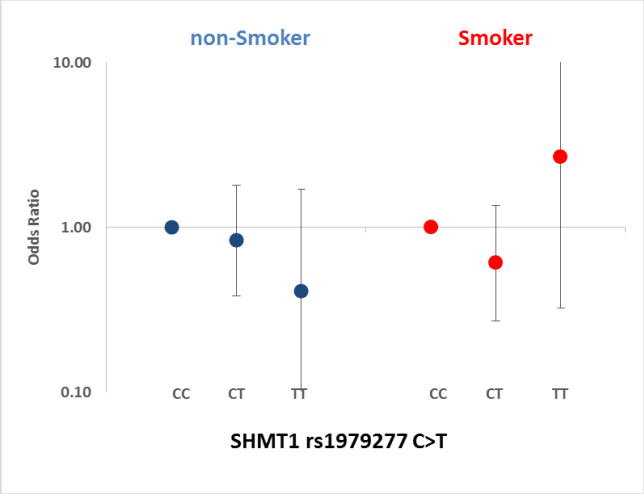

Figure 3.123. Association of SHMT1 rs1979277 SNP with drug response according to patients' smoking status.

The results of genotyping for SLC19A1 rs1051266 showed an almost null associations of TC heterozygous (OR=1.11, 95% CI=0.51-2.43, p=0.80) and an association of CC homozygous (OR=2.49, 95% CI=0.66-9.33, p=0.18) in non-smokers and also a trend of reverse association of TC heterozygous (OR=0.68, 95% CI=0.24-1.91, p=0.47) and CC homozygous (OR=0.49, 95% CI=0.16-1.52, p=0.22) in smokers with response to the therapy. However, none of the observed results were significant (Figure 3.124).

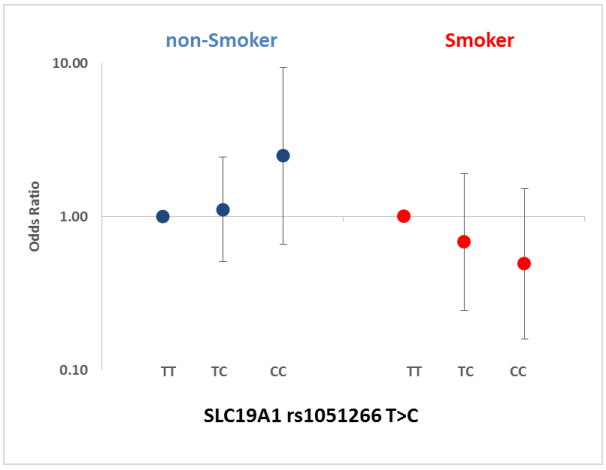

Figure 3.124. Association of SLC19A1 rs1051266 SNP with drug response according to patients' smoking status.

The results of genotyping stratified by smoking status for MTHFR rs1801133 showed almost lack of association of GA heterozygous (OR=1.25, 95% CI=0.54-2.85, p=0.61) and AA homozygous (OR=1.09, 95% CI=0.37-3.22, p=0.89) with response to the therapy in non-smokers and also in GA heterozygous (OR=1.23, 95% CI=0.50-3.05, p=0.66) and AA homozygous (OR=1.08, 95% CI=0.36-3.24, p=0.90) in smoker patients. However, none of the observed associations were significant (Figure 3.125).

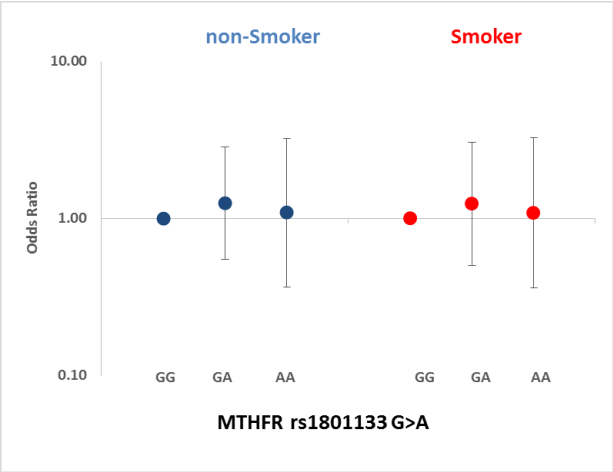

Figure 3.125. Association of MTHFR rs1801133 SNP with drug response according to patients' smoking status.

As shown in figure 3.126, MTHFR rs1801131 had reverse association of TG heterozygous (OR=0.64, 95% CI=0.29-1.40, p=0.26) and GG homozygous (OR=0.54, 95% CI=0.15-1.88, p=0.34) in non-smokers and reverse association of TG heterozygous (OR=0.49, 95% CI=0.22-1.12, p=0.09) and almost null association of GG homozygous (OR=1.22, 95% CI=0.24-6.05, p=0.82) in smoker patients with response to the therapy. None of the observations were statistically significant.

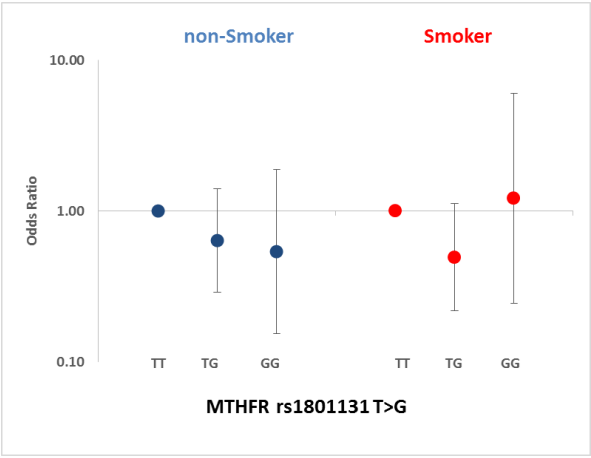

Figure 3.126. Association of MTHFR rs1801131 SNP with drug response according to patients' smoking status.

Pharmacogenetic of Early Rheumatoid Arthritis-BMI

The results of genotyping for 6 candidate SNPs stratified by BMI are presented in Table 3.12 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	BMI < 30			BMI > 30			BMI < 30			BMI > 30		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	111	137	22	21	23	5	20	32	2	7	5	1
MTRR rs1801394 A>G	84	125	61	15	24	10	15	29	10	5	5	3
SHMT1 rs1979277 C>T	164	88	14	26	17	5	27	23	4	9	4	0
SLC19A1 rs1051266 C>T	78	134	51	16	20	11	17	26	10	2	8	3
MTHFR rs1801133 A>G	76	140	46	11	25	11	16	25	12	5	7	1
MTHFR rs1801131 T>G	130	109	24	28	15	4	21	27	5	5	7	1

Table 3.12. Genotyping data of non-responder and responder patients to the therapy stratified by BMI. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed a reverse association of CG heterozygous (OR=0.77, 95% CI=0.42-1.42, p=0.41) and an association of GG homozygous (OR=1.98, 95% CI=0.43-9.10, p=0.39) in patients with BMI below 30 kg/m^2 and also a trend of association of CG heterozygous (OR=1.53, 95% CI=0.42-5.57, p=0.53) and GG homozygous (OR=1.68, 95% CI=0.16-16.81, p=0.68) in patients with BMI above 30 kg/m^2 with drug response. However, none of the observed results were significant (Figure 3.127).

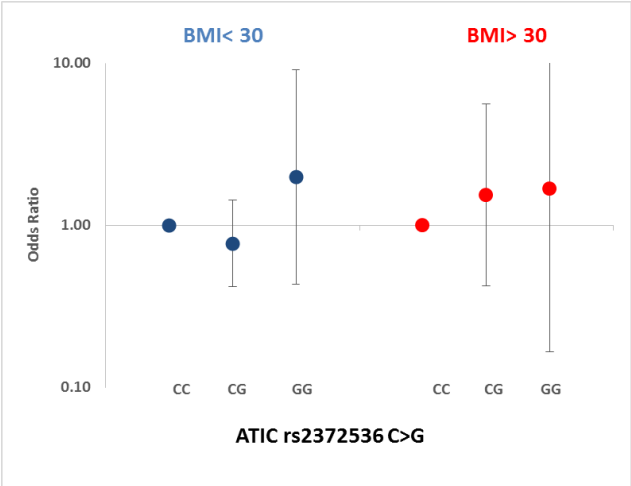

Figure 3.127. Association of ATIC rs2372536 SNP with drug response according to patients' BMI.

The results of genotyping for MTRR rs1891394 showed a reverse association of AG heterozygous (OR=0.77, 95% CI=0.39-1.52, p=0.46) and null association of GG homozygous (OR=1.09, 95% CI=0.46-2.59, p=0.86) in patients with BMI below 30 kg/m^2, and also association of AG heterozygous (OR=1.60, 95% CI=0.40-6.47, p=0.52) and almost null association of GG homozygous (OR=1.11, 95% CI=0.22-5.73, p=0.91) in patients with BMI above 30 kg/m^2 with response to the therapy. Although, none of the observed associations were significant (Figure 3.128).

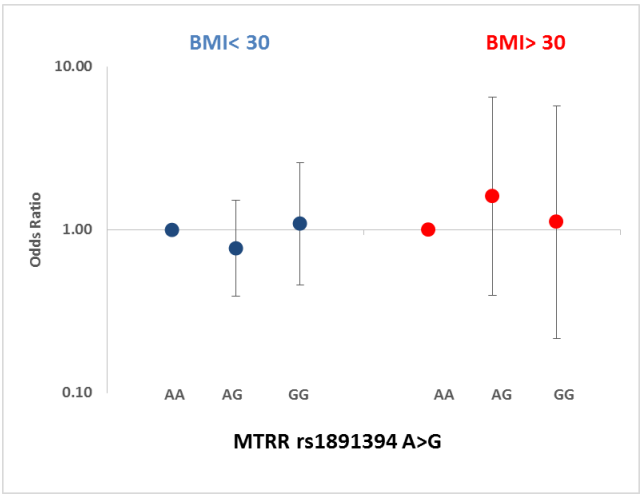

Figure 3.128. Association of MTRR rs1891394 SNP with drug response according to patients' BMI.

As shown in figure 3.129, SHMT1 rs1979277 had a reverse association of CT heterozygous (OR=0.63, 95% CI=0.34-1.16, p=0.14) and TT homozygous (OR=0.58, 95% CI=0.18-1.88, p=0.37) in patients with BMI below 30 kg/m^2 and association of CT heterozygous (OR=1.47, 95% CI=0.39-5.55, p=0.58) in patients with BMI above 30 kg/m^2 with response to the therapy. Due to lack of TT homozygous evaluation association was not possible. However, none of the observations were statistically significant.

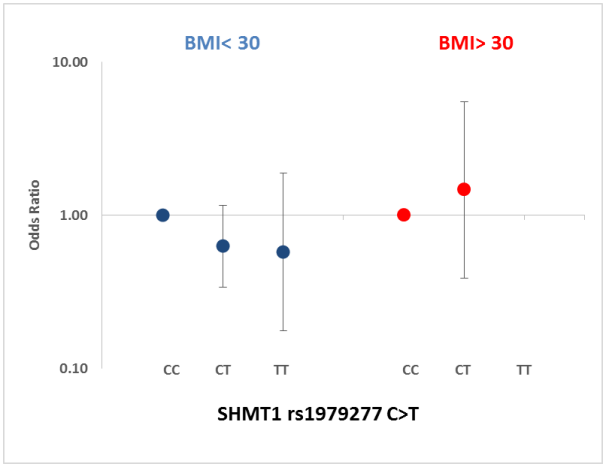

Figure 3.129. Association of SHMT1 rs1979277 SNP with drug response according to patients' BMI.

The results of genotyping for SLC19A1 rs1051266 showed an almost null associations of TC heterozygous (OR=1.12, 95% CI=0.57-2.20, p=0.75) and CC homozygous (OR=1.11, 95% CI=0.47-2.62, p=0.82) in patients with BMI below 30 kg/m^2 and reverse association of TC heterozygous (OR=0.31, 95% CI=0.06-1.68, p=0.18) and CC homozygous (OR=0.46, 95% CI=0.06-3.21, p=0.44) in patients with BMI above 30 kg/m^2 with response to the therapy. However, none of the observed results were significant (Figure 3.130).

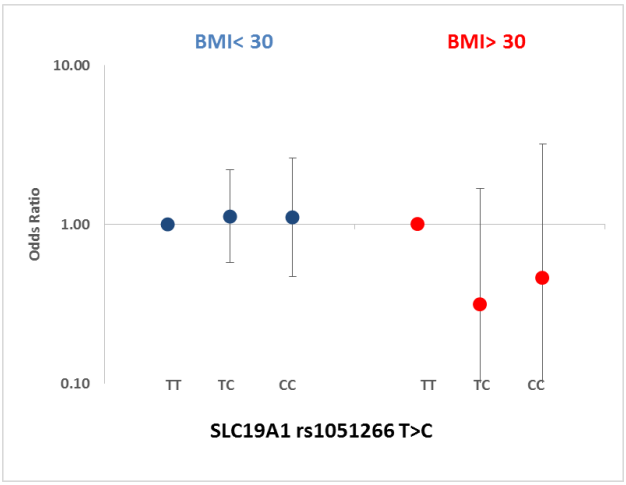

Figure 3.130. Association of SLC19A1 rs1051266 SNP with drug response according to patients' BMI.

The results of genotyping stratified by smoking status for MTHFR rs1801133 showed almost lack of association of GA heterozygous (OR=1.18, 95% CI=0.59-2.34, p=0.65) and AA homozygous (OR=0.81, 95% CI=0.35-1.86, p=0.63) with response to the therapy in patients with BMI below 30 kg/m^2 and also a trend of association of GA heterozygous (OR=1.62, 95% CI=0.42-6.26, p=0.49) and AA homozygous (OR=5.00, 95% CI=0.50-50.07, p=0.17) in patients with BMI above 30 kg/m^2. However, none of the observed associations were significant (Figure 3.131).

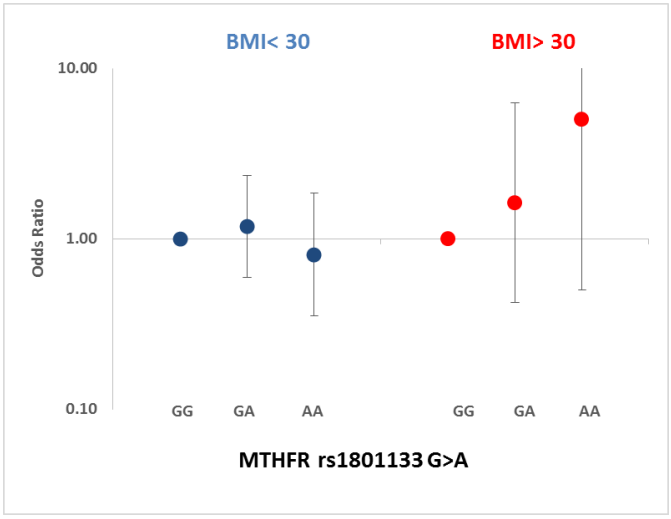

Figure 3.131. Association of MTHFR rs1801133 SNP with drug response according to patients' BMI.

As shown in figure 3.132, MTHFR rs1801131 had reverse association of TG heterozygous (OR=0.65, 95% CI=0.35-1.22, p=0.18) and GG homozygous (OR=0.77, 95% CI=0.27-2.26, p=0.65) in patients with BMI below 30 kg/m^2 and reverse association of TG heterozygous (OR=0.38, 95% CI=0.10-1.41, p=0.15) and GG homozygous (OR=0.71, 95% CI=0.07-7.79, p=0.79) in patients with BMI above 30 kg/m^2 with response to the therapy. None of the observations were statistically significant.

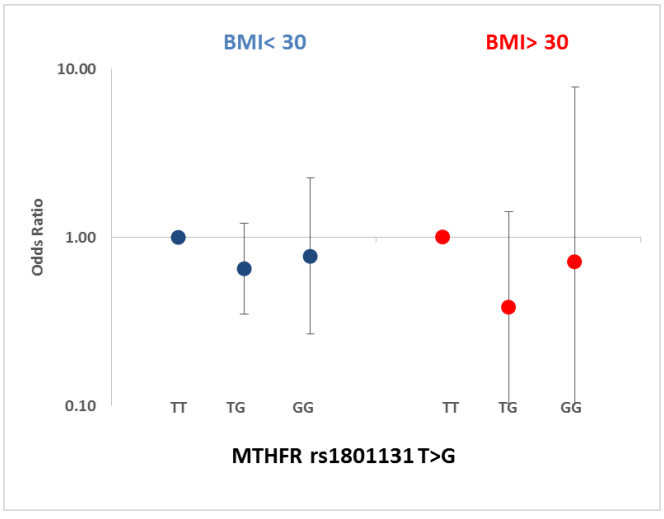

Figure 3.132. Association of MTHFR rs1801131 SNP with drug response according to patients' BMI.

Pharmacogenetic of Early Rheumatoid Arthritis-RF

The results of genotyping for 6 candidate SNPs stratified by rheumatoid factor are presented in Table 3.13 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	RF -			RF +			RF -			RF +		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	82	88	21	50	71	6	11	16	1	14	19	2
MTRR rs1801394 A>G	54	93	44	44	56	27	9	16	3	11	16	8
SHMT1 rs1979277 C>T	122	56	11	68	48	8	16	10	2	18	15	2
SLC19A1 rs1051266 C>T	62	88	36	32	65	26	10	16	2	7	16	11
MTHFR rs1801133 A>G	44	103	39	42	62	18	7	13	8	12	17	5
MTHFR rs1801131 T>G	97	72	17	61	52	10	13	13	2	11	20	3

Table 3.13. Genotyping data of non-responder and responder patients to the therapy stratified by rheumatoid factor. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed a reverse association of CG heterozygous (OR=0.74, 95% CI=0.32-1.68, p=0.48) and an association of GG homozygous (OR=2.82, 95% CI=0.34-23.06, p=0.34) in RF negative patients and also almost lack of association of CG heterozygous (OR=1.05, 95% CI=0.48-2.28, p=0.92) and GG homozygous (OR=0.84, 95% CI=0.15-4.63, p=0.85) in RF positive patients with drug response. However, none of the observed results were significant (Figure 3.133).

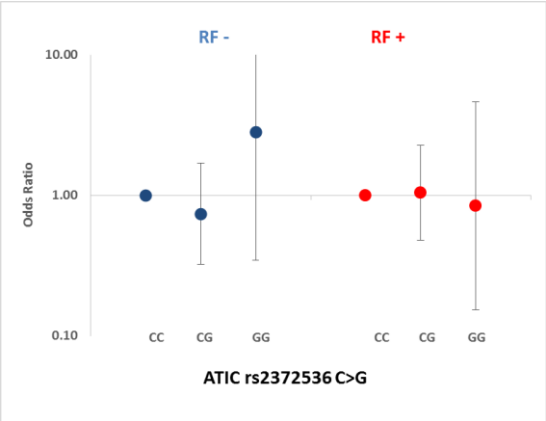

Figure 3.133. Association of ATIC rs2372536 SNP with drug response according to patients' rheumatoid factor.

The results of genotyping for MTRR rs1891394 showed a null association of AG heterozygous (OR=0.97, 95% CI=0.40-2.34, p=0.95) and an association of GG homozygous (OR=2.44, 95% CI=0.62-9.58, p=0.20) in RF negative patients, and also almost lack of association of AG heterozygous (OR=0.87, 95% CI=0.37-2.07, p=0.77) and GG homozygous (OR=0.84, 95% CI=0.30-2.36, p=0.76) in RF positive patients with response to the therapy. Although, none of the observed associations were significant (Figure 3.134).

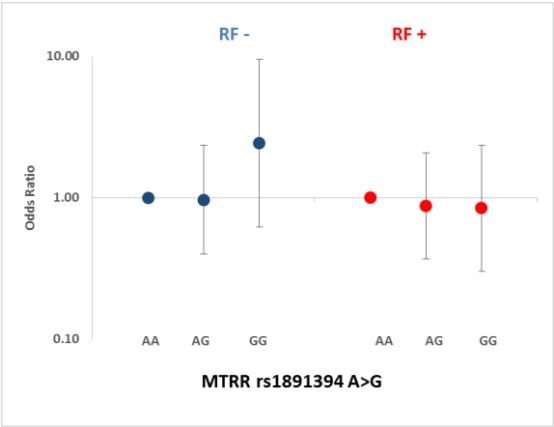

Figure 3.134. Association of MTRR rs1891394 SNP with drug response according to patients' rheumatoid factor.

As shown in figure 3.135, SHMT1 rs1979277 had a reverse association of CT heterozygous (OR=0.73, 95% CI=0.31-1.72, p=0.49) and TT homozygous (OR=0.72, 95% CI=0.15-3.55, p=0.70) in RF negative patients and almost lack of association of CT heterozygous (OR=0.85, 95% CI=0.39-1.84, p=0.69) and TT homozygous (OR=1.06, 95% CI=0.21-5.43, p=0.95) in RF positive patients with response to the therapy. However, none of the observations were statistically significant.

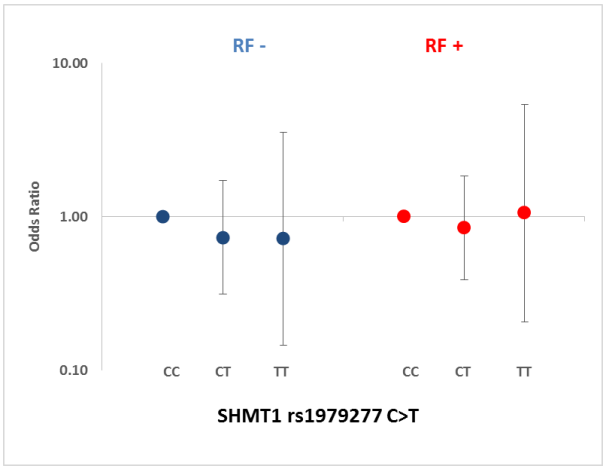

Figure 3.135. Association of SHMT1 rs1979277 SNP with drug response according to patients' rheumatoid factor.

The results of genotyping for SLC19A1 rs1051266 showed an almost null associations of TC heterozygous (OR=0.89, 95% CI=0.38-2.08, p=0.80) and an association of CC homozygous (OR=2.90, 95% CI=0.60-13.99, p=0.18) in RF negative patients and a trend of reverse association of TC heterozygous (OR=0.89, 95% CI=0.33-2.38, p=0.83) and CC homozygous (OR=0.52, 95% CI=0.18-1.52, p=0.23) in RF positive patients with response to the therapy. However, none of the observed results were significant (Figure 3.136).

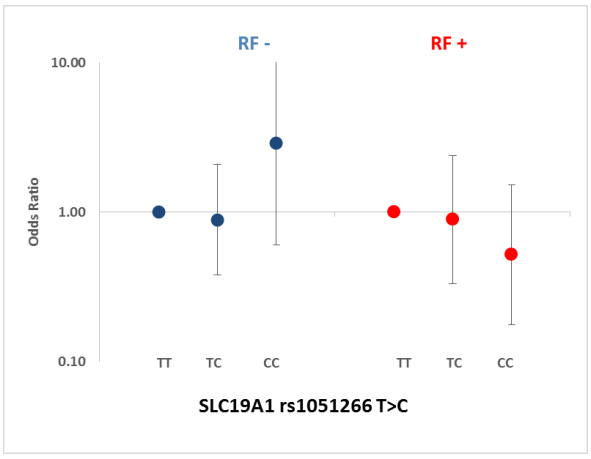

Figure 3.136. Association of SLC19A1 rs1051266 SNP with drug response according to patients' rheumatoid factor.

The results of genotyping stratified by RF status for MTHFR rs1801133 showed almost lack of association of GA heterozygous (OR=1.26, 95% CI=0.47-3.37, p=0.66) and AA homozygous (OR=0.78, 95% CI=0.26-2.33, p=0.66) in RF negative patients and also in GA heterozygous (OR=1.04, 95% CI=0.45-2.40, p=0.93) and AA homozygous (OR=1.03, 95% CI=0.32-3.35, p=0.97) in RF positive patients with response to the therapy. However, none of the observed associations were significant (Figure 3.137).

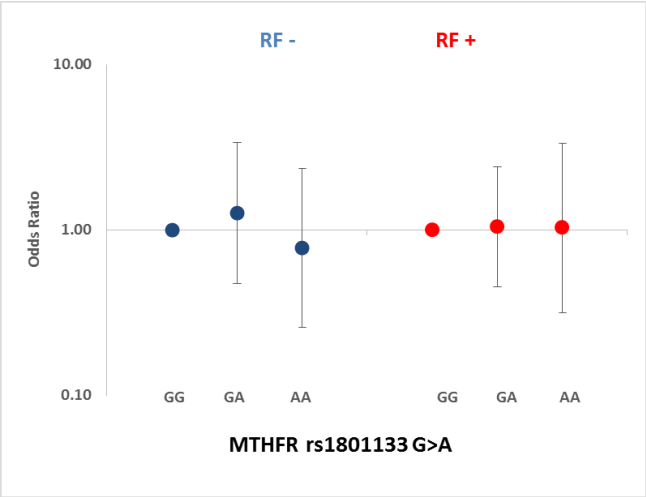

Figure 3.137. Association of MTHFR rs1801133 SNP with drug response according to patients' rheumatoid factor.

As shown in figure 3.138, MTHFR rs1801131 had reverse association of TG heterozygous (OR=0.74, 95% CI=0.32-1.70, p=0.49) and almost null association of GG homozygous (OR=1.14, 95% CI=0.24-5.50, p=0.88) in RF negative patients and reverse association of TG heterozygous (OR=0.47, 95% CI=0.21-1.07, p=0.07) and GG homozygous (OR=0.60, 95% CI=0.14-2.54, p=0.50) in RF positive patients with response to the therapy. None of the observations were statistically significant.

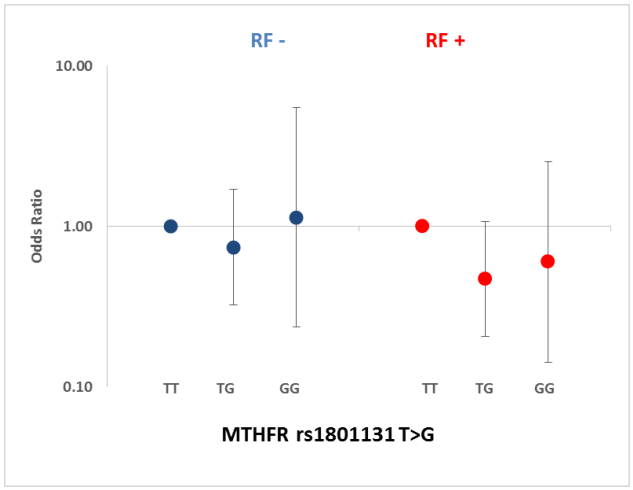

Figure 3.138. Association of MTHFR rs1801131 SNP with drug response according to patients' rheumatoid factor.

Pharmacogenetic of Early Rheumatoid Arthritis-ACPA

The results of genotyping for 6 candidate SNPs stratified by anti-Citrullinated Protein Antibody are presented in Table 3.14 and subsequently followed by the association graphs.

Gene variant	non-Responder						Responder					
	ACPA -			ACPA +			ACPA -			ACPA +		
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3
ATIC rs2372536 C>G	77	100	21	54	57	6	13	18	2	12	16	1
MTRR rs1801394 A>G	50	104	44	46	44	27	10	17	6	10	14	5
SHMT1 rs1979277 C>T	123	60	12	66	42	7	22	9	2	11	16	2
SLC19A1 rs1051266 C>T	61	87	44	33	63	18	11	20	2	5	12	11
MTHFR rs1801133 A>G	45	111	36	41	53	19	8	17	8	10	13	5
MTHFR rs1801131 T>G	104	71	17	52	52	10	15	14	4	8	19	1

Table 3.14. Genotyping data of non-responder and responder patients to the therapy stratified by anti-Citrullinated Protein Antibody. Genotype 1: Wild-type homozygous; **Genotype 2**: heterozygous; **Genotype 3**: Rare homozygous.

The results of genotyping for ATIC rs2372536 showed null association of CG heterozygous (OR=0.94, 95% CI=0.43-2.03, p=0.88) and an association of GG homozygous (OR=1.77, 95% CI=0.37-8.48, p=0.48) in ACPA negative patients and also almost lack of association of CG heterozygous (OR=0.79, 95% CI=0.34-1.83, p=0.60) and GG homozygous (OR=1.33, 95% CI=0.15-12.12, p=0.81) in ACPA positive patients with drug response. However, none of the observed results were significant (Figure 3.139).

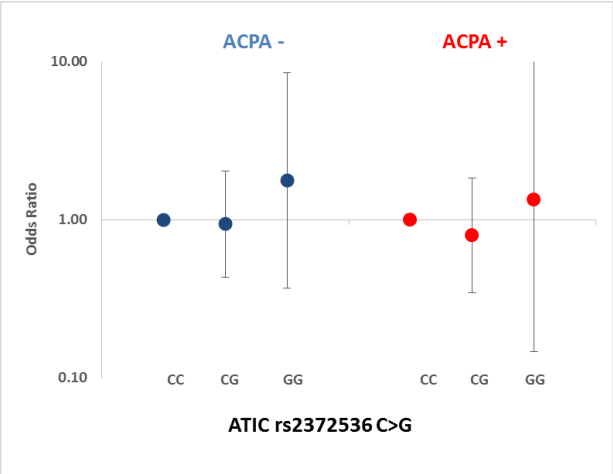

Figure 3.139. Association of ATIC rs2372536 SNP with drug response according to ACPA presence.

The results of genotyping for MTRR rs1891394 showed a trend of association of AG heterozygous (OR=1.22, 95% CI=0.52-2.86, p=0.65) and GG homozygous (OR=1.47, 95% CI=0.49-4.36, p=0.50) in ACPA negative patients, and also a reverse association of AG heterozygous (OR=0.68, 95% CI=0.27-1.70, p=0.42) and almost null association of GG homozygous (OR=1.17, 95% CI=0.36-3.79, p=0.80) in ACPA positive patients with response to the therapy. Although, none of the observed associations were significant (Figure 3.140).

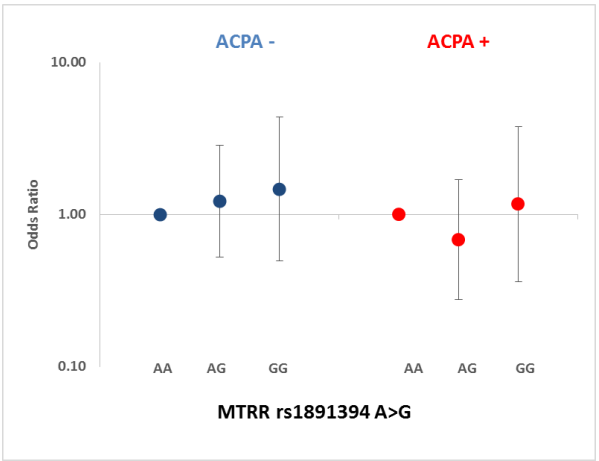

Figure 3.140. Association of MTRR rs1891394 SNP with drug response according to ACPA presence.

As shown in figure 3.141, SHMT1 rs1979277 had almost null association of CT heterozygous (OR=1.19, 95% CI=0.52-2.75, p=0.69) and TT homozygous (OR=1.07, 95% CI=0.22-5.13, p=0.93) in ACPA negative patients and reverse associations of CT heterozygous (OR=0.44, 95% CI=0.18-1.03, p=0.06) and TT homozygous (OR=0.58, 95% CI=0.11-3.18, p=0.54) in ACPA positive patients with response to the therapy. However, none of the observations were statistically significant.

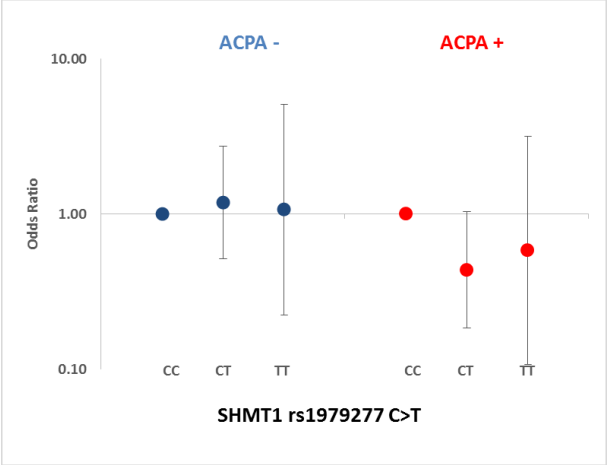

Figure 3.141. Association of SHMT1 rs1979277 SNP with drug response according to ACPA presence.

The results of genotyping for SLC19A1 rs1051266 showed an almost null association of TC heterozygous (OR=0.78, 95% CI=0.35-1.75, p=0.57) and an association of CC homozygous (OR=3.97, 95% CI=0.84-18.80, p=0.08) in ACPA negative patients and a trend of reverse association of TC heterozygous (OR=0.79, 95% CI=0.26-2.45, p=0.70) and CC homozygous (OR=0.25, 95% CI=0.07-0.83, p=0.02) in ACPA positive patients with response to the therapy. Association of CC homozygous in ACPA positive patients with drug response was significant (Figure 3.142).

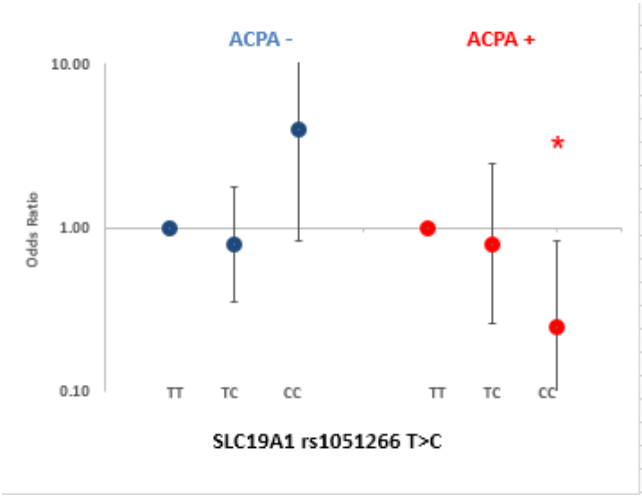

Figure 3.142. Association of SLC19A1 rs1051266 SNP with drug response according to ACPA presence.

The results of genotyping stratified by ACPA for MTHFR rs1801133 showed almost lack of association of GA heterozygous (OR=1.16, 95% CI=0.47-2.88, p=0.76) and AA homozygous (OR=0.80, 95% CI=0.27-2.34, p=0.70) in ACPA negative patients and also in GA heterozygous (OR=0.99, 95% CI=0.40-2.49, p=0.99) and AA homozygous (OR=0.93, 95% CI=0.28-3.09, p=0.91) in ACPA positive patients with response to the therapy. None of the observed associations were significant (Figure 3.143).

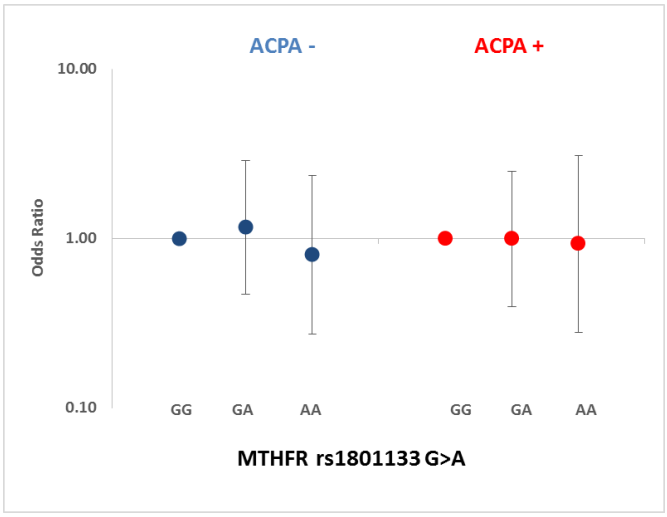

Figure 3.143. Association of MTHFR rs1801133 SNP with drug response according to ACPA presence.

As shown in figure 3.144, MTHFR rs1801131 had a trend of reverse association of TG heterozygous (OR=0.73, 95% CI=0.33-1.61, p=0.44) and GG homozygous (OR=0.61, 95% CI=0.18-2.07, p=0.44) in ACPA negative patients and a reverse association of TG heterozygous (OR=0.42, 95% CI=0.17-1.05, p=0.06) and an association of GG homozygous (OR=1.54, 95% CI=0.17-13.69, p=0.71) in ACPA positive patients with response to the therapy. None of the observations were statistically significant.

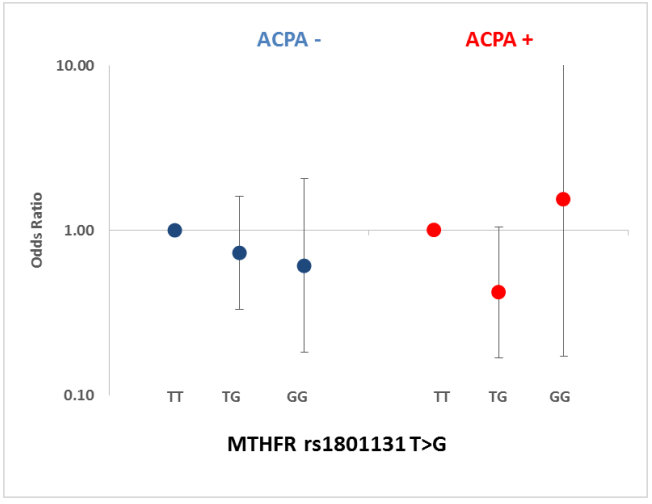

Figure 3.144. Association of MTHFR rs1801131 SNP with drug response according to ACPA presence.

MDR Analysis

The result of MDR analysis revealed an interaction between SHMT1 rs1979277 and smoking in response to the therapy (figure 3.145). According to the figure 3.146, the percentage of responders to the therapy was higher in CC homozygous (94.62%) comparing to CT heterozygous (98.09%) and TT homozygous (50%) among non-smokers. The same trend was present in smokers that indicates the role of C allele in response to the therapy. The probability of responding to the therapy in CC homozygous and CT heterozygous non-smokers or TT homozygous smokers was significantly different comparing to CC homozygous and CT heterozygous smokers or TT homozygous non-smokers (OR=0.27, 95% CI=0.13-0.55, p=4.01E-04).

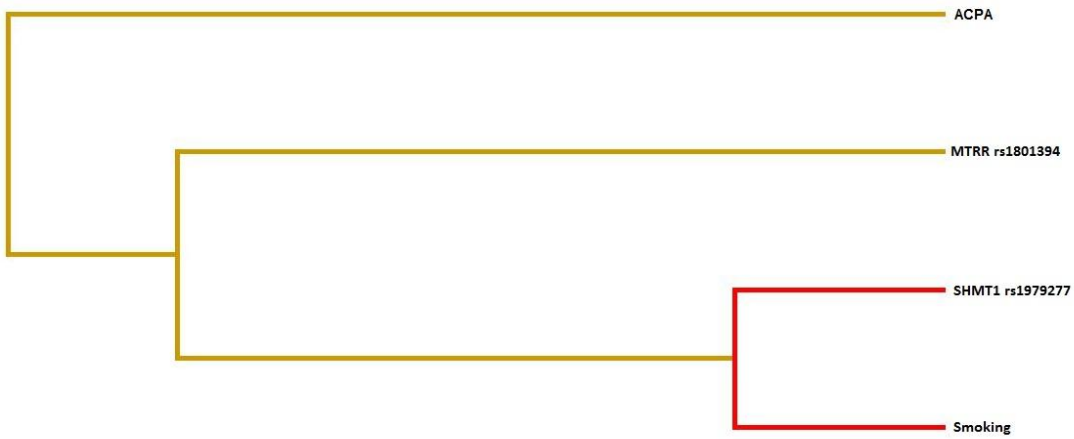

Figure 3.145. MDR analysis. Interaction of SHMT1 rs1979277 and smoking in response to the therapy.

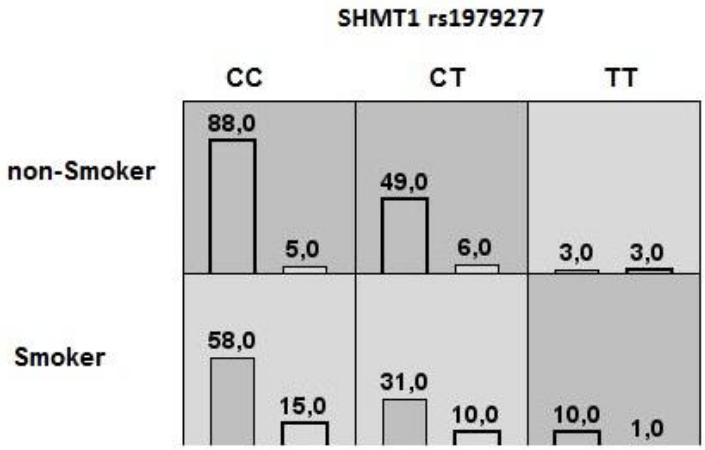

Figure 3.146. MDR analysis. Interaction of SHMT1 rs1979277 and smoking in response to the therapy.

Dark gray bar: Responders, Light gray bar: non-Responders.

Epigenetic of Tissue

The third part of the present study focused on differences between epigenetic of 1) different tissues from 6 RA patients, 2) fifty healthy controls, fifty ERA and thirty RA patients, and 3) response to the therapy in patients who developed early rheumatoid arthritis.

Table 3.15 shows the result of methylation level in LINE-1 of gDNA extracted from white blood cells and synovial cells. As shown in figure 3.147, synovial cells are hypomethylated compared to white blood cells. However, this difference was not significant. Therefore, gDNA from white blood cells had been considered for further epigenetic study.

Case	$\lvert\Delta\rvert$ blood-tissue	± mean SD	Paired t-student test	*p*
RA001	0.070	0.371	1.457	0.891
RA002	0.044	0.061	1.275	0.864
RA003	0.002	0.020	0.035	0.513
RA004	0.246	0.006	0.515	0.683
RA005	0.023	0.034	0.447	0.661
RA006	0.056	0.011	1.308	0.870

Table 3.15. Intra-patient comparison of LINE-1 methylation between white blood cells and synovial cells.

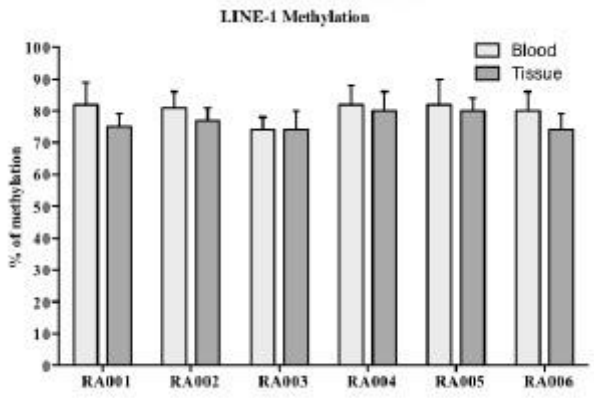

Figure 3.147. Intra-patient comparison of LINE-1 methylation between white blood cells and synovial cells.

Epigenetic of Rheumatoid Arthritis

The LINE-1 methylation analyses showed that there were no significant differences between healthy controls (80.0±15.63%) and ERA T0 (79.6±9.91%), ERA T6 (81.1±9.60%) and RA (80.1±5.93%) patients. In addition, there were no significant differences between ERA T0 and ERA T6. The methylation difference between RA and both ERA T0 and ERA T6 was not significant (Table 3.16 and Figure 3.148).

Comparison	|Δ| means	±Δ SEM	unpaired t-student test	*p*
CTRL vs ERA T0	0.005	0.039	0.124	0.9017
CTRL vs ERA T6	0.009	0.025	0.377	0.7077
CTRL vs RA	0.0003	0.023	0.0140	0.9890
ERA T0 vs ERA T6	0.013	0.024	0.568	0.5765
ERA T0 vs RA	0.005	0.059	0.077	0.9390
ERA T6 vs RA	0.010	0.036	0.265	0.7923

Table 3.16. LINE-1 methylation comparison between healthy controls and ERA and RA groups.

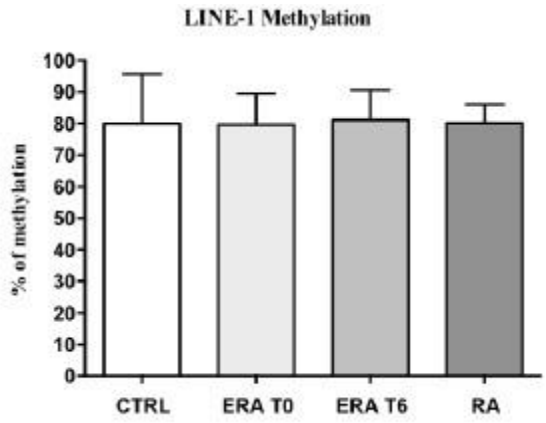

Figure 3.148. LINE-1 methylation comparison between healthy controls and ERA and RA groups.

Epigenetic of response to therapy

As presented in table 3.17 and figure 3.149, the different level of LINE1 methylation considering the response to the therapy was not statistically significant in non-responders (83.1±5.96%) compared to ERA low-responders (78.1±7.87%) and ERA high-responders (82.8±11.20%) after 6 months of treatment with methotrexate.

Comparison	|Δ| means	±Δ SEM	unpaired t-student test	*p*
ERA T6 No Responders vs ERA T6 Low Responders	0.050	0.051	0.983	0.34
ERA T6 No Responders vs ERA T6 High Responders	0.005	0.056	0.082	0.94
ERA T6 Low Responders vs ERA T6 High Responders	0.045	0.031	1.453	0.15

Table 3.17. LINE-1 methylation comparison between ERA non-responders, ERA low-responders and ERA high responders after 6 months of treatment with methotrexate.

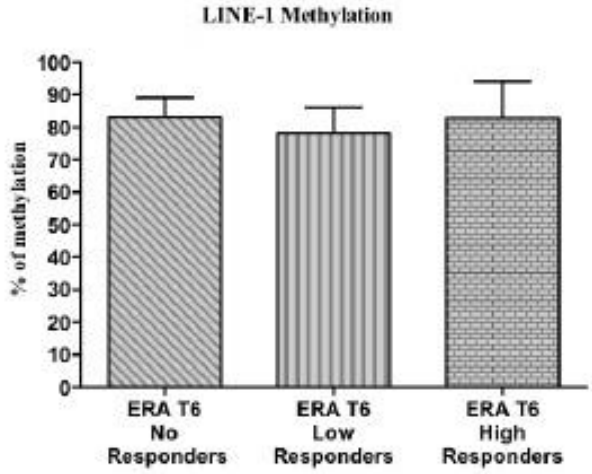

Figure 3.149. LINE-1 methylation comparison between ERA non-responders, ERA low-responders and ERA high responders after 6 months of treatment with methotrexate.

Chapter 4

Discussion

Genetics of Rheumatoid Arthritis

Undifferentiated arthritis is a common inflammatory type of arthritis which is known by joint swelling, pain, and stiffness and is not classified as a specific rheumatologic disease. It is estimated that 45% to 55% of patients will reach remission spontaneously; however, approximately 32% of them will develop rheumatoid arthritis (Wevers-de Boer et al., 2013). Considering the significant overlap with RA, UA is considered as a promising window of opportunity in order to manage the pathology before being established.

Due to nonspecific signs, undifferentiated arthritis is usually an exclusion condition before meeting criteria for RA. Considering the 80% to 85% sensitivity of 2010 ACR classification criteria for RA, there is possibility of overlooking initial diagnosis of patients with RA features which is supported by reports mentioning that about one-third of UA patients could subsequently diagnosed as having rheumatoid arthritis (Aletaha *et al.*, 2010; Pile, 2012) that according to our study, 20% of patients developed RA.

As reviewed by Pile, primary criteria for diagnosis UA patients with high risk of developing RA, according to the Leiden Early Arthritis Clinic Prediction Rule, includes: age, female sex, family history of RA, early morning stiffness, increased C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) and swollen joint count, positive RA and ACPA, pattern of join involvement and 8 weeks or longer duration of first signs. As much as manifestation of these features is evident, the likelihood to develop RA is increased (Pile, 2012). The ACR/EULAR guidelines and van der Helm-van Mil prediction model are considered promising in distinguishing UA patients with higher risk of RA development (Pile, 2012; van der Halm-van Mil *et al*., 2007; Bedran *et al.*, 2013).

Persistence of inflammatory arthritis especially in the presence of RF and ACPA is an important factor in developing RA that among the UA patients in this study, the percentage of RA and ACPA positivity were 31% and 24%, respectively. The presence of these two factors is associated with long morning stiffness, increased tender and swollen joint count, elevated ESR, erosive changes, and inefficient results of treatment (Aletaha *et al*., 2010; Mjaavatten *et al*., 2010).

Currently, the challenge is to distinguish UA patients with high risk of RA development in order to initiate early DMARD therapy specific to RA.

Since 2007, rapid progresses in genome-wide association study (GWAS) have contributed to findings of more than 100 genetic loci associated with rheumatoid arthritis development which belong to HLA and non-HLA loci (Okada *et al.*, 2019).

Our study focused on 14 SNPs, seven belonging to HLA and the other seven belonging to non-HLA genes, for association with early rheumatoid arthritis development in a cohort of undifferentiated arthritis patients. No significant associations of putative SNPs within the genes: HLA, PTPN22, PADI4,

STAT4, CTLA4, TRAF1, IL-10 and IL-6 with ERA development under also different stratifications were found (Owen *et al*., 2013).

It has been reported that the Major Histocompatibility Complex (MHC) region located in chromosome 6 contributes to 30% to 50% of overall genetic risk of rheumatoid arthritis (van der Woude *et al*., 2009).

In MHC region, there are three classes of Human Leucocyte Antigen (HLA) genes which are responsible for surface antigen production on lymphocytes and manage immune response by discrimination of self and non-self antigens and their presentation to T lymphocytes (Gough SCL *et al.*, 2007).

HLA-G is among susceptible genes for immune diseases because of its important role in immunosuppression. HLA-G belongs to class I and has low polymorphism and encodes molecules either for cell surface or as soluble form (Verbruggen et al., 2006). It acts reciprocally with immune system cells and suppresses their response by different pathways (Veit TD et al., 2014). It impedes cytotoxic $CD8^+$ T-lymphocytes activity by acting on their cell mediated cytolysis. It decreases the cytotoxic activity of natural killer cells and their proliferation. Beside, HLA-G molecules could suppress $CD4^+$ T-cell which act in allo-proliferative response that is initiated by external factors and is able to induce a certain immune response under specific conditions (Bahri R et al., 2006). HLA-G molecules also change the physiological function of the APCs and their proliferation (Horuzsko et al, 2001). Beside, it is reported that HLA-G mRNA undergoes alternative splicing and forms 7 different protein isoforms, four of which bind to membrane and three are soluble and secreted (sHLA-G) (Murdaca et al., 2016)

Except 5' and 3' UTRs, HLA-G is not very polymorphic. The 3' UTR region has an important function in post-transcriptional regulation of HLA-G expression and 14bp insertion/deletion (14bp indel, rs16375) and rs1063320 SNP variant within this region may modify gene expression. The 14bp in/del variant is correlated with messenger RNA stability. The stability of mRNA in presence of insertion (+14bp 5'-ATTTGTTCATGCCT-3') reduces which results in lower production of HLA-G leading to decreased levels of soluble HLA-G. Besides, the variant contributes to alternative splicing; the insertion-containing transcript has additional splicing site that excludes 92 bases including the insertion region itself which result in decreased HLA-G expression. Besides, the microRNAs are affected by presence or absence of 14bp variant which in turn modifies HLA-G expression and subsequent processes (Rebmann et al., 2014).

Rs1063320 SNP is mapped at position +3142, approximately 200bp far from the 14bp in/del variant It modifies the binding of some miRNAs. The allele G is a target of 3 miRNAs, namely miR 148a, 148b and 152, which contribute to reduction of HLA-G expression (Tan et al., 2007) and it has been shown that homozygous GG contributes to RA development (Veit et al., 2014)

On the other hand, the regulatory HLA-G 5' region contains CpG islands. The rs1233334 G>A variant has been found in upstream of transcription site (-725bp). The G allele contributes to the creation of a CpG dinucleotide island that has been reported to be methylated in the DNA sequence of blood mononuclear cells and potentially results in a different methylation pattern. It has been suggested that -725G allele is associated with HLA-G expression because of different methylation pattern that interfere with binding of Interferon Factor-1 (IRF-1) to Interferon Specific Regulatory Element (ISRE) sequence (Ober et al., 2003). The -725G allele is likewise correlated with higher levels of promoter activity that result in an increase of HLA-G transcription. In contrast with allele G, the -725C allele correlated with lower activity of the promoter. (Jassem et al., 2013).

HLA-DRB1 locus which belongs to HLA class II, is correlated with RA development. It provides peptides to lymphocytes and is transcribed and expressed in Antigen Presenting Cells (APC). The heterodimer molecule includes α-chain (DRA) and β-chain (DRB), both have extracellular and trans-membrane domains. Shared epitope (SE) in the third hypervariable region of HLA-DR β chain, is highly considered in RA development (Pratesi F *et al*., 2013). The most accepted hypothesis suggests that autoimmunity begins by molecular mimicry or by specific peptides presentation to lymphocyte T receptor (TCR), which results in modification of T $CD4^+$ cells response (Beri et al, 2005). In addition, HLA-DRB1 rs660895 A>G and rs6910071 A>G SNPs have been shown to associate with RA development. Both of them are in considerable linkage disequilibrium with HLA-DRB1 SE with a 300 kb distance. (Mosley et al., 2016).

HLA-DQB2 has been known to be associated with RA development independent of HLA-DRB1 SE (Kochi *et al*., 2004) and an interaction between HLA-DQA2 (rs9275595) and HLA-DQB2 (rs10807113) has been reported with 11-fold higher risk of RA development (Liu et al., 2011). Both genes belong to class II and encode HLA molecules which are composed of 2 α-chains (DQA2) and 2 β-chains (DQB2). HLA-DQA2 codes a protein for APCs that involve in processing and presenting antigens to $CD4^+$ T-lymphocytes. HLA-DQB2 is suggested either to play a role similar to HLA-DQA2 or acts as a pseudogene (Pera et al., 2000).

Genetic risk factor located outside the MHC region

PTPN22: it has been found that the rare allele of SNP rs2476601 in the *PTPN22* gene, mapped in chromosome 1p13, contributes to RA development. The *PTPN22* was increased in RA patients versus healthy controls in studies of multiple North American and European Caucasian populace, but not in Koreans (Kochi *et al*. 2009). The *PTPN22* gene is highly considered in autoimmune diseases and is expressed in a class of immunological-related tissues (Begovich *et al*. 2004) and encodes the intracellular protein lymphoid tyrosine phosphatase (LYP). Protein tyrosine phosphatases (PTPs) has an important role in signal transduction and are necessary for the T-cell antigen receptor (TCR) signaling pathway. LYP is found to act as an efficient suppressor of T-cell activation (Hill *et al*. 2002).

PADI4: The genetic variant, *PADI4* gene is located on chromosome 1 (1p36). The *PADI4* gene encodes the type 4 peptidylarginine deiminase enzyme that by catalyzing the posttranslational modification of arginine to citrulline contributes to the production of citrullinated proteins (Vossenaar *et al*. 2004). It has been found that antibodies against these citrullinated peptides are significantly specific for RA and generally occur before the disease development that indicate an important role in RA pathogenesis. *PADI4* was the first non-HLA genetic risk factor found to be associated with RA, particularly in Japanese population (Suzuki *et al*. 2003). Association has likewise been reported in North American and Korean populations (Plenge *et al*. 2005; Kang *et al*. 2006). Lack of evidence for association of *PADI4* with RA in Swedish, Spanish, and UK populations has been reported (Caponi *et al*. 2005; Martinez *et al*. 2005). The rs2240340 SNP in *PADI4* gene located in intron 3 showed strongest association and a meta-analysis found a significant association between RA and the SNP in Asian population (Takata *et al*. 2008).

Other genetic or environmental factors that interfere with the genetic factor may be present in a certain population and could affect disease susceptibility in one population but not in the other. Despite of involvement of peptidylarginine deiminases in creation of ACPA, there is no evidence to reinforce that *PADI4* genotypes are associated with ACPA levels (van der Helm-van Mil and Huizinga 2008).

STAT4: The signal transducer and activator of transcription 4 (*STAT4*) gene is located on the q arm of chromosome 2 is another non-*MHC* gene correlated with RA development (Remmers *et al*. 2007). The JAK/STAT pathway is the signaling target of several cytokines which play important biological roles in rheumatoid synovial inflammation (Walker and Smith 2005). Particularly, STAT4 transfers induced signals from multiple cytokines such as IL-12, IL-23, and type I interferons (IFNs) (Watford *et al*. 2004). STAT family members during response to growth factors and cytokines are phosphorylated by the receptor-related kinases, and later create homodimers or heterodimers which by translocating to the cell nucleus function as transcription activators. Association of *STAT4* with RA was identified by a combination of linkage and candidate gene investigations. Unlike *HLA-DRB1* and *PTPN22*, the association of *STAT4* with RA development is not strong. Four closely-related polymorphisms, including rs11889341, rs7574865, rs8179673 and rs10181656, create a susceptibility haplotype that is tagged by the T allele (rs7574865), showed the strongest association with RA development (Remmers *et al*.2007). Association of *STAT4* SNP rs7574865 with RA development was proved in European, North American and Asian populations (Zervou *et al*. 2008; Lee *et al*. 2010). Europeans found to have the lowest (21.4%) and Asians the highest (32.0%) prevalence of the rs7574865 variant among their population (Lee *et al*. 2010). Stratification of RA patients based on presence of ACPA antibody revealed a significant association between the rs7574865 SNP and RA in both $ACPA^+$ and $ACPA^-$ RA cases (Orozco *et al*. 2008). It has been reported that T allele homozygous of rs7574865 patients with early arthritis, could develop a more severe form of the disease with enhanced disease activity and disability (Lamana *et al*. 2012).

CTLA4: Genes involved in the regulation of T-cell responses could mainly determine the susceptibility to RA. *CTLA4* is mapped on chromosome 2q33 and polymorphisms within it found to be associated with RA (Lee *et al*. 2003; Zhernakova *et al*. 2005; Suppiah *et al*. 2006).A large cohort from the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) collections and the North American Rheumatoid Arthritis Consortium (NARAC) have reported an association of *CTLA4* with the development of RA (Plenge *et al*. 2005). These findings correlated well with a meta-analysis that confirmed an association of *CTLA4* gene polymorphism with RA in Caucasians which also revealed that *CTLA4* enhance the development of ACPA-positive as compared with ACPA-negative RA (Daha *et al*. 2009). Similar to *PTPN22* and *HLA-DBR1* SE, these studies clearly demonstrate that *CTLA4* affects the development of RA only in $ACPA^+$ patients and also point to a divergence in pathology dependent ACPA status.

TRAF1-C5: The TNF receptor-associated factor 1 (TRAF1) and complement component 5 (*C5*) genes are located on chromosome 9 is a member of the TNF receptorassociated factor (TRAF) family, a group of adaptor proteins that bond TNF receptor family members (e.g. TNF-*α)* to downstream signalling (Arch *et al*. 1998). The molecules play a role in signaling pathways which involved in cell proliferation and differentiation, apoptosis, bone remodeling and activation or suppression of cytokines (Speiser *et al*. 1997). It is reported that the GG homozygotes at the *TRAF1-C5* SNP rs3761847 in cases with RA have a significantly increased risk of death from malignancy or sepsis, conceivably leading to identification of patients for appropriate screening (Panoulas *et al*. 2009).

In 2007, the SNP has been identified to be significantly associated with RA in Europeans (Plenge *et al.*, 2007). Several studies aimed to survey the association in different populations among which North Americans, Swedish (Plenge *et al.*, 2007) Japanese 9Nishimoto *et al*., 2010) and Han Chinese Asians (Zhu *et al*., 2011) showed an association for developing RA; however, lack of association has been reported in Korean (Han *et al.*, 2009) and Chinese (Huang *et al.*, 2019). Although, our study was investigating the association of the SNP with conversion of UA to RA, non-significant evidence was obtained. After stratifying by sex, a significant reverse association between allele G and RA development in males was found. After evaluating under different genetic models, the significance was observed under dominant model. It has been shown that disease-associated SNP of *TRAF1* by changing its protein level critically regulates signaling pathways and subsequently the level of proinflammatory cytokines in human and animal models (Abdul-Sater *et al.*, 2017). Although some evidence have been presented for pathogenesis role of TRAF1 (Abdul-Sater *et al.*, 2017) our understanding of exact mechanism by which this genetic variation contributes to RA development is limited.

There are several major factors that integratedly influence overall immune response in males and females differently which include: 1) sex chromosomes, 2) hormonal mediators and 3) environmental factors (Klein and Flanagan, 2016). These aspects opens windows of opportunity to further investigate the observed different association results between opposite sexes with *TRAF1* G allele in RA development. As it has been reported, males and females possess different level of certain immune cell

populations, among which men have lower T cells (Bernin *et al.*, 2016); as such, this highlights the effect of TRAF1 in regulating T cells and proportional subsequent of cytokines dysregulation (Huang *et al.,* 2019), which is strongly correlated with the pathogenesis of UA and its progression to RA (Brzustewicz *et al*., 2017).

IL-10: IL-10 is one of the main immunoregulatory cytokines which is usually produced by Th2 lymphocytes, B lymphocytes or macrophages (Munsh *et al*., 2010) and contributes significantly to the pathogenesis of RA (Schotte *et al*., 2005). IL-10 can control the down-regulation of the inflammatory response in RA. It has been found that IL-10 inhibits joint swelling and deformation as well as necrosis of cartilage in RA animal model (Ying *et al.*, 2010; Ji *et al*., 2005).

The human IL-10 gene is located on 1q31-32, and some polymorphisms in the promoter region including rs1800896 A>G, rs1800871C>T and rs1800872C>A have been found (Hee *et al.,* 2007; Bartelds *et al.,* 2009; Won *et al.,* 2010). The rs1800896 A>G polymorphism which is located at putative regulatory regions in the promoter of the IL-10 gene can modify the binding sites of different transcription factors that in turn could change the production of IL-10 protein and has been considered as a potential susceptible risk factor for RA (Hee *et al*., 2007). Several investigations have reported the association between rs1800896 A>G polymorphism in the IL-10 gene and the risk of RA (Nemec *et al.,* 2009; Pawlik *et al.,* 2005; Hee *et al*., 2007; Menegatti *et al.,* 2009; Cantagrel *et al*., 1999; Trajkov *et al.,* 2009; Ates *et al.,* 2008; Paradowska-Gorycka *et al.,* 2010; de Paz *et al.,* 2010; Padyukov *et al*., 2004), however the results were consistent. This could happen due to an underpowered study especially when the sample size is relatively small (Lui *et al*., 2010).

The rs1800896 A>G polymorphism lies within a Ets transcription factor binding site that can change the production of IL-10 protein (Hee *et al*., 2007). A study reported that the G allele was correlated with increased *in vitro* IL-10 production and the A allele was associated with decreased levels (Martinez *et al*., 2003). Elevated levels of IL-10 were observed in serum and synovial fluid from RA patients that indicates association between the G allele and the increased risk of RA. However, a meta-analysis reported that the G allele could have a correlation with decreased risk of RA.

IL-6 has a function in induction of the immunological abnormalities and in the progression of joint and systemic inflammation in RA (McInnes and Schett, 2011; Furst and emery, 2014). Excessive levels of IL-6 are found in RA patients, especially in thin tissue layer which cover the joints, and involve in the painful and persistent damage of the joints and chronic inflammation (Yoshizaki *et al*., 1998; Lipsky, 2006). IL-6 has been demonstrated to contribute to the production of RA autoantibodies including RF and ACPA, by affecting plasmablasts (Suematsu *et al*., 1989). IL-6 upregulates the nuclear factors of activated T cells (NFAT)c2 and c-maf that enhances type 2 T helper (Th2) cell differentiation and which in turn affects T cell effector functions, whereas by inhiniting interferon gamma (IFN-g)-signaling, it suppresses type 1 T helper (Th1) cell differentiation (Rincon *et al.,* 1997). Elevated levels of IL-6 result in a range of complications such as fatigue, depression and mood disorders, weight loss, anemia, high risk of cardiovascular disease and osteoporosis in RA patients (Yoshizaki *et al.,* 1998; Lipsky, 2006;

Maggio *et al.*, 2006). The SNP rs1800795 C>G in the promoter region of IL-6 and the risk of RA development has been investigated in different populations across the world with conflicting results (Li *et al.*, 2014; Li *et al.*, 2015; Shafia *et al*., 2014). The exact and ethnicity-specific nature of this genetic association still remains unknown. There are several studies that investigated the polymorphism and its association with activity and susceptibility to RA (Li *et al.*, 2014; Li *et al.*, 2015; Shafia *et al*., 2014), and demonstrated its significant association mainly with the Asian population (Li *et al.*, 2014; Li *et al.*, 2015; Shafia *et al*., 2014; You *et al.*, 2013; Huang *et al.*, 2007). Nevertheless, some studies in European population have likewise correlated this polymorphism with the risk of RA development (Trajkov *et al.*, 2009; Palwik *et al.*, 2005); however, most of the studies in Caucasians has resulted in no association at all (Zavaleta-Muniz *et al.*, 2013; Arman *et al*., 2012; Emonts *et al*., 2011; Palomino-Morales *et al*., 2009). Overall, these reports show a lack of global consensus among different populations.

Pharmacogenetics of Rheumatoid Arthritis

All inflammatory arthritis patients including undifferentiated arthritis should begin DMARD therapy along with more assessment for infectious, crystalline, malignant, and metabolic etiologies. It has been found that treatment with DMARD based on serological markers decreases disease progression within 1 year (Pile, 2012). Once DMARD therapy is started, methotrexate with other DMARDs or corticosteroids is the first choice (Wevers-de Boer *et al*., 2013; Smolen *et al*., 2014). Currently, it is recommended that the therapy should be started as early as the disease is diagnosed (Wevers-de Boer *et al*., 2013; Pile, 2012; Smolen *et al*., 2014; Lopez-Olivo *et al.*, 2018). Some studies have reported the evidence of delayed treatment in decreasing remission induction.

Methotrexate that is folic acid antagonist is the most used DMARD in the treatment of RA, either as a monotherapy or in combination with other DMARDs (Kremer, 2004; Smolen *et al*., 2007). Apart from its use in RA treatment, MTX is routinely prescribed in the treatment of other malignant diseases, likewise (Gonen and Assaraf, 2012; Karnofsky, 1993). The anti-inflammatory effects originated from low-dose MTX treatment of RA can engage mechanisms that are different from those involved with high-dose MTX treatment of different cancers. Although, considering the cellular pharmacologic influences of MTX, there are overlapping mechanisms of action in RA and cancer. MTX inhibits proliferation of malignant cells by suppressing the de novo biosynthesis of purines and pyrimidines (Gonen and Assaraf, 2012). In addition, MTX blocks the homocysteine and methionine cycle engaged in methylation of DNA, RNA, and proteins. MTX induces blocking of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARTF/ATIC) that in turn increases the recreation of the endogenous anti-inflammatory mediator adenosine (Gonen and Assaraf, 2012; Cronstein, 2005; Cutolo *et al*., 2001). MTX has also been demonstrated to have further pharmacologic effects such as generation of reactive oxygen species through JNK activation (Spurlock *et al.*, 2012; Phillips *et al*., 2003), induction of T cell apoptosis (Genestier *et al.*, 1998), and suppression of NF-κB (Majumdar and Aggarwal, 2001), for which the contribution of its therapeutic effectiveness is not fully understood. The

treatment with MTX mainly interferes with folate homeostasis, which is strictly controlled at several levels such as: 1) pathways of cellular uptake and efflux of MTX/folate; 2) intracellular metabolism and maintaining of MTX/folate; and 3) expression of key regulatory folate-dependent enzymes ((Gonen and Assaraf, 2012; Van der Heijden *et al*., 2007). The proton-coupled folate transporter (PCFT/ *SLC46A1*) absorbe oral MTX in the upper small intestine and have a better functions at the acidic pH of the duodenum and jejunum that both them have predominant expression of PCFT (Gonen and Assaraf, 2012; Qiu *et al.*, 2006). Immune cells uptake MTX by the reduced folate carrier (RFC/*SLC19A1*), which is a third transport route and include a receptor-mediated process via folate receptor β (FRβ), is expressed mainly in macrophages and can be functional in activated macrophages in inflamed synovial tissue (Nakashima-Matsushita *et al.*, 1999; Van der Heijden *et al*., 2009). When MTX enters into the cell, it becomes metabolically active by the enzyme folylpolyglutamate synthetase (FPGS) to a polyanionic polyglutamate form which is kept highly within cells (Stark *et al.*, 2009). γ-glutamyl hydrolase (GGH) can reverse polyglutamylation by cleaving off the polyglutamyl chain from folates and polyglutamatable antifolates like MTX (Rots *et al.*,1999). MTX inhibits the important enzyme dihydrofolate reductase (DHFR) while MTX polyglutamates (MTXGlu) strongly suppress thymidylate synthase (TYMS) that both of them are essential enzymes for the de novo biosynthesis of purines and pyrimidines which are needed for DNA replication and cellular proliferation. After DHFR suppression, MTXGlu and dihydrofolate polyglutamates are increased and can block other folate-dependent enzymes downstream of DHFR like glycinamide ribonucleotide formyltransferase (GART) and methylenetetrahydrofolate reductase (MTHFR) (Rots *et al*., 1999; Allegra *et al*., 1085) that likewise play a role in de novo purine biosynthesis and methylation of DNA, RNA, and proteins, respectively. At the end, folate/MTX are discarded as the nonpolyglutamated form by ATP-binding cassette (ABC) transporters including ABCC1 and ABCG2 (Gonen and Assaraf, 2012; Hooijberg *et al.*, 1999). In spite of playing a key role in RA therapies, mechanism of action of MTX remains unknown, and lack of a clinical response and the risk of toxicity in a considerable fraction of patients are still to solve.

Our study has focused on 6 SNPs in five key MTX/folate metabolic pathway genes for association with MTX efficacy in this cohort of ERA patients. No significant associations of SNPs within the genes: ATIC, MTRR, SHMT1, SLC19A1, and MTHFR with MTX efficacy under also different stratifications were found. There have been several studies performed to determine the response to MTX in RA patients by applying candidate gene approach, genotyping isolated SNPs within the gene and evaluating the association. Our study focused likewise on candidate genes approach in the MTX/folate metabolic pathway due to success of this approach in a number of other common treatments (Owen *et al.*, 2013). There is a report for association of rs2372536 SNP in *ATIC* with response to MTX in RA patients. The SNP is mapped on exon 5 of the gene, and it is a threonine to serine substitution at position 116 of the *ATIC* gene. There is an increase in supporting the role of the *ATIC* gene in the response to MTX treatment. ATIC is the enzyme that is highly blocked by MTX polyglutamates and involve in the

conversion of AICAR to formyl-AICAR. AICAR and its metabolites suppress two crucial enzymes in adenosine metabolism causing intracellular accumulation of adenosine. Adenosine is a strong anti-inflammatory mediator and it is believed that maybe this pathway leads to the functionality of MTX (Chan and Cronstein, 2010). Defects in ATIC can potentially cause low enzyme activity and affect AICAR accumulation and adenosine secretion (Hinks *et al.,* 2011).

MTRR gene is located at 5p15.31 and the rs1801394 A>G polymorphism in the gene results in the replacement of isoleucine with methionine at codon 22 (Wilson *et al.,* 1999). There are some reports demonstrating that AA homozygous cases had increased homocysteine levels compared with those who had minor genotypes (Gaughan *et al.,* 2001; Geisel *et al*., 2001); on the other hand, one investigation has reported that genotype was not an important predictor of homocysteine level (O'Leary *et* al., 2002).

SHMT1 mapped on chromosome 17p11.2 and encodes a vitamin B6-dependent enzyme. This enzyme transfers reversibly the hydroxymethyl group of serine to tetrahydrofolate to produce 5,10-methylene tetrahydrofolate and glycine. It also catalyzes the reversable conversion of 5,10-methylene tetrahydrofolate to 5-formyl tetrahydrofolate. Production of 5-formyl tetrahydrofolate contributes to the one-carbon homeostasis during the fast cell proliferation steps (Fu *et al.,* 2005; Niclot *et al.,* 2006). The non-synonymous rs1979277C>T SNP located in the 12th exon of the *SHMT1* gene causes an amino acid substitution from leucine to phenylalanine. This common polymorphism contributes to reduced plasma and red blood cell folate levels in homozygous CC individuals (Heil *et al.,* 2001), and can lead to reduction of one carbon moieties for both remethylation of homocysteine and DNA synthesis (Zhao *et al*., 2015).

SLC19A1 gene encodes the solute carrier family 19 member 1 (SLC19A1) protein that transports the vital nutrient folate into an out of the cell and therefore contributes to the intracellular regulation of folate concentrations. It is main folate transporter and thus is generally expressed in all tissues of the body (Lapenta *et al.,* 1998). The rs1051266 A>G SNP in the *SLC19A1* gene is associated with poor health outcomes and the risk allele 'A' is correlated with increased homocysteine levels in the blood (Liang *et al.*, 2014; DeVos *et al.,* 2008). Increased homocysteine level could damage tissues especially the specialized endothelial tissue that covers the blood vessels and heart. Therefore, it contributes to increased cardiovascular risks (Cattaneo, 1999) along with various neurological issues (Morris, 2003). In spite of being associated with increased homocysteine level, the risk allele 'A' has not been described with direct health effects. It proposes that the risk 'A' allele of rs1051266 may not be enough to cause adverse health outcomes.

The *MTHFR* gene is mapped on 1p36.3 (Goyette *et al.,* 1994) and its enzyme mediates the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF) (Choi and Mason, 2000; Yadav *et al.,* 2015) and has several polymorphisms which the most studied ones are: 1) rs1801133 C>T at nucleotide 677 which cause an alanine to valine substitution in the protein

(Frosst *et al.*, 1995), and 2) rs1801131 A>C at nucleotide 1298 that results in an alanine to glutamate substitution in the protein. These polymorphisms are located 2.1 kb apart. It has been found in rs1801133 C>T polymorphisms that heterozygous CT cases have 65% and homozygotes TT individuals have 30% activity in their MTHFR enzyme activity compared with the normal homozygous CC cases (Rozen, 1997). The investigation showed that compared with CC homozygotes, heterozygotes have 10% and TT homozygotes have 18% lower red cell folate levels (Molloy *et al*., 1997). TT homozygous cases have also shown decreased level of plasma folate and vitamin B12 and elevated homocysteine levels (Ma *et al*., 1997, 1999). Considering DNA methylation, it has been reported that DNA from TT homozygous cases had a significant higher methyl group acceptance capacity compared to DNA from CC homozygous subjects (Stern *et al*., 2000) although, this finding was not confirmed in a larger study (Narayanan, 2001). Similarly for rs1801131 SNP, compared with the wild type homozygotes AA, enzyme activity has been found to be decreased moderately in heterozygotes (AC) and highly in homozygote variants (CC) (van der Put *et al*.,1998). However, reports for rs1801131 and plasma folate and homocysteine are inconsistent (Chen *et al.*, 2002; Friedman *et al.*, 1999; Lievers *et al.*, 2001; Weisberg *et al.*, 1998).

Epigenetics of Rheumatoid Arthritis

DNA methylation is a process by which a methyl groupbiochemically binds with the cytosine at position 5 of ring carbon to form 5-methylcytosine (5-mC). DNA methylation happens preferentially in CpG dinucleotides located across the whole gene either as single dinucleotide or concentrated into CpG-islands close to gene promoters. When a promoter is hypermethylated, it means the DNA is in heterochromatin conformation that by blocking the binding of transcription factors to DNA suppresses gene transcription. Hypomethylation of promoters is correlated with open chromatin conformation and active transcription of the gene (Eden and Cedar, 1994). Since DNA methylation is a reversible process, it can be considered as a therapeutic target. It has been confirmed that global DNA hypomethylation in T-cells and monocytes of RA patients compared to healthy individuals had been observed (de Andres *et al*., 2015). Genome-wide analysis of DNA methylation has found its modifications in B-cells on the early RA cases who have not yet started the treatment (Glossop *et al*., 2016).

Cribbs *et al.* (2014) investigated an irregular function of regulatory T cells in RA patients and reported a particular region in the promoter of the *CTLA4* (-658 CpG) that was hypermethylated compared to healthy controls. DNA hypermethylation blocks binding of the nuclear factor of activated T cells with cytoplasmic one that results in decrease of *CTLA4* expression. Therefore, regulatory T cells were not able to express and activate the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase which subsequently led to a failure in activating the immunomodulatory kynurenine pathway (Cribbs et al., 2014). In addition, it has been reported that methotrexate cause DNA hypomethylation of *FoxP3* locus in regulatory T cells. This contributes to the gene upregulation with further increase of CTLA4

concentration and normalization of regulatory T cells function in rheumatoid arthritis. These reports explain exactly how irregular DNA methylation can influence cell functions and how epigenetic mechanisms could be applied in therapy (Cribbs *et al.*, 2015). In order to identify differentially methylated regions as candidate epigenetic markers of RA predispositions, Liu *et al*. (2013) carried out an epigenome-wide association study. They investigated more than 485,000 CpG sites in blood of 354 RA patients and 337 healthy controls which ended in identification of 10 differentially methylated CpG sites. All of them are mapped on 6p12.1 and also contain the genes of the major histocompatibility complex (Raychaudhuri *et al*., 2012). This approved the function of DNA methylation as a further mechanism leading to RA development. Considerably, the heterogeneity existed in cell populations of blood can result in diverse methylation profile that should be considered in designing of the experiment.

Some of these observations were reported by other studies. Aberrant DNA methylation was detected in peripheral blood mononuclear cells of RA cases. For instance, it has been found that cg23325723 site was significantly associated with RA in peripheral blood mononuclear cells. Although not statistically significant, four other CpG sites (cg16609995, cg19555708, cg19321684, and cg25949002) have been reported to have similar different methylation in peripheral blood mononuclear cells of RA cases compared to healthy controls (van Steenbergen *et al.* (2014))

Other reports have demonstrated aberrant methylation of one cytosine in the *IL-6* promoter in RA cases which was correlated with reduction of its transcription (Nile *et al.*, 2008). On the other hand, hypomethylation of cytosine in the promoter of *IL-10* associated with higher expression of IL-10 in peripheral blood mononuclear cells (Chen *et al.*, 2011).

LRPAP1 gene is expressed in peripheral blood mononuclear cells and encodes the chaperone of low density lipoprotein receptor-related protein 1 which modifies the activity of transforming growth factor beta (Kolker *et al.*, 2012). It was reported that 4 CpG-dinucleotides in exon 7 of *LRPAP1* had decreased methylation levels in patients who failed in TNF inhibitors (etanercept) therapy compared to responders. In that study, the locus of cg04857395 shares structures which involved in alternative splicing: the region correlated with trimethylation of histone H3 at lysine 36 (H3K36me3) and the binding site of CCCTC-binding factor, which is a methyl-sensitive transcriptional repressor (Lev Maor *et al.*, 2015).

Julià *et al.* (2017) investigated the methylation patterns of B lymphocytes in patients with RA. They have found differentially methylated CpG sites in the CD1C, TNFSF10, PARVG, NID1, DHRS12, ITPK1, ACSF3, and TNFRSF13C genes and two intergenic regions (10p12.31). (Julià et al., 2017).

Conclusion

As described earlier, significant progress has been made in understanding the genetics of RA. Early diagnosis and initiation of DMARD therapy are essential to avoid damage from occurring or becoming clinically significant. However, the exact pathogenic mechanism of RA is yet to be fully elucidated.

Folate plays a key role in several metabolic processes including DNA synthesis and methylation. Thus, modifications in folate status may affect the DNA stability and integrity or change the methylation patterns in some tissues and predispose it to the development of RA. Polymorphic variants of the enzymes engaged in folate metabolism likewise have an important function in leading the susceptibility of an individual to RA. Genetics-based drug response prediction is currently challenging partially due to the lack of fully understanding of responsible variants. As such, there is a need to move forward applying the various approaches to identify the missing links.

Reports that highlight epigenetic modifications correlated with response to treatment not only have significant clinical uses in terms of optimizing patient care, but could also help in reduction of economic burden related to unnecessary drug prescriptions. In spite of the complications, studying epigenetic markers is strongly a great achievement of molecular biology and molecular medicine. Epigenetic alterations are the earliest parameters which are related with the development of the disease before its clinical signs and symptoms. They can be applied for prevention and monitoring of patient status and also are the earliest to show the influence of the therapy at the cellular level.

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