Università degli Studi di Ferrara

Ph.D. Course in

"MOLECULAR MEDICINE AND PHARMACOLOGY"

CYCLE XXX

DIRECTOR Prof. Francesco Di Virgilio

MULTIDISCIPLINARY APPROACHES AND INTERACTION NETWORK IN THE DIAGNOSIS AND TREATMENT OF RHEUMATOID ARTHRITIS

Scientific Disciplinary Sector (SDS) MED/03

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Years 2014/2017

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MOLECULAR MEDICINE AND PHARMACOLOGY

PhD Thesis, University of Ferrara, Ferrara, Italy

The research in this thesis has been performed at the Medical Genetics Unit, Department of Biomedical and Specialty Surgical Sciences, University of Ferrara, Italy.
Patients recruitment took place in the Rheumatology Unit (Prof. Govoni) and Obstetrics and Gynecology Unit (Dott.ssa Capucci) of Sant'Anna University Hospital of Ferrara and in CTO Hospital of Milan (Dott.Fossali).

The studies presented in this thesis have been partly supported by:
- Gisea "*Identification of the gene interaction network based on the response / toxicity to Methotrexate therapy used as "first line therapy" in rheumatoid arthritis*"
- Ministry of Health Project (RF-2010-2317168, PI Meliconi-IOR).
- Regional Project (PRUA1GR-2013-00000220, PI Dr. Graziano-AOU Ferrara).
- Regional Project (PRUA1GR-2013-00000203; PI Dr. Assirelli-IOR).
- Spontaneous Study, (no-profit DM 17/12/2004) "*Genetics and Pharmacogenetics of Rheumatoid Arthritis: interaction between genetic profile and risk factor associated to obstetric history*" (2016).
- Spontaneous Study case-only, (no-profit DM 17/12/2004) "*Fetal Microchimerism in rheumatoid arthritis female subjects: casual or causal factor?"* (2017).

Financial support for the publication of thesis was kindly provided by the Department of Biomedical and Specialty Surgical Sciences, University of Ferrara, Italy.

Cover and Design:

Printing:

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TABLE OF CONTENTS

CHAPTER 1

GENERAL OVERVIEW
ON RHEUMATOID ARTHRITIS DISEASE

LONG PATHWAY AND UNKNOWN WAY

"Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity"

-World Health Organization-

CHAPTER 1

GENERAL OVERVIEW
ON RHEUMATOID ARTHRITIS DISEASE:
LONG PATHWAY AND UNKNOWN WAY

Table of Contents

1.1 Overview on Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an inflammatory, autoimmune and chronic progressive disease, which affects predominantly diarthrodial joints causing pain, swelling and articular stiffness (Gonzalez-Gay MA et al., 2005). RA onset is extremely variable, usually gradually and slowly with nonspecific symptoms, such as asthenia, arthromyalgia, prostration and general unhealthy conditions. Subsequently, symptoms are more defined showing pain, tumefaction joints and swelling and they could variate for intensity and severity with generally recurrent course (Wasserman AM. 2011).
RA development is characterized in three pathogenic steps:

- ***Induction***: response to several pathogenic stimuli, with release of local cytokines and nonspecific dendritic cells and macrophages activation.
- ***Inflammation***: development of synovitis, B and T-lymphocytes and macrophages massive activation, production of rheumatoid factor and several cytokines.
- ***Destruction***: presence of erosions, articular and cartilage damages (Burmester GR et al., 2014).

The phases reflect the pathophysiological scenario: firstly, the ***beginning of autoimmune process*** characterized by the interaction between antigen-presenting cells (APCs) and T and B lymphocytes, inducing the inflammatory mediators and antibodies production. Subsequently, the mechanism involves the ***inflammatory factors infiltration and amplification in synovial localization*** with the synovitis development and leading to chronic process. Progressively, pannus starts growing followed by cartilage erosion and exacerbated phlogosis. The condition provokes a general unhealthy status, morning stiffness and swelling joints appearance, causing initial functional articular impairment. Then, several processes such as ***bone erosion, massive inflammatory cells infiltration and synovial pannus severe enhancement*** are associated to functional joint activity reduction, leading to the disability and ankylosis. Lastly, pannus overruns in cartilage location, subchondral bone function is compromised with alteration of tendons and ligaments integrity (Figure 1.1) (Alam J et al., 2017).

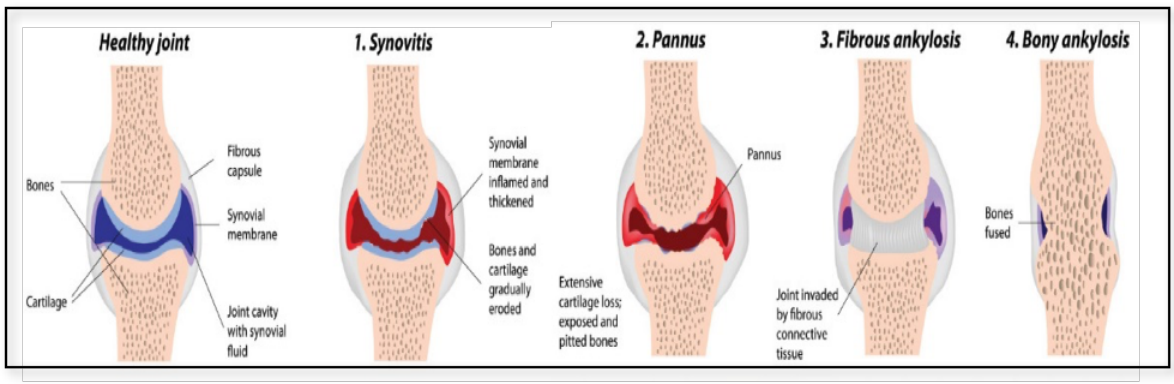


Figure 1.1 *Stages of Rheumatoid Arthritis*

Damage progression leads to a complete disability, with the development of rheumatoid nodules, typical feature of RA advance and chronic phase. Full-blown disease showing symmetric polyarthritis, which affects metacarpus-phalanges and proximal interphalangeal joints of hands and wrists, metatarsus-phalanges and proximal interphalangeal joints of feet and anklebones. In more severe cases, it could involve extra-articular locations, such as skin, respiratory, ocular and cardiovascular systems (Soubrier M et al., 2014).

Epidemiology

Studies of RA have reported during last decades that Northern European and North American areas estimate a prevalence of 0.5-1.1% (Scott DL et al., 2010). The geographical distribution could reflect the urbanization levels, considering higher prevalence in developed countries respect developing countries as Western Africa (Kalla AA et al., 2003) or different ethnicity, with a lower prevalence in Japanese and Chinese population (Silman AJ, Pearson JE, 2002). In Italy, RA prevalence is included within 0.33% and 0.46 % (De Waure C et al., 1010) (Figure 1.2). Annual incidence rates of RA are various between 20-50 cases/100.000 inhabitants and it increases by people age (Carbonell J et al., 2008; Pedersen JK et al., 2009) with a female-to-male ratio of 3/4:1 (Alamanos Y et al., 2005). Several studies reported RA prevalence in Italy and stratification by gender (Table 1.1). In addition, RA was ranked as the 42nd highest contributor to global disability (Cross M et al. 2014), continuing to induce significant excess mortality (Minichello E et al., 2016).

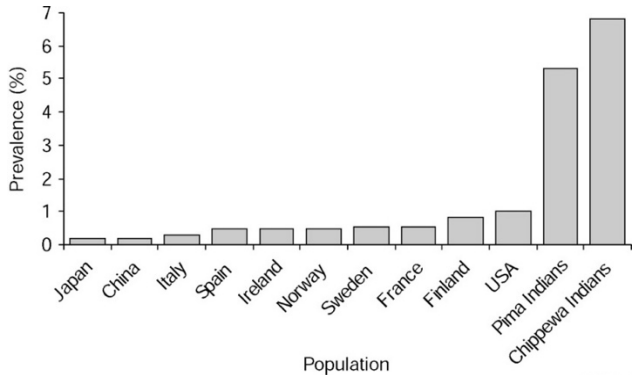


Figure 1.2 *RA prevalence in different population (from Silman AJ, Pearson JE, 2002)*

First Author, paper year	Place	Study length	Subjects (n°)	Average Age (years)	RA prevalence (%)	Gender stratyfication (%)
Cimmino et al., 1998	Liguria	1991-1992	3294	48.3	0.33	F: 0.51 / M: 0.13
Marotto et al., 2005	Sardinia	2002-2003	30264	62	0.46	F: 0.73 / M: 0.19
Salaffi et al., 2005	Marche	2004	2155	57.8	0.46	/
Della Rossa et al., 2010	Tuscany	2006-2007	26709	66.5	0.40	F: 0.63 / M: 0.14

Table 1.1 *Summary of studies in Italian population*

Etiology

RA development causes are still not completely understood. The major hypothesis is the multifactorial etiology, caused by a combination of several and different factors generally classified in two categories, genetics and environmental (Scott DL et al., 2010).

The contribution of genetics factors has been estimated around 50-60%, with a relevant role performed by Human Leukocyte Antigen (HLA) complex genes, expressed on the leucocytes surface and deputed to immune response control (Gregersen PK et al., 2009, Figure 1.3). Among HLA genes, the presence of the Shared Epitope (SE) amino acid sequence at position 70-74 of the third hypervariable region (HVR3) of the HLA-DRB1 is associated with high susceptibility to rheumatoid arthritis. More recently, non-HLA-related genes have been identified with RA susceptibility (Viatte S et al., 2013), such as STAT4, TRAF1 and C5, PTPN22, PADI4, genes associated with the immune system activation, RA progression and chronic inflammation process (Snir O et al., 2014; Mohan VK et al., 2014).

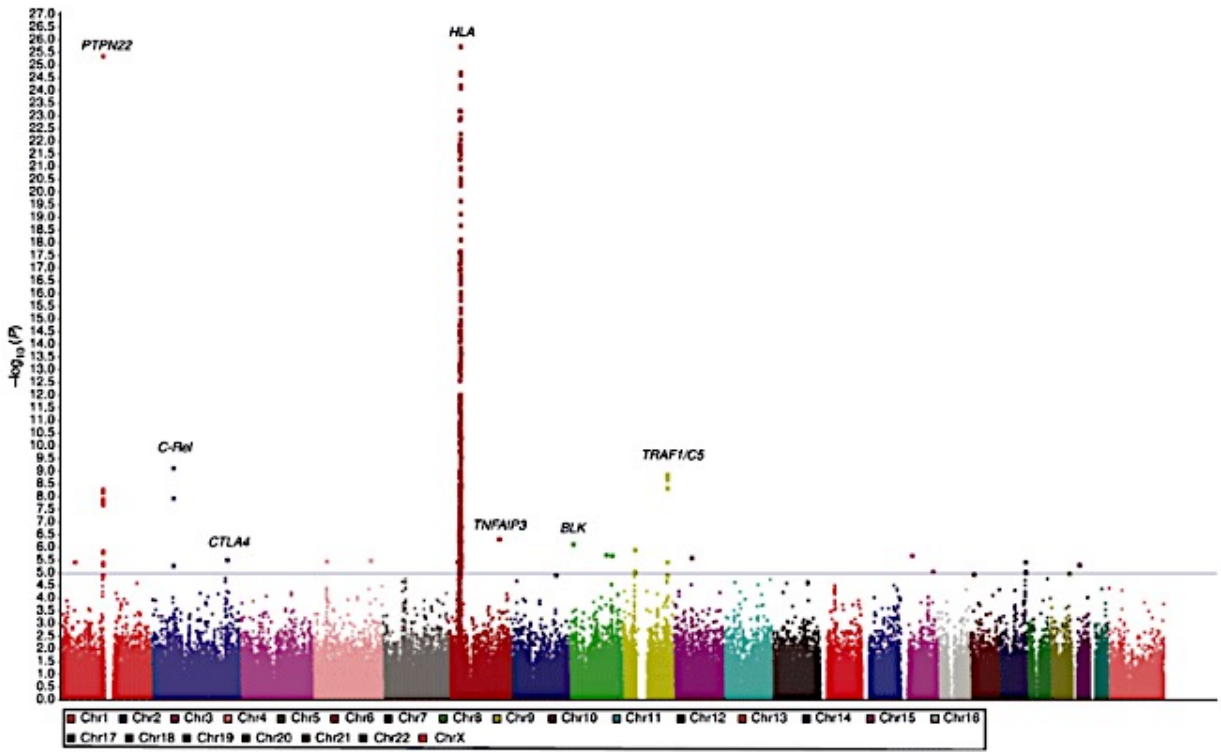


Figure 1.3 *Manhattan Plot from a genome-wide association study (GWAS). Chromosomes, depicted in alternating colors, are reported in X-axis and each dot corresponds to a SNP. Statistical significance is represented on the Y-axis [$-\log^{10}$(P-value)] and SNPs above the horizontal blue line (at $P=1\times10^{-5}$) represent statistically significant results in this GWAS. Depending on the peak points height, significant association value increases, a region (HLA) belongs to 6-chromosome results strongly associated. Figure reproduced from Gregersen PK et al., 2009.*

Besides, several environmental factors occur in RA etiopathology contributing to the disease onset:

- ***Female gender***: women are more affected, with an F/M ratio of about 3-4/1 in adulthood, tending to decrease with age advancement (Oliver JE et al., 2009). In general, a high estrogen/testosterone ratio appears to be predisposed to RA (Masi AT et al., 2005). The role of hormones seems to explain the women RA highest prevalence: estrogen has a stimulating effect on the immune system (Alamanos Y et al., 2005) and contributes to the activation and chronicity of the general inflammatory state, while testosterone, on the contrary, has immunosuppressive properties.
- ***Perinatal factors***: during pregnancy, the two immune systems of mother and fetus are in close contact. Exposure to particular immune molecules, called NIMA (Non Inherit HLA-antigen from the Mother), could exert an immunomodulatory effect, which might last for a lifetime and play a role in autoimmune disease etiology, such as RA (van der Horst-Bruinsma IE et al, 1998).
- ***Comorbidities*:** population studies have shown that patients with RA have a life expectancy of 8 to 12 years fewer than healthy subjects (Turesson C, 2006). Cardiovascular affections are significantly higher in subjects with RA (Roman MJ et al., 2006) and the frequency of infections, especially related to the affections of the osteoarticular and connective systems, is almost double compared to control subjects (Doran MF et al., 2002)
- ***Smoking***: it is one of the factor mostly influencing RA onset, it induces pro-inflammatory cytokines production, contributing to the inflammation and synovium affection and increasing the proteins citrullation (Klareskog L et al., 2006; Linn-Rasker SP et al., 2006).
- ***Infectious agents:*** RA development has been associated with bacterial and viral infections such as Epstein-Barr (EBV) virus and Hepatitis B virus (HBV) (Meron MK et al., 2010). Established theories seek the initial RA cause in the presence of "super-antigens" in joints,capable of activating multiple clones of T-lymphocytes through an independent MHC process (Edwards CJ et al., 2006).
- ***Obesity and diet:*** Pedersen M et al. (2006) have found a strong association starting with obesity classified as mild (body mass index<30), probably due to adipokines secretion, able to mediate and stimulate systemic inflammation (Ferraz-Amaro I et al., 2013). Diet habits could make effort on RA susceptibility: omega-3 and vitamin D seem to have a protective effect on the development of the disease, while a high intake of coffee is related to increased susceptibility (Tobón GJ et al., 2010; Källberg H et al., 2009).
- ***Estroprogestinic intake***: progesterone role is controversial. It would seem to have a protective function, but it has failed the periodic monitoring visits to track the course of RA disease and to verify the effectiveness of therapies, arguing that effectively the role of progesterone is not a protection, but it is limited to producing a delay in clinical disease manifestation (Hannaford PC et al. 1990).

Pathogenesis

Nowadays, the pathogenic model mostly accredited assumes the activation of immune system due to a contact between a genetic predisposed subject (background condition) and occasional environmental causative antigens (acquired factors). The interaction would trigger the immune process, whose ultimate outcome is the development of chronic inflammation predominantly located in the synovial membrane (synovitis) (Figure 1.4, modified from Benson RA et al. 2005).

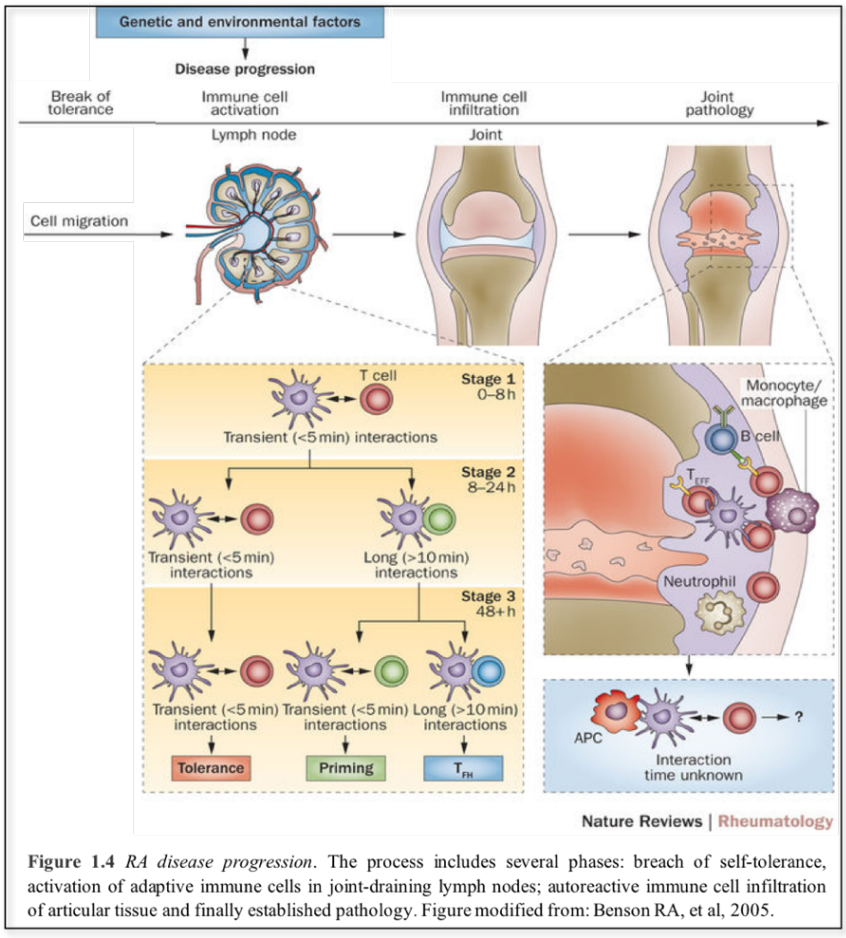


Figure 1.4 *RA disease progression*. The process includes several phases: breach of self-tolerance, activation of adaptive immune cells in joint-draining lymph nodes; autoreactive immune cell infiltration of articular tissue and finally established pathology. Figure modified from: Benson RA, et al, 2005.

Immediately, organism shows acute inflammation due to the activation and co-stimulation mechanisms between Antigen-Presenting Cells (APCs) and T-lymphocytes, through the involvement of costimulatory molecules (especially CD80/86 system). This condition is sustained by both innate and adaptive systems, which maintain a continue inflammation and lead to chronicity.

The innate immune pathway consists in nonspecific response due to the activation of APCs (dendritic cells, macrophages and B lymphocytes) by exogenous and endogenous factors (Smolen JS et al., 2016). Antigens considered as RA causative are several and they include: bacterial agents (ex: streptococcus, E. Coli), some mycobacteria and mycoplasma, viral agents (ex: Epstein-Barr, Herpes Simplex Virus), super-antigens (ex: proteins synthetized by streptococcus, staphylococcus and mycoplasma) (de Waure C et al., 2010; Iebba F et al., 2011). Moreover, some endogenous antigens, such as collagen epitopes, proteoglycans, Fc of Ig-G are involved in the RA pathogenesis during the chronicity process (Firestein GS et al., 2012). Infective or others environmental factors are implicated in auto-reactivity through the process called "molecular mimicry": during infection, the immune system produce a cross-reactive response against self-proteins with structure similar to infective

agents and so T cell is induced to proliferate and to start the immune process. Even after the infection eradication, this autoreactive response could continue due to those proteins action (Taneja V, 2014). Globally, these conditions lead to unstopped inflammatory process and to immune-mediated response through cytokines production. B and T activated lymphocytes contribute to RA pathogenesis producing pro-inflammatory chemokines, cytokines, such as TNF-α, interleukin, such as IL-1 and IL-6 (Smolen JS et al., 2003; Smolen JS et al., 2007) and antibodies, as rheumatoid factor (RF) and antinuclear antibodies (ANA), causing inflammation immune complexes-mediated.

The molecules infiltration increases vascularization and synovial membrane hyperplasia: intimal layer, normally constituted by 1-2 cellular layers, results thickened (up to 4-10 layers) with even cartilage involvement. Cellular subtypes are mostly synovial macrophages and Fibroblast-like Sinoviocytes (FLS) implicated as well in pro-inflammatory cytokines production (Choy E, 2012). Macrophages and RANKL-RANK system are involved in osteoclasts genesis, consisting in bone reabsorption and more severe phenotype of RA disease and the activation of several metalloproteases caused the cartilage damage. The synovitis affection is sustained through neo-angiogenesis process allowing neutrophils and others molecules, due to chemotactically agents secreted by endothelium and fibroblasts, to get out from vessels, adhere and accumulate in synovial membrane. In joint, they interact with immune-complexes through Ig-Fc fragment, the inflammation and synovial proliferation lead to pannus development and lastly severe phenotype consists in ankylosis and cartilage/bone disruption.

Clinical Evaluation

RA diagnosis is essentially clinical. In the evolved forms, the symmetrical polyarticular involvement is highly evocative: the disease appears as a chronic destructive arthritis with joint swelling and spasms, deformities, subluxations, tenosynovitis and muscle hypotrophy, resulting in typical deformity, such as fingers "en boutonnière", "swan neck" o the classical ulnar deviation "wind blow" (Erol K et al., 2017) (Figure 1.5). These are late aspects of poor utility for the purpose of the therapeutic result.

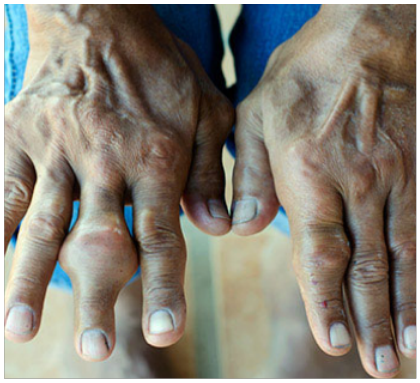


Figure 1.5 *Typical deformations of RA hand affection.*

Classical classification criteria for RA has been proposed by America Rheumatism Association (ARA), with the purpose to distinguish rheumatoid arthritis patients from others with similar disorders. However, these criteria (Table 1.2, modified from Arnett FC et al., 1988) manifest important limitations for identifying early forms.

Recently, new classification criteria have been published (Table 1.3, modified from Aletaha D et al., 2010), inspired to the necessity to identify patients in very early stage, with negative prognostic parameters and with higher probability to develop a persistent and aggressive forms. New criteria expect to evaluate 4 different characteristics: articular affection, serological autoantibodies pattern, acute phase reactants and symptoms duration.

Criterion	Definition
1) Morning stiffness	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement
2) Arthritis of three or more joint areas	At least three joint areas simultaneously have had soft tissue swelling or fluid observed by a physician (PIP, MCP, wrist, elbow, knee, ankle, and MTP joints)
3) Arthritis of hand joints	At least one area swollen in a wrist, MCP, or PIP joint
4) Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body
5) Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxta-articular regions
6) Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor
7) Radiographic changes	Posteroanterior hand and wrist radiographs showing juxta-articular bony decalcification or erosions
For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least four or these seven criteria. Criteria one through four must have been present for at least six weeks.	

Table 1.2 *1987 ARA Classification Criteria for Rheumatoid Arthritis. Modified from Arnett FC et al., 1987.*

2010 ACR/EULAR Criteria		
	Criteria	Score
Joint Involvement	1 Large Joint	0
	2-10 Large Joints	1
	1-3 Small Joints	3
	>10 Joints (at least 1 small joint)	4
Serology	Negative RF and anti-CCP	0
	Low-Positive RF or anti-CCP	2
	High-Positive RF or anti-CCP	3
Acute-Phase Reactants	Normal CRP and ESR	0
	Abnormal CRP or ESR	1
Duration of Symptoms	<6 weeks	0
	≥6 weeks	1
***Total score of greater than 6 is classified as RA**		

Table 1.3 *2010 ACR/EULAR Classification Criteria. Total of greater than 6 out of the total 10 suggests RA diagnosis. Modified from Aletaha D et al. 2010.*

Concerning the disease activity score evaluation and the functional impact assessment, it has been recommended to assume several clinical, quantitative and semi-quantitative parameters, such as DAS (Disease Activity Score) and HAQ (Health Assessment Questionnaire). DAS, in the more common version DAS28 (based on the evaluation of 28 joints), provides the estimation of 4 parameters: 1) swollen joints count; 2) painful joints count; 3) ESR (Erythrocytes Sedimentation Rate) value; d) the patients global health status measured through a visual-analog scale. The result is a numeric value that defines different states of disease activity (Table 1.4 modified from Bakker MF et al., 2012). HAQ is a simply questionnaire filled by the patient, that provide a numeric indication (0-3) on the disease functional impact: 3 expresses the more severe condition.

DAS28 at End Point	Improvement in DAS28 From Baseline		
	>1.2	>0.6 and ≤1.2	≤0.6
≤3.2	Good	Moderate	None
>3.2 and ≤5.1	Moderate	Moderate	None
>5.1	Moderate	None	None
DAS28 = Disease Activity Score assessing 28 joints.			

Table 1.4 *EULAR Response Criteria by using Disease Activity Score 28 (DAS28). Modified from Bakker MF et al. 2012.*

Laboratory Evaluation

Laboratory exams of practical and diagnostic utility include *Rheumatoid Factor* (RF), *Citrullinated cyclic anti-peptide antibodies* (anti-CCP), and *Anti-Nucleus antibodies* (ANAs), related to the activation of immune-complexes pathway and inflammation exacerbation (Koopman VJ et al., 1985; Jansen LM et al., 2003). RF is found in 2/3 of RA patients, but its detection is not pathognomonic, resulted positive in many other condition. Presence of absence of RF allows to distinguished two main disorder subsets: positive autoantibodies serological forms (RA+) and seronegative (RA-), usually less aggressive. RF importance, apart from the diagnosis, concerns in its prognostic value: the positivity is associated with a greater probability of persistent arthritis, higher severity and extra-articular manifestation. Even ANAs are present especially in patients with extra-articular and RF positive manifestations (Turkiewicz AM et al., 2006).

Moreover, some others parameters were evaluated to identify and to diagnose RA disorder, reflecting severity grade of systemic inflammation, in particular:

- ***Erythrocyte sedimentation rate (ESR or VES):*** it indicates the rate of red blood cells, isolated from a blood sample that has made not coagulable, to settle and to precipitate at the bottom of the test tube. In healthy condition, red blood cells remain in suspension, separated from each other, whereas during established inflammation, the increased blood concentration leads to aggregating erythrocytes. Faster sedimentation rate is referred to the more coarse formations. ESR increases quite slowly as a response to inflammatory stimuli, as it is a consequence of the acute phase protein increase (Silva I et al., 2010).
- ***Activated protein C (APC or PCR):*** APC and others acute phase positive proteins are molecules produced in the liver due to immune system activation. Blood values are generally rather low but rapidly increase during inflammation and APC values also correlate with radiographic RA progression (Corrao S et al., 2009).

Biopsy Evaluation

Arthrocentesis is a commonly performed medical procedure for the diagnosis and treatment of some joint diseases, as RA. The aim of the technique is to extract, by suction, a sample of synovial fluid contained in its articular capsule; the sample thus collected is examined in the laboratory, to assess the degree of inflammation and the presence of crystals or pathogens, especially in the case of synovial discharge of unknown origin.

Moreover, arthrocentesis allows the possibility to make a joint injection, which involves intra-articular administration of a drug useful to provide relief from pain and swelling. The most commonly used drugs for this purpose are steroidal anti-inflammatory drugs or corticosteroids. Synovial fluid can be tested for the presence of blood, pus, crystals, proteins and glucose, as well as it can be cultured to determine the presence of pathogenic microorganisms. The sample appearance

(color, viscosity, turbidity, volume of synovial fluid, etc.) is also evaluated and the cellular count (number of white or red cells) is performed. Each of these parameters can be useful in defining the cause of a particular pathology. In case of RA diagnosis, synovial liquid analysis reveals inflammatory-like fluid, full of immune system cells, with a prevalence of neutrophils and this survey is particularly useful for excluding any infectious complications. The synovial joints provide a unique opportunity for studies of microenvironmental pattern of local inflammation, especially for patients in the chronic arthritis condition (Simkin PA, 2015). The synovial fluid and the correspondent tissue specimen, obtained through arthrocentesis, permit quantitative and qualitative study in a specific site, evaluating hallmarks of phlogosis.

Imaging Techniques

Instrumental exams include the X-ray, articular ultrasound and magnetic resonance imaging (RMI or RMN) (Figure 1.6).

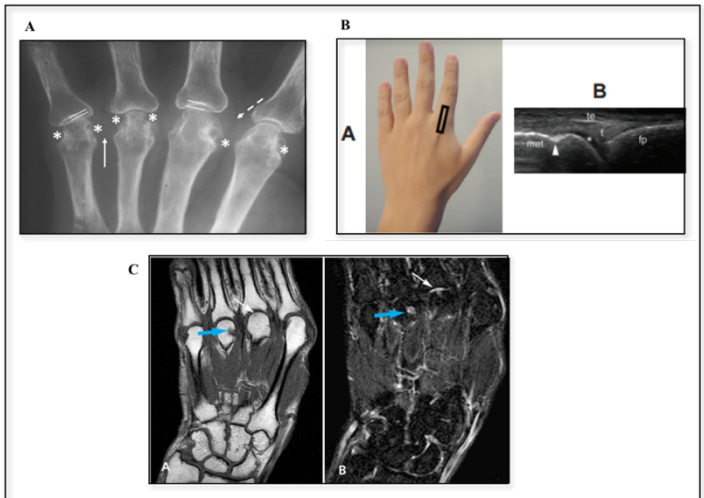


Figure 1.6 *Imaging techniques in RA* **A.** Radiography (from Wick MC et al., 2012); **B.** Ultrasonography evaluation (from Arend CF et al., 2013); **C.** Magnetic Resonance (from Mak W and Hunter JC, 2009)

Radiology examination is the inquiry of the first instance. It is normally used for assessing and monitoring over time joint damage; however, it is not a very sensitive technique since it is not able to highlight the typical alterations of the early phases of RA (Llopis E et a., 2017). Articular ultrasound is useful for RA diagnosis in early stages as it could early identify the presence of radiologically not detectable articular erosions. RMI is useful for early articular injury and to confirm the presence of pre-erosive alterations such as bone edema, usually corresponds to inflammatory and hyperemic areas where erosions will develop.

Therapy

One of the fundamental aspects of RA therapeutic management is to establish the disabling potential of the disease and then to set a more or less aggressive therapeutic strategy (Wijbrandts CA, Tak PP, 2017). Among clinical prognostic indicators, a large number of painful and swollen joints, female gender, length of disease, foot involvement and smoking habit are the mains related to bad prognosis. On the laboratory point of view, negative prognosis indicators are RF and ACPA positivity (especially if contemporary and high concentrated) and the persistent increase for reactant acute

phase. Considering the imaging, the early detection of erosive phenomena characterizes the most aggressive forms, reputed a progression marker that alone can justify changes in therapeutic treatment. Accurate patient and disease profile allow to maximize the personalization of treatment: in the initial forms, the primary target of therapy should be to achieve a state of remission, whereas in the more advanced forms it is reasonable to reach the low level of disease activity. The current clinical practice using of the previously mentioned clinometric indexes, is the most suitable instrument for establishing and verifying whether or not the therapeutic target is obtained. Pharmacological therapy includes symptomatic drugs (NSAIDs and Coxib), corticoids, Disease Modifying anti-rheumatic drugs (DMARDs) and other useful choices for the prevention of specific disease complications, such as osteoporosis and accelerated atherosclerosis. Corticoid is a fundamental drug, if used at low doses: it is particularly useful in initial phases as "bridge therapy" while waiting for DMARDs effect and in the disease acute flares (Paolino S et al., 2017). Side effects are well-known and several, such as diabetes, hypertension, osteoporosis, myopathy and weight gain and they require adequate monitoring and appropriate preventive measures. DMARDs, distinguished in conventional (csDMARDs) and biological (bsDMARDs), are not only effective on symptoms control but have the ability to interfere with the progression of anatomic damage by modifying the course and outcomes of the disease.

csDMARDs are characterized by multiple pharmacodynamical mechanisms and slow action, requiring from several weeks (6-8) to a few months (4-6) to be effective. The commonly used are: methotrexate (MTX), leflunomide, sulfasalazine, cyclosporine A, synthesis antimalarials (chloroquine, and hydroxychloroquine) and gold. First-line cDMARD is MTX, with 7.5-15mg/week initial dose and it is considered as anchor drug in the RA treatment. Leflunomide can represent valid alternative, especially in patient who cannot tolerate MTX or have contraindication to its use. Cyclosporine A is used mostly in the initial phase and gold, once widely used, is nowadays reserves for a few selected cases. Azathioprine, nonetheless its efficacy, is a rarely used immunosuppressive molecule.

bsDMARDs (Table 1.5), obtained using modern and complex molecular biology techniques, are characterized by much more selective targets (cytokines, surface molecules, cellular markers), but are burdened by high costs and the need for careful monitoring (Takaechi T, 2017). They are powerful and very effective

Compound	Structure	Target
Infliximab	Chimeric monoclonal antobody	TNF-**α**
Adalimumab	Human monoclonal antobody	TNF-**α**
Etanercept	Human fusion protein	TNF-**α**
Golimumab	Human monoclonal antobody	TNF-**α**
Certolizumab	PEGylated Humanized Fab fragment	TNF-**α**
Anakinra	Recumbinant human IL-1R antagonist	IL-1
Tocilizumab	Humanized monoclonal antobody	IL-6R
Abatacept	Human fusion protein (Fc-CTLA4)	CD80/86 (T-cells)
Rituximab	Chimeric monoclonal antobody	CD20 (B-cells)

Table 1.5 Currently available bsDMARDs for Rheumatoid Arthritis treatment.

immunosuppressants that have been shown to significantly reduce radiological progression of joint damage; their use involves careful preliminary screening aimed primarily at the recognition of latent infections as TBC and hepatitis HBV, that could be reactivated by such therapies. Following the more recently recommendations, the bsDMARD to use firstly is anti-TNFα (nowadays 5 molecules are available). In the event of failure of a first anti-TNFα, is allowed the switch or substitution to a different anti-TNF, whereas in case of further non-response, is indicated the use of another bDMARD, belonging to a different subgroup ("swap"), such as Rituximab (anti-CD20), Tocilizumab (anti-IL6R) or Abatacept (anti-CD80/86).

Early Arthritis

The term refers to all forms of arthritis of recent onset, regardless of their etiology. Within this non-specific terminology, is also included rheumatoid arthritis at the initial stage; in this case, if there are already enough suspicious elements, the term preferred to use is "early rheumatoid arthritis" (ERA) (Di Giacinto G, 2014). Indeed, early diagnosis of rheumatoid arthritis is quite difficult since, in very early stages, clinical phenomena may still be modest and very unspecific (Undifferentiated Arthritis, UA). Early diagnosis is the essential basis for setting up timely pharmacological therapy able to counteract the progression of the disease. It is therefore necessary to take advantage of the so-called window of opportunity, the interval time from the symptoms onset (3-6 months) within the treatment may potentially stop the phlogosis and modulate promptly the disease course (Franceschi PL et al., 2008; Breedveld FC, 2004). The presence of alarm indicators of early stages of disease, called red flags, allow to recognize risk subjects, who are premonitory to a bad prognosis and evolution of artropathy (Emery P et al., 2002). Red flags are following: 1) swelling of three or more joints lasting more than 6 weeks; 2) involvement of metacarpophalangeal or metatarsophalangeal joints; 3) positive compression test (gutter sign); 4) morning stiffness for over 30 minutes.

Rheumatoid Arthritis and Pregnancy

Pregnancy is a biological process that occurs physical, hormonal and immune system changes in the maternal body, due to the development and growth of a new life. RA does not directly affect fertility, but therapeutic treatments involving teratogenic agents may interest the proper fetus development and cause irreversible damage, resulting in complications, malformations or fetus death. Other issues affecting pregnancy could be the conception delay due to reduced sexual function, ovulation dysfunction, hormonal alterations, and possible production of sperm-directed antibodies (Ince-Askan H, Dolhain RJ, 2015).

The Italian Society of Rheumatology (SIR) suggests to undertake a pregnancy during lowest DAS condition, allowing to discontinue treatment assumption, as MTX, at least 6 months before

conception. The therapy will be shifted to one considered safe, cause it does not cross the placenta, such as Cyclosporine, Hydroxychloroquine and steroids in case of flares. During pregnancy, the patient should be followed by a specialized team, with monthly clinical and laboratory controls. The condition should be monitored and extended even after childbirth, especially on the baby, to detect any consequences.

Approximately 70% of RA patients observed symptoms improvement and disease remission during pregnancy. This condition is presumed to be determined by hormonal changes in the mother and above all due to higher cortisol production, which would act on joints phlogosis process. Estrogens and progesterone substances could exert a potential anti-inflammatory effects, due to the shift of cytokines produced by Th1 cells (T-helper $CD4^+$ type 1 lymphocytes) to those produced by Th2 cells (T-helper $CD4^+$ type 2 lymphocytes). However, within 3-4 months after childbirth, 90% of women experienced a disease exacerbation caused by the drastic reduction of these substances production (Prevoo ML et al., 2005; de Man YA et al., 2014; Barrett JH et al., 1999).

During pregnancy, the fetus-maternal interface guarantees the positive gestational environment, allowing the nutritive transition to the fetus; at the same time it acts as a barrier to separate the two immune systems (Du MR et al., 2014). On one side, mother develops immune tolerance due to role of the several molecules recovered only in placenta, as HLA-G and NK cells; on the other side, fetus promotes tolerance through fetal cells migration and cell-free fetal DNA (cffDNA) in maternal bloodstream, creating a state called microchimerism, persistent even many years after childbirth.

The microchimerism condition in mother (fMC) could exert different roles, explained in the following three hypotheses:

1. ***fMC induces graft-versus-host reaction (GVH)*** (Figure 1.7): fetal chimeric T lymphocytes (graft) recognize maternal cell (host) as extraneous, stimulating specific antibodies production (Chosidow O et al., 1992).

2. ***fMC induces host-versus-graft reaction (HVG)*** (Figure 1.8): fetal chimeric cells are the target of maternal immune response, as result of direct action against fetus antigens (Figure 1.8 A) or due to a molecular mimicry mechanism (Figure 1.8 B).

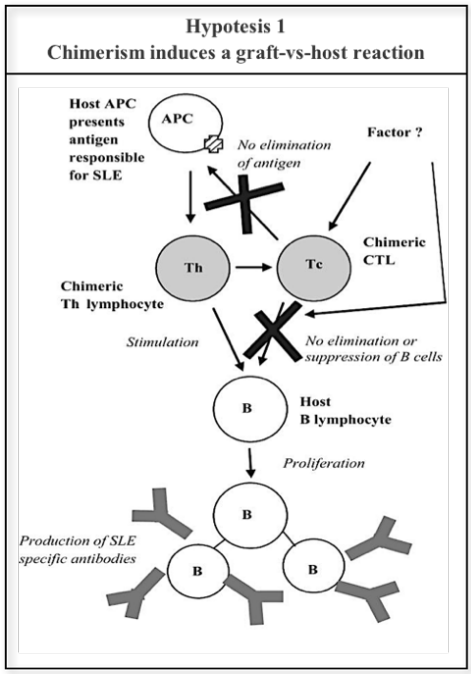


Figure 1.7 Graft-vs-host reaction pathway.

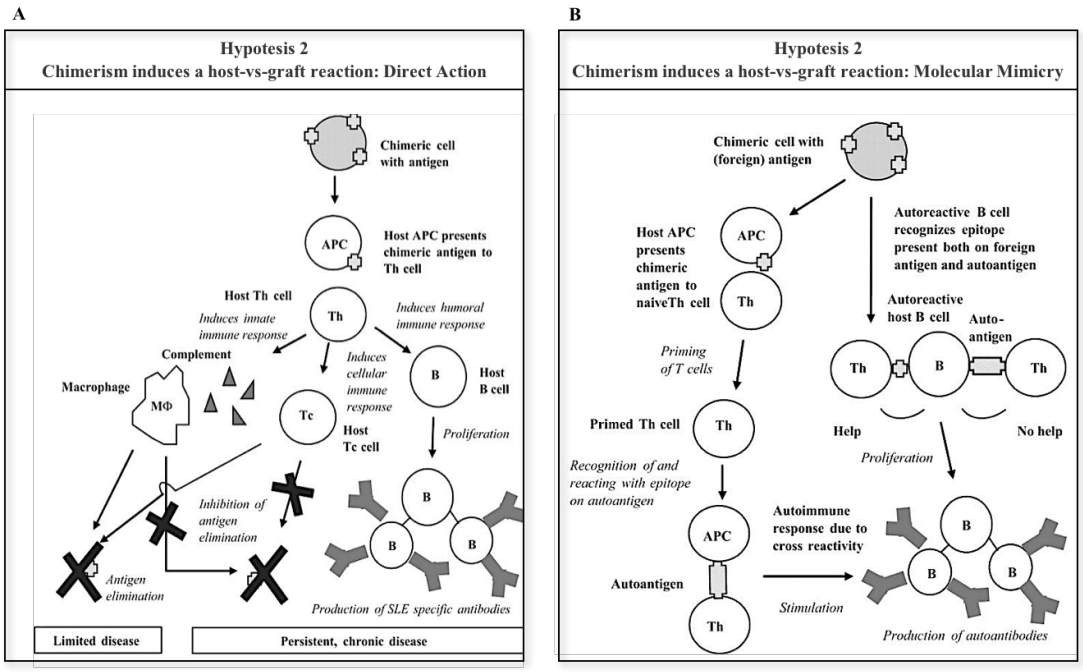


Figure 1.8 Host-vs-graft reaction induced by fMC through two different pathways . **A.** Direct action, **B.** Molecular mimicry.

3. ***fMC cells repair damaged tissues*** (Figure 1.9):
   fetal chimeric cells differentiate from progenitors to distinct phenotypes, included endothelial cells (Mahmood U et al., 2004), neurons (Tan KH et al., 2011), smooths muscle cells and cardiomyocytes (Bayes-Genis A et al., 2005; Kara RJ et al., 2012).
   Considering the evidence of mesenchymal cells from fetus have been found in the bone marrow of women with at least one son, pregnancy should be considered as a physiological mechanism to acquire fetal cells population, able to repair maternal injured tissues (Bianchi DW et al., 1996).

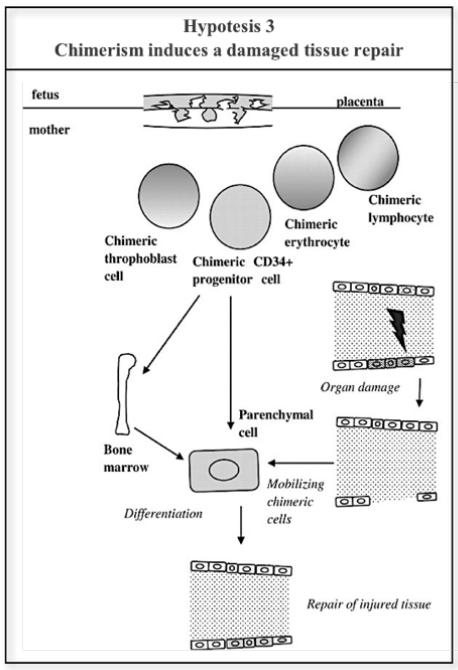


Figure 1.9 fMC mechanism to damaged tissue repair.

The fetal microchimeric cells have been found in the bloodstream of autoimmune diseases affected women, as Systemic Lupus Erythematosus (SLE) patients (Gannage M et al., 2002) and even in synovial tissue of RA female subjects (Hromandnikova I et al., 2008) and rheumatoid nodules (Chan WF et al., 2012), suggesting a cross-talk interaction between mother and fetus and a potential causative role in the autoimmune etiopathology.

1.2 General objectives of the thesis

The above background reflects that even after many years of investigation on Rheumatoid Arthritis disease, the mode of the onset and development remains difficult and challenging to assess in advance, probably due to the absence of ascertained and specific biomarkers. Moreover, the occurrence risk of RA is more concentrate in female gender rather than male and the RA development is different accordingly to geographical distribution. Progressively, many approaches have been employed for identification of genes contributing to RA susceptibility, ranging from linkage studies to genome wide association studies. Although the higher success of these studies, results seem to be restricted to RA established patients and the genetic approach has been evaluated only for single candidate-gene variant and without considering the cross-linked effects of gene variants interactions. Then, it can be reasoned to ignorance of sexual dimorphism, while identifying the genes contributing to these etiologies and involved in the pharmacogenetics pathways.

In addition, just a few studies evaluated the impact of environmental factors, which ideally are linked and could influence the development and progression to a severe RA phenotype.

In order to gain insights of several facets of RA disease, the present study aims to elucidate the following mainly objectives:

- Identification of a genetic occurrence risk profile of RA patients in a complete Italian case-series, performing a retrospective case-control study. Results obtained on associated gene variants were evaluated in an Italian ERA case-series, to assess the potential predictive role.
- Pharmacogenetics approach, evaluating candidate gene variants association in terms of efficacy and toxicity of MTX, the anchor-drug treatment used as first-line therapy.
- Focus on gender-medicine, considering specific women parameters and to relate them to the development of autoimmune disease.
- Evaluation of biopsies tissues samples, exploring differences in the pattern of some immune system cells using histological images and linked them to the treatment assumed.
- Epigenetic regulation, evaluating the DNA methylation, in terms of global LINE-1 and particular gene targets in RA and ERA samples.

1.3 Outlines of the Thesis

After the general overview and the introduction on several facets of RA disease, the workflow of the thesis starts from CHAPTER 2. It focuses on RA susceptibility and association study of several selected candidate-gene variants, considered as singles and as combinations. Subsequently, CHAPTER 3 deals with gene variants, serological parameters and their interactions, respectively in two different case-series: in UA group has been evaluated the impact on evolution to RA, versus stable condition; in ERA cohort has been considered the disease progression, in terms of DAS28

improvement. The target to personalized and predictive response to treatment in terms of MTX efficacy and toxicity is presented in CHAPTER 4, focusing on the association of specific gene variants and MTX response in RA administration. A new direction to better identification of the profile of RA patient MTX non-responder has been given in CHAPTER 5, directing the view on gender-medicine approach and considering women and deeper, a particular subgroup of female patients with specific characteristics, as a cohort with different hallmarks from the entire RA affected participants.

CHAPTER 6 gives a deeper observation of RA features involving the synovial tissue, to evaluate immune system cells in the localized inflammation. It has used an inherent and easy-to-use imaging tool from image analysis software, Fiji (part of Image J).

The final work of this thesis, CHAPTER 7 focuses on the epigenetic modulation of DNA methylation in global LINE-1 and specific genes (HLA-G and MTHFR) in RA and ERA patients.

The last and final part of the thesis CHAPTER 8 is a concise discussion on outcome of each study presented in this thesis and its implications.

References

Alam J, Jantan I, Bukhari SNA. Rheumatoid arthritis: Recent advances on its etiology, role of cytokines and pharmacotherapy. Biomed Pharmacother. 2017 Aug; 92:615-633.

Alamanos Y, Drosos AA, Epidemiology of adult rheumatoid arthritis. Autoimmun Rev. 2005 Mar;4(3):130-6.

Aletaha D et al., 2010. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum;2010. 62:256981.

Arend CF, Ultrasonography in rheumatoid arthritis: what rheumatologist should know, Rev Bras Reumatolo. 2013 Feb;53(1):88-100.

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; 31:315-24.

Bayes-Genis A, Bellosillo B, de la Calle O, Salido M, Roura S, Ristol FS, Soler C, Martinez M, Espinet B, Serrano S, et al. Identification of male cardiomyocytes of extracardiac origin in the hearts of women with male progeny: male fetal cell microchimerism of the heart, J Heart Lung Transplant , 2005, vol. 12 (pg. 179-183)

Bakker MF et al., Low-Dose Prednisone Inclusion in a Methotrexate-Based, Tight Control Strategy for Early Rheumatoid Arthritis: A Randomized Trial. Annals of Internal Medicine, 6 March 2012.

Bang S-Y, Han T-U, Choi C-B, Sung Y-K, Bae S-C, Kang C. Peptidyl arginine deiminase type IV (PADI4) haplotypes interact with shared epitope regardless of anti-cyclic citrullinated peptide antibody or erosive joint status in rheumatoid arthritis: a case control study. Arthritis Res Ther. 2010;12(3):R115.

Baricordi OR, Stignani M, Melchiorri L, Rizzo R, HLA-G and inflammatory diseases. Inflamm Allergy Drug Targets. 2008 Jun;7(2):67-74.

Barrett JH, Brennan P, Fiddler M, et al. Does rheumatoid arthritis remit during pregnancy and relapse postpartum? Results from a nationwide study in the United Kingdom performed prospectively from late pregnancy. Arthritis Rheum, 42 (1999), pp. 1219-1227.

Begovich AB et al. 2004. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am. J. Hum. Genet*. 75:330–337.

Benson RA, McInnes IB, Brewer JM, Garside P. Cellular imaging in rheumatic disease. Nature Reviews Rheumatology 11,357–367(2015).

Beri R, Peterlana D, Puccetti A, et al. Alleli HLA-DRB1 e artrite reumatoide nel nord Italia : assenza di correlazione con la gravità della malattia e con manifestazioni extra-articolari. 2005;96:139-143.

Bianchi DW, Zickwolf GK, Wil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA1996 Jan 23; 93(2): 705–708.

Breedveld FC, Kalden JR. Appropriate and effective management of rheumatoid arthritis. Ann Rheum Dis. 2004 Jun;63(6):627-33

Burmester GR, Feist E, Dörner T. Emerging cell and cytokine targets in rheumatoid arthritis. Nature Reviews Rheumatology 2014; 10: 77-88.

Carbonell J, Cobo T, Balsa A, Descalzo MA, Carmona L, and the SERAP Study Group. The incidence of rheumatoid arthritis in Spain: results from a nationwide primary care registry. Rheumatology (Oxford) 2008; 47: 1088–92.

Castelli EC, Ramalho J, Porto IO, Lima TH, Felicio LP, Sabbagh A, et al. Insights into HLA-G Genetics provided by worldwide haplotype diversity. Front Immunol. 2014 Oct6;5:476.

Chan WFN, Gurnot C, Montine TJ, Sonnen JA, Guthrie KA, Nelson JL (2012) Male Microchimerism in the Human Female Brain. PLoS ONE7(9): e45592.

Chosidow O, Bagot M, Vernant JP, Roujeau JC, Cordonnier C, Kuentz M, Wechsler J, André C, Touraine R, Revuz J. Sclerodermatosus chronic graft-versus-host disease. Analysis of seven cases. J Am Acad Dermatol. 1992 Jan;26(1):49-55.

Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology 2012;51:v3v11

Corrao S, Pistone G. Artrite reumatoide. Manuale pratico per la diagnosi in reumatologia 2009; (3). Pensiero scientifico.

Cross M et al. The global burden of rheumatoid arthritis. Ann Rheum Dis. 2014 Jul;73 (7): 1316-22.

de Man YA, Dolhain RJ, Hazes JM. Disease activity or remission of rheumatoid arthritis before, during and following pregnancy. Curr Opin Rheumatol, 26 (2014), pp. 329-333

De Waure C, Sferrazza A, Gualano MR, La Torre G, Montecucco C, Ricciardi W. Epidemiologia e burden of disease dell'artrite reumatoide. Italian Journal of Public Health 2010; 7: S3-13.

Di Giacinto G. Artrite reumatoide: diagnosi e terapia nella medicina generale. M.D. Medicinae Doctor; Anno XXI 2014; 2: 41-6.

Donadi EA, Castelli EC, Amaiz-Villena A, Roger M, Rev D, Moreau P, Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease assicuation. Cell Mol Life Sci. 2011.

Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. Arthritis Rheum 2002;46:87-93M.

Du MR, Wang SC, Li DJ. The integrative roles of chemokines at the maternal-fetal interface in early pregnancy. Cell Mol Immunol. 2014 Sep;11(5):438-48.

Edwards CJ, Goswami R, Goswami P, et al. Growth and infectious exposure during infancy and the risk of rheumatoid factor in adult life. Ann Rheum Dis 2006; 65: 401-4.

Emery P, Breedveld FC, Dougados M, Kalden JR, Schiff MH, Smolen JS. Early referral recommendation for newly diagnosed rheumatoid arthritis: evidence based development of a clinical guide. Ann Rheum Dis 2002; 61: 290-7.

Erol K, Gok K, Cengiz G, Ozgocmen S. Hand functions in systemic sclerosis and rheumatoid arthritis and influence on clinical variables. Int J Rheum Dis. 2017 Mar 21.

Ferraz-Amaro I, González-Juanatey C, López-Mejias R, Riancho-Zarrabeitia L, González-Gay MA. Metabolic Syndrome in Rheumatoid Arthritis. Mediators of Inflammation 2013; 710928.

Firestein GS, Budd RC, Gabriel SE, McInnes IB, O'Dell JR. Etiology and Pathogenesis of Rheumatoid Arthritis. Kelley's Textbook of Rheumatology 9th ed. 2012; 1035-86.

Franceschi PL, Mannoni A. L'artrite reumatoide in medicina generale. Collana Disease Management 2008; 24: 7-27.

Gannage M, Amoura Z, Lantz O, Piette LC, Caillat-Zucman S. Feto-maternal microchimerism in connective tissue diseases Eur J Immunol, 32 (2002), pp. 3405-3413.

Gonzalez-Gay MA, Gonzalez-Juanatey C, Martin J. The increased risk of ventricular diastolic dysfunction and congestive heart failure in patients with rheumatoid arthritis is independent of the duration of the disease. Semin Arthritis Rheum. 2005 Oct;35(2):132-3.

Gough SCL, Simmonds MJ. The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. Curr Genomics. 2007;8(7):453-465.

Gregersen PK, et al. REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet. 2009; 41:820–823.

Hannaford PC, Kay CR, Hirsch S. Oral contraceptives and rheumatoid arthritis: new data from the Royal College of General Practitioners' oral contraception study. Ann Rheum Dis 1990; 49:744-6

Hromadnikova I, Zlacka D, Hien Nguyen TT, Sedlackava L, Zejskova L, Sosna A. Fetal cells of mesenchymal origin in cultures derived from synovial tissue and skin of patients with rheumatoid arthritis. Joint Bone Spine. 2008 Oct;75(5):563-6.

Iebba F, Di Sora F, Tarasi A, Leti W, Montella F. L'artrite reumatoide come modello di malattia genetica multifattoriale: una rassegna della letteratura. Recenti Prog Med 2011; 102: 175-82.

Ince-Askan H, Dolhain RJ. Pregnancy and rheumatoid arthritis. Best Pract Res Clin Rheumatol. 2015 Aug-Dec;29(4-5):580-96.

Kalla AA, Tikly M. Rheumatoid arthritis in the developing world. Best Pract Res Clin Rheumatol 2003; 17: 863–75.

Källberg H, Jacobsen S, Bengtsson C, et al. Alcohol consumption is associated with decreased risk of rheumatoid arthritis: results from two Scandinavian case-control studies. Ann Rheum Dis 2009; 68: 222-7.

Kara RJ, Karakikes I, Bolli P, Matsunaga I, Tripodi J, Tanweer O, Altam P, Shachter NS, Nakano A, NajfeldV, Chaudhry HW. Fetal Cells Traffic to Injured Maternal Myocardium and Undergo Cardiac Differentiation-Fetal Cell Differentiation in Maternal Hearts. Circ Res. 2012 Jan 6; 110(1): 82-93.

Klareskog L, Stolt P, Lundberg K, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope) - restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum 2006; 54: 38-46.

Kochi Y, Yamada R, Kobayashi K, Takahashi A, Suzuki A, Sekine A, Mabuchi A, Akiyama F, Tsunoda T, Nakamura Y and Yamamoto K: Analysis of single-nucleotide polymorphisms in Japanese rheumatoid arthritis patients shows additional susceptibility markers besides the classic shared epitope susceptibility sequences. Arthritis Rheum2004,50:63–71.

Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. Ann Rheum Dis 2006; 65: 366-71.

Llopis E, Kroon HM, Acosta J, Bloem JL. Conventional Radiology in Rheumatoid Arthritis. Radiol Clin North Am. 2017 Sep;55(5):917-941.

Mahmood U, et al. Microchimeric fetal cells play a role in maternal wound healing after pregnancy. Chimerism. 2014;5(2):40-52.

Mak W, Hunter JC, MRI for Earlier Diagnosis of Inflammatory Arthritis. Imaging in Rheumatology Arthritis, Juvenile Arthritis, 2009 dec 10.

Masi AT, Aldag JC. Integrated neuroendocrine immune risk factors in relation to rheumatoid arthritis: should rheumatologists now adopt a model of a multiyear, presymptomatic phase? Scand J Rheumatol 2005;34:342-52

Meron MK, Amital H, Shepshelovich D, Barzilai O, Ram M, Anaya JM. Infectious aspects and the etiopathogenesis of rheumatoid arthritis. Clin Rev Allergy Immunol 2010:38:287-91.

Minichello E, Semeraro L, Boisser MC. Time trends in the incidence, prevalence, and severity of rheumatoid arthritis: A systematic literature review. Joint Bone Spine 2016; Dec; 83(6): 625-30.

Mohan VK, Ganesan N, Gopalakrishnan R. Association of susceptible genetic markers and autoantibodies in rheumatoid arthritis. J Genet. 2014;93(2):597-605.

Liu C, Ackerman HH, Carulli JP. A genome-wide screen of gene-gene interactions for rheumatoid arthritis susceptibility. Hum Genet. 2011;129(5):473-485.

Mariaselvam CM, Chaaben AB, Salah S, Charron D, Krishnamoorthy R, Tamouza R, et al. Human leukocyte antigen-G polymorphism influences the age of onset and autoantibody status in rheumatoid arthritis. Tissue Antigens. 2015 Mar;85(3):182-9.

Moore JH et al., 2006. A flexible computational framework for detecting, characterizing, and interpreting statistical patterns of epistasis in genetic studies of human disease susceptibility. J Theor Biol.,241:252–261.

Mosley JD, Witte JS, Larkin EK, Bastarache L, Shaffer CM, Karnes JH, Stein CM et al. Identifying genetically driven clinical phenotypes using linear mixed models. Nat Commun. 2016; 7:11433

Mueller RB et al., 2014. Is radiographic progression of late-onset rheumatoid arthritis different from young-onset rheumatoid arthritis? Result from the Swiss prospective observational cohort. Rheumatology (Oxford), 2014. Apr;53(4):671-7

Oliver JE, Silman AJ. Why are women predisposed to autoimmune rheumatic diseases? Arthritis ResTher 2009;11:252

Paolino S, Cutolo M, Pizzorni C. Glucocorticoid management in rheumatoid arthritis: morning or night low dose? Reumatologia. 2017;55(4):189-197.

Pedersen M, Jacobsen S, Klarlund M, et al. Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides. Arthritis Res Ther 2006; 8: R133.

Pedersen JK, Kjaer NK, Svendsen AJ, Hørslev-Petersen K. Incidence of rheumatoid arthritis from 1995 to 2001: impact of ascertainment from multiple sources. Rheumatol Int 2009; 29: 411–15.

Pratesi F et al., 2013. HLA shared epitope and ACPA: just a marker or an active player?. Autoimmun Rev., 12(12):1182-7.

Prevoo ML, van't Hof MA, Kuper HH, et al. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum, 38 (1995), pp. 44-48.

Roman MJ, Moeller E, Davis A, Paget SA, Crow MK, Lockshin MD, et al. Preclinical carotid atherosclerosis in patients with rheumatoid arthritis. Ann Intern Med 2006;144:249-56

Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid Arthritis. Lancet 2010;376: 1094-1108.

Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis Res 2002;4:S265-72.

Silva I, Mateus M, Branco JC. Assessment of erythrocyte sedimentation rate (ESR) and C- reactive protein (CRP) on rheumatoid arthritis activity prediction. Acta Reumatol Port. 2010 Oct-Dec;35(5):456-62.

Simkin PA. The human knee: A window on the microvasculature. Tissue Barriers. 2015 Apr 3;3(1-2): e970465.

Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2003;2:47388.

Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. Lancet 2007;370:186174

Smolen JS, Landewe´ R, Breedveld F et al EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. Ann Rheum Dis 2010, 69:964–975

Snir O, Gomez-Cabrero D, Montes A, et al. Non-HLA genes PTPN22, CDK6 and PADI4 are associated with specific autoantibodies in HLA-defined subgroups of rheumatoid arthritis. Arthritis Res Ther. 2014;16(4):414.

Soubrier M., Barber Chamoux N., Tatar Z., Couderc M., Dubost JJ., Mathieu S. Cardiovascular risk in rheumatoid arthritis. Joint Bone Spine. 2014 Jul;81(4):298-302.

Speiser DE et al. 1997. A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. J. *Exp. Med.* 185:1777–1783.

Suzuki A et al., 2003 Functional haplotypes of PADI4, encoding citrulli- nating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet*. 34:395–402.

Takaechi T. Treatment of rheumatoid arthritis with biological agents - as a typical and common immune-mediated inflammatory disease. Proc Jpn Acad Ser B Phys Biol Sci. 2017;93(8):600-608.

Tan KH, Zeng XX, Sasajala P, Yeo A, Udolph G. Fetomaternal microchimerism. Chimerism. 2011; Jan-Mar; 2(1): 16–18.

Tan Z, Randall G, Fan J, Camoretti-Mercado D, Brockman-Schneider R, Pan L, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. Am J Hum Genet. 2007 Oct;81(4):829-34.

Taneja V. Cytokines pre-determined by genetic factors are involved in pathogenesis of Rheumatoid arthritis. Cytokine. 2014 Dec 22. pii: S1043-4666(14)00611-5

Tobón GJ, Youinou P, Saraux A. The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. J Autoimmun 2010; 35: 10-4.

Turesson C. Comorbidity in rheumatoid arthritis. Swiss Med Wkly. 2016 Apr 5;146:w14290.

van der Horst-Bruinsma IE, Hazes JM, Schreuder GM, et al. Influence of non-inherited maternal HLA-DR antigens on susceptibility to rheumatoid arthritis. Ann Rheum Dis 1998; 57: 672-5.

Viatte S, Plant D, Raychaudhuri S. Genetics and epigenetics of rheumatoid arthritis. Nat Rev Rheumatol. 2013 March ; 9(3): 141–153.

Wasserman AM. 2011. Diagnosis and management of rheumatoid arthritis. Am Fam Physician. 2011 Dec1;84(11):1245-52.

Watford WT et al., 2004. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.* 202:139–156.

Wick MC, Klauser AS; Radiological differental diagnosis of rheumatoid arthritis, Radiologe, 2012 Feb;52(2):116-23.

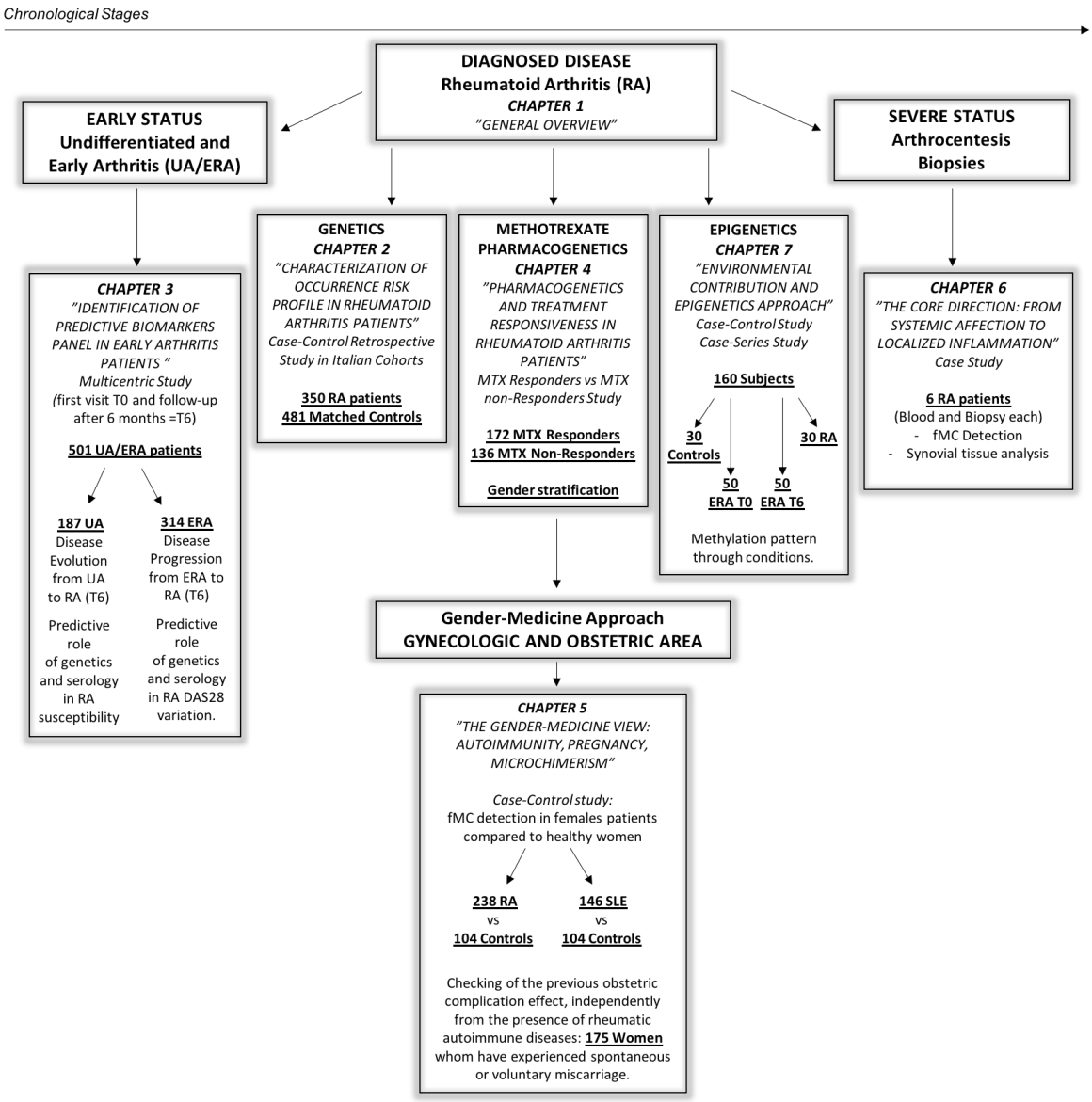
Wijbrandts CA, Tak PP. Prediction of Response to Targeted Treatment in Rheumatoid Arthritis. Mayo Clin Proc. 2017 Jul;92(7):1129-1143.

Wisniewski A, Kowal A, Wyrodek E, Nowak I, Majorczyk E, Wagner M, et al. Genetic polymorphisms and expression of HLA-G and its receptor, KIR2DL4 and LILRB1, in non-small cell lung cancer. Tissue Antigens 2015 Jun;85(6):466-75.

Yie SM, Xiao R, Librach CL, Progesterone regulates HLA-G gene expression through a novel progesterone response element. Hum Reprod, 2006.

Zambra FM, Biolchi V, de Cerqueira CC, Brum IS, Castelli EC, Chies JA, Immunogenetics of prostate cancer and bening hyperplasia-the potential use of an HLA-G variant as a tag SNP for prostate cancer risk. HLA. 2016 Feb;87(2):79-88.

THESIS' FLOWCHART



CHAPTER 2

CHARACTERIZATION OF THE OCCURRENCE RISK PROFILE IN RHEUMATOID ARTHRITIS PATIENTS

FROM HEALTH TO DISEASE

"The laws of genetics apply
even if you refuse to learn them"

Alison Plowden

CHAPTER 2

CHARACTERIZATION OF THE OCCURRENCE RISK PROFILE IN RHEUMATOID ARTHRITIS PATIENTS

FROM HEALTH TO DISEASE

Table of Contents

Abstract

Background: Rheumatoid Arthritis (RA) is a common autoimmune disease which affects up to 1% of the general adult worldwide population. Genetic studies have identified multiple major histocompatibility complex (HLA) region alleles involved in RA susceptibility, and several variants belonging to non-HLA loci. Nowadays, is still not understood how these variants are involved in RA risk, as well as the effect of gene-gene interactions. In addition, the contribution of genetic variant in RA occurrence could be variable into different ethnicities, indicating that in-depth studies in particular population might provide more insights in variants risk carriers.

Objective: Taking advantage of a complete Italian case-series, the aim is to individuate RA risk gene variants specific for Italian patients. The evaluation of effect in terms of a single gene SNP o through interaction study could help to identify a more complete risk profile and to predict in advance the RA susceptibility.

Materials and methods: A total of 350 RA patients and a matched group of 481 healthy controls have been enrolled, along with peripheral whole blood sample and clinical data. From the whole blood, genomic DNA was extracted and identified genetic variants in HLA and non-HLA regions were assessed by allelic discrimination. Statistical analyses of genetic association were carried out following a case-control study model. Gene-gene interaction and predict combined model elaboration have been identified using Multifactorial Dimensionality Reduction (MDR) software.

Results: HLA-DQA2 rs9275595 was been identified as the main variant involved in RA occurrence in Italian patients, following a dose allele effect: homozygotes C/C showed around double significant risk respect heterozygotes T/C patients (p-values respectively 2E-04, 1E-05). Significant sex difference was observed for HLA-DQA2 rs9275595, HLA-DQB2 rs10807113 and PTPN22 rs2476601. STAT4 rs7574865 showed RA risk only in male cohort, whereas HLA-DRB1 rs660895 and PADI4 rs2240340 acted as RA risk only in female.
Interaction study revealed significant combined effect between T/T for rs9275595 and A/A for rs660895 variants, showing 2.45-fold decreased RA risk compared to any others combination (p value=7E-09).

Conclusion: SNP × SNP results suggested to explore deeper the interaction between variants belonging to HLA complex. Further analysis might be performed in early rheumatoid arthritis patients, in order to investigated HLA-DQA2 rs9275595 variant as a predictive genetic biomarker.

2.1 Introduction

Rheumatoid Arthritis (RA) is an inflammatory, autoimmune and chronic progressive disease, which affects predominantly diarthrodial joints causing pain, swelling and articular stiffness (Gonzalez-Gay MA et al., 2005). RA onset is extremely variable, usually gradually with nonspecific symptoms, as asthenia, arthromyalgia and general unhealthy conditions. Subsequently symptoms are more defined showing pain and tumefaction joints and they could variate for intensity and gravity with generally recurrent course (Wasserman AM. 2011).

RA is a multifactorial disorder, caused by a combination both genetics and environmental factors (Scott DL et al., 2010). It is estimated that genetics factors contribute to RA risk around 50-60% (Iebba F et al., 2011) and epidemiological studies have been reported higher risk of RA development in partecipants with familiarity (Stack RJ et al., 2016). The main loci involved in the disease susceptibility belonged to the Human Leucocyte Antigen (HLA), localized on the surface of lymphocytes and deputed to the immune response control through the discrimination of self and non-self-antigens and to their presentation to T lymphocytes (Gough SCL et al., 2007). HLA is located on p arm of chromosome 6 (position 6p21.1-23.3), it is longer 3500Kb and, from the centromere, it is constituted by classes II, III, I:

- HLA I class (2000Kb): expressed on nuclear cells surface and constituted by α (α_1, α_2, α_3) and β_2-microglobulin polypeptide chains. It contains classical HLA (HLA-A, HLA-B, HLA-C) which present endogenous peptides to CD8+ T cytotoxic lymphocytes, and non-classical HLA (HLA-E, HLA-F, HLA-G), encoding for minor proteins.
- HLA II class (500Kb): expressed on APC membrane and constituted by α (α_1, α_2) and β (β_1, β_2) polypeptide chains; the function is presented exogenous peptides to CD4+ T helper lymphocytes. It contains D locus, subdivided in DP, DQ, DR, DM.
- HLA III class (1000Kb) encode for several proteins and cytokines involved in inflammation, as C2 and C4 complement fragments and TNF-α and –β (Tumor Necrosis Factor).

HLA genes are extremely polymorphic and strongly associated together and they are able to bond huge number of different antigens (Williams TM. 2001).

HLA-DRB1, belonged to HLA II class**,** represents the locus mainly associated in RA occurrence. It presents exogenous peptides to lymphocytes and it is expressed in Antigen Presenting Cells (APC). The molecule is a heterodimer composed by α-chain (DRA) and by β-chain (DRB), both show extracellular region and trans-membrane portion. The presence of alleles that share a consensus sequence, known as the 'shared epitope' (SE), in 70-74 positions of the third hypervariable region belonged to HLA-DR β chain, is implicated in higher susceptibility for RA risk (Pratesi F et al., 2013: Table 1). Mechanisms through the predisposing risk of the amino-acidic sequence SE (^{70}QRRAA^{74}, ^{70}QKRAA^{74}) (Bang SJ et al, 2010; Konda Mohan V et al. 2014) are still controversial.

The most sustained hypothesis proposes that autoimmunity induction is carried out by molecular mimicry or through particular peptides presentation to lymphocyte T receptor (TCR), causing a modification of T $CD4^+$ cells response (Beri R et al., 2005). Moreover, variants HLA-DRB1 rs6910071 A>A/G (position 6p21.32, occurring within an intron of C6orf10 gene, 263 Kb upstream HLA-DRB1 gene) and rs660895 A>A/G (intergenic region 30,8 Kb downstream HLA-DRB1 gene) have been associated to RA risk (Mosley JD et al., 2016). Both are in strong linkage disequilibrium with HLA-DRB1 SE and between them the distance is around 300Kb.

A study on linkage disequilibrium found out HLA-DQB2 gene as associated to RA occurrence independently to HLA-DRB1 SE (Kochi Y et al., 2004) and an interaction between HLA-DQA2 (rs9275595) and HLA-DQB2 (rs10807113) has been identified, with 11-fold higher risk to develop RA (Liu C et al., 2011). Both genes are HLA molecules of class II, composed by 2α-chain (DQA2) and by 2β-chain (DQB2); HLA-DQA2 encodes for a protein localized in APCs, which contributes to process and to present antigens to $CD4^+$T-Lymphocytes. HLA-DQB2 function is still controversial, some studies suggest it plays a role as HLA-DQA2, some others report it acts as a pseudogene (Pera C et al., 2000).

Moreover, HLA-G is a candidate gene for the susceptibility of immune mediated diseases, such as RA, due to its fundamental role in immunosuppression (Bortolotti D et al., 2014). Belonging to HLA class I, it present low polymorphism and it is expressed on several cellular types surfaces, but also in soluble form (Verbruggen LA et al., 2006). It interacts with immune system cells and prevents their response through different mechanisms (Veit TD et al., 2014). In fact, it inhibits the activity of cytotoxic $CD8^+$ T-Lymphocytes, acting on their cell-mediated cytolysis (CTL), it reduces the cytotoxic action of natural killer cells (NK) as well as their proliferation. In addition, HLA-G molecules may inhibit $CD4^+$ T-cell involved in the allo-proliferative response, activated by external factors and capable of inducing a specific immune response under appropriate conditions (Bahri R et al., 2006). Another function of the HLA-G molecules is to alter the physiological function of the APCs by also inhibiting proliferation (Horuzsko A et al., 2001). Moreover, it is known HLA-G mRNA goes to alternative splicing, forming 7 different protein isoforms, 4 bond to membrane and 3 soluble and secreted (sHLA-G) (Murdaca G et al., 2016).

HLA-G is not very variable, but the regulatory regions at 5 'and 3' UTRs are, on the contrary, highly polymorphic. The 3'UTR region plays an important role in the post-transcriptional regulation of HLA-G expression and the 14bp insertion/deletion (14bp indel, rs16375) and the one single nucleotide variant rs1063320 occurred in this region, may result in alteration of gene regulation.

The 14 bpindel variant is associated with the stability of the messenger RNA (mRNA): the presence of insertion (+14 bp 5′-ATTTGTTCATGCCT-3′) makes the mRNA more unstable, resulting in reduced production of HLA-G associated with low levels of sHLA-G (soluble HLA-G). Moreover, the variant regulates alternative splicing: the transcript with the insertion is able to have additional

splicing that removes 92 bases, including the insertion region, leading to lower HLA-G expression. In addition, the presence or absence of 14bp variant alters the microRNA set (miRNAs), affecting their regulation on HLA-G and the turnover of RNAs (Rebmann V et al., 2014).

The rs1063320 variant (C>C/G) is located at about 200bp from the 14bp indel variant, at +3142 position. It affects the binding of some miRNAs: more precisely, the variant carrying the G is a target of 3 miRNAs (miR 148a, 148b and 152), causing a reduction of HLA-G expression (Tan Z et al., 2007) and it has been reported that genotype +3142 G/G is related to the RA developing risk (Veit TD et al., 2014).

On the other side, the regulatory HLA-G 5' region shows the presence of CpG islands: portions of DNA characterized by CG repetitions, which could undergo to epigenetics methylation process, affecting the gene expression. Variants occurring in these sites may alter the binding of promoter transcription and/or methylation factors resulting in modulation in the transcription rate (Nilsson LL et al., 2014). Upstream the transcription site (-725bp), in fact, it has been found the rs1233334 G>G/C variant: the presence of G allele on this site leads to the creation of a CpG dinucleotide island, which is methylated in the DNA sequence in the blood mononuclear cells and potentially occurs to a different pattern of methylation. Authors have hypothesized that the -725G allele is associated with a variation of HLA-G expression due to its methylation, interfering to the bind of IRF-1 (Interferon Factor-1) with the ISRE (interferon specific regulatory element) sequence located nearby (Ober C et al., 2003). The -725G allele was also associated with higher levels of promoter expression leading to an increase in HLA-G transcription. On the contrary, the -725C allele carriers show a lower expression of the promoter. The underlying mechanism is not fully understood, but studies suggested the G or C allele presence in -725 position might influence differently the gene expression and even in different cellular populations (Jassem RM et al., 2012).

Besides, some studies have reported non-HLA genes related to RA etiology (Viatte S et al., 2013).

Protein tyrosine phosphatase non-receptor type 22 (PTNP22), localized in the chromosome 1p13.2, represents the second most important genetic factor responsible for the onset of RA after HLA-DRB1, in fact mutation in this gene are involved in the onset of several autoimmune diseases including RA, type 1 diabetes and systemic lupus erythematosus (SLE) (Korczowska I, 2014). More precisely, PTPN22 gene, expressed mostly in lymphoid tissues, codes for tyrosine lymphoid phosphatase (Lyp) responsible for the activation and differentiation of lymphocytes (Gianchecchi E et al., 2013). Under normal conditions, the enzyme acts as a negative regulator, preventing the hyperactivity of the immune cells. The PTPN22 variant involved in RA pathophysiology is rs2476601 (G>A/G, C1858T): it codes for a hyperactive phosphatase Lyp R620W, which replaces the amino acid Arginine (R) with the amino acid Tryptophan (W) at codon 620 of the gene. This substitution induces lymphocyte hyper activation and the production of autoreactive T and B subpopulation. Partecipants carrier one allele variant showed 2-fold higher RA risk, while patients

A/A homozygous presented 3/4-fold increase RA susceptibility (Begovich AB et al., 2004; Lee YH et al., 2007). There seems to be an association with HLA-DRB1 SE (Kurkò J et al., 2013).

Based on recent GWAS studies, the TNF receptor-associated factor 1 (TRAF1) gene located on chromosome 9q33-q34 has been identified as the third locus associated with RA occurrence risk after HLA-DRB1 and PTPN22. Overall, TRAF proteins represent a class of cytoplasmic adapters that binds tumor necrosis factor receptor (TNFR) and CD40, able of activate APCs associated with the development of RA. TRAF1 is involved in proliferation, differentiation, apoptosis, bone remodeling and modulation of cytokines production (Speiser DE et al., 1997).The variant associated is rs3761847 (G> A / G), mainly involved in the radiological progression of the disease (Konda Mohan V et al., 2014).

Peptidyl-arginine deaminase type 4 (PADI4) gene was firstly identified as one of the major RA risk factors in Asian population, recently it has been also associated in European population (Snir O et al., 2014). The gene is located on chromosome 1 (1p36.13), it encodes for the Peptide Enzyme Arginine Type IV Deaminase, which is involved in the catalytic process of arginine desulfurization to neutral citrulline, resulting in the production of citrullinated proteins (anti-CCP) (Raffaele G, 2014). The enzyme finds its highest expression in bone marrow and in peripheral blood leucocytes, but also appears in synovial tissue. The PADI4 mRNA is over-expressed in the RA patients blood and its activity is modulated by Ca^{2+} and pH concentration. During the apoptotic process, detected by fibrin deposits presence in the synovium of patients, Ca^{2+} cell levels increased to stimulate the activation of PADI4. Among its 4 single variants, the rs2240340 (T>C/T) appears to be more associated with RA, due to its main involvement in producing high amount of citrullinated proteins, acting as autoantigens (Suzuki A et al., 2003). Moreover, PADI4 seems to interact with HLA-DRB1 SE (Bang SJ et al., 2010). Signal Transducer and Activator of Transcription 4 (STAT4), localized in 2q32.2-q32.3 has been found associated with RA in Europe, Asia and Latin America populations and in particular it is overexpressed in RA patients with more sever phenotypes (Konda Mohan V et al., 2014). The gene codes for the STAT4 protein, implicated in intracellular signals activation through IL-12, IL-23 and IL-27 cytokines, leading to the production of IFNγ (interferon gamma) (Watford VT et al., 2004) and involved in the cell differentiation process of Th1 and Th2, both play a crucial role in autoimmune pathologies (Rodrìguez-Elìas AK et al., 2016). The main variant involved in RA pathogenesis is rs7574865 (T>G/T) located on intron 3 of the STAT4 gene. Studies have been observed treatment with DMARDs reduce the gene expression in the synovial dendritic cells and suggested STAT4 as a possible therapeutic target.

Nowadays, the single evaluation of RA genetic causative variants is useful in order to provide a panel of inherited risk, as well the association between genotypes and personal characteristics of RA patients, such as smoking and BMI. However, the great and remarkable value should be obtained from the interactions and combination of those variants.

2.2 Rationale and aims

The main purpose of this case-control study is to identify genetic variants associated to the etiopathology of Rheumatoid Arthritis (RA) in a complete Italian case-series. The result could be linked to a determination of new or stronger genetic biomarkers and it should be specific and population-based. Second objective is the individuation of some particular genetic variants associated to the sex and to assess the sex-compared difference. Thirdly, it will be investigated the complex interaction among different gene variants and how variants could influence each other. In other words, if RA susceptibility could be related to the combination of the variants effects and if their interaction follows synergic, additive or redundant model.

The outcome might suggest the good direction to develop a model based on the patient's personal profile and to potentiate the value of the P4 medicine consisting on predictive, preventive, personalized and participatory approach.

2.3 Materials and Methods

Subjects

The study included a cohort of 350 Italian patients with diagnosis of RA, satisfying the American College of Rheumatology and the European League Against Rheumatism (2010 ACR/EULAR) criteria (Aletaha D et al., 2010). Participants were recruited in the Rheumatology Unit of Sant'Anna University Hospital of Ferrara, with the collaboration of Prof. *M. Govoni*. The study was reviewed and approved by the committee for Medical Ethics in Research of Ferrara and the written informed consent was taken from all patients enrolled. Among them, 81 were males and 269 females. On the basis of age of onset, the disease was classified into young onset RA when onset was ≤60 years and late onset RA when onset was >60 years of age (Mueller RB et al., 2014). The study comprised a group of 481 healthy control individuals, matched for age, sex, geographical origin and ethnicity. To each subject enrolled was been assigned a code and sensible data were separated from biological sample. Biological samples were storage at -20°C until processed for genomic DNA extraction.

Genomic DNA isolation from blood samples

Whole venous blood was taken from each patient with Vacutainer (BD, United States) containing ethylendiaminetetraacetic acid (EDTA). Genomic DNA (gDNA) was extracted from peripheral blood leucocytes from 1.5ml of fresh or frozen blood using Nucleon™ DNA Extraction kit (Amersham Biosciences, part of GE Healthcare Europe, CH), following the manufacturer's instructions.

Briefly, the first step consisted in cell lysis followed by nucleic acid separation from all others components. The water-phase extraction was possible using alcohol/chloroform mixture. After

emulsion centrifugation, organic phase was discarded and water-phase, containing gDNA was obtained.

In case of less amount of blood, gDNA was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manifacturer's protocol. Basically, after the sample lysis with protease or proteinase K, the DNA was bound and adsorbed onto silica membrane of the specific spin column during a brief centrifugation. The sample was washed using two different wash buffer which ensured complete removal of any residual contaminants affecting DNA binding. Final step was the purified DNA elution from the spin column in mQ water.

Genomic DNA titration and normalization

All genomic DNA was quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA). After titration, each gDNA was inserted in Matrix 2D-Barcoded (Thermo Fisher Scientific) and they made possible set up a DNA-Biobank located in -80°C freezer equipped with Access Key and constant monitoring of use and function conditions.

Working conditions took place using genomic DNA at concentrations of 10ng/ul or 1ng/ul, depending on methodology, in order to normalize results.

Genotyping

Gene variants analyzed, belonging to Human Leucocyte Antigen genes (HLA) and not HLA were followed:

- *HLA-G 14bp indel D>I*
- *HLA-G rs1063320 C>G*
- *HLA-G rs1233334 G>C*
- *HLA-DRB1 rs6910071 A>G*
- *HLA-DRB1 rs660895 A>G*
- *HLA-DQA2 rs9275595 T>C*
- *HLA-DQB2 rs10807113 C>A*
- *PADI4 rs2240340 C>T*
- *TRAF1 rs376184 G>A*
- *PTPN22 rs247660 G>A*
- *STAT4 rs7574865 T>G*

Using Genome Browser (http://genome.ucsc.edu/), has been obtained the nucleotidic sequence of the corresponding region of interest (GRCh38) and the relative MAF.

Real-Time PCR

HLA-G +3142 G>C (rs1063320) and HLA-G +725C>G>T (rs1233334) variants were analyzed using the TaqMan™ Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific,

Foster city, CA, USA) and Custom-designed 5'-nuclease TaqMan™ SNP Genotyping Assays (Real Time PCR System, Applied Biosystems by Thermo Fisher Scientific, Foster city, CA, USA), with allele-specific fluorogenic oligonucleotide probes allowing allele discrimination. All the others variants (except HLA-G 14bp indel) were analyzed using the TaqMan™ Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific, Foster city, CA, USA) and Predesigned 5'-nuclease TaqMan™ SNP Genotyping Assays (Real Time PCR System, Applied Biosystems by Thermo Fisher Scientific, Foster city, CA, USA).

PCR conditions for all reaction were as follows: 50°C for 2 min, 95°C for 10 min and (95°C for 15 s, 60°C for 1 min) x 50 cycles for both variants. Plates were read and analyzed on a 7300 Real Time PCR System (Applied Biosystems by Thermo Fisher Scientific).

PCR-PAGE

The HLA-G 14bp indel variant was detected by a polymerase chain reaction (PCR) sequence-specific primer method, already described (Castelli EC et al., 2014).

Briefly, the reaction is based on restriction fragment length process and the polymorphic region of interest was amplified using forward primer: 5'-GTGATGGGCTGTTTAAAGTGTCACC-3' and reverse primer: 5'-GGAAGGAATGCAGTTCAGCATGA-3' (Wisniewski A et al. 2015). The amplification was performed by PCR with a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA) in a 25ul reaction mixture containing 100ng of genomic DNA, 10XPCR buffer, 50mM $MgCl_2$, 10mM dNTPs, 20pmol of each primer and 1U of Taq polymerase (Invitrogen Co., Carlsbad, Ca). The PCR conditions comprised initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 15 s, annealing at 64°C for 30 s and extension at 72°C for 30 s, then 25 cycles of denaturation at 94°C for 15 s, annealing at 63°C for 30 s and extension at 72°C for 30 s, and final extension at 72°C for 5 min. The purified PCR products size were analyzed using an 8% polyacrylamide gel. The product size was 224 bp for Ins/Ins (I/I) and 210 bp for Del/Del (D/D) and both 224 bp and 210 bp for Del/Ins (D/I) genotypes. The PCR products were visualized using silver staining.

Statistical Analyses

For each SNP, were assessed genotypic frequencies, allelic frequencies and Hardy Weinberg equilibrium. Chi-squared test χ^2 with Yates continuity correction will be performed using R statistical software version 3.0.1, in order to evaluate the difference between the data series and to verify null hypothesis of independency between variables. Statistical analyses of odd ratio (ORs) with 95% Confidence Interval (95% CI) have been performed using MS™Excel and GraphPad packages. The comparison between two ORs has been evaluate (ex: in sex stratification analyses) with the

correspondent p-value of the comparison, reported only if the value is significant. As we undertook multiple comparisons for the association study on 11 genetic variants, a Bonferroni adjustment was done and results were considered significant for a probability (p) value of 0.0045 (=0.05/11).

To evaluate SNP-SNP interaction and to individuate epistasis phenomenon, it was used a genetic model-free method called multifactor dimensionality reduction (MDR) (Moore JH et al., 2006). MDR reduces the dimensionality of multi-allelic loci and recognizes the genetic combinations given greatest risk of developing the disease. It has been designed to analyze the interaction in datasets composed by independent categorical variables, as SNPs, which have the endpoint as a dependent variable that must be dichotomous, as case/control.

MDR outputs were in form of histograms with main interactions and dendrograms, where darker and closer lines were referred to the strongest interaction.

2.4 Results

A total of 350 patients, 269 females and 81 males, were enrolled in the study. The baseline characteristics of the patients included in the study are summarized in Table 2.1.

Young onset RA when onset was ≤60 years and late onset RA when onset was >60 years of age (Mueller RB et al., 2014). Besides, a group of 481 matched healthy controls has been identified, in order to perform the case-control study.

From a subgroup of 100 RA patients have been collected personal characteristic regarding weight, height and smoking habits, to carried out further analyses.

Characteristics		RA (n=344)
Demographic		
	Female : Male	3.32:1
	Age (years) (mean ± SD)	58.89 ± 12.76
Clinical		
	Young onset RA (%)	75.65
	Late onset RA (%)	24.35
Antibodies		
	RF+ (%)	66.76
	ANA+ (%)	34.21
	Anti-CCP+ (%)	55.67
Characteristics		RA (n=100)
Personal		
	Smokers (%)	32.5%
	BMI (kg/m^2) (mean ± SD	25.48 ± 4.39

Table 2.1 *Characterization of RA Italian subjects studied.*

2.4.1 Case-Control Genetic Association Study

Single variant association analysis has been carried out considering 11 candidate genes variants: 7 belonged to HLA complex and 4 in candidate-genes non-HLA. Both RA and control cohorts were in Hardy-Weinberg equilibrium (data not shown). OR, 95% C.I. and the corresponding p value concerning the codominant genetic model for each variant, were reported in Table 2.2.

Candidate Gene Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G 14 bp (rs66554220)				
	DI/DD	0.912	0.667-1.246	0.574
	II/DD	**1.551**	**1.504-2.322**	**0.029**
HLA-G rs1063320				
	GC/GG	0.763	0.550-1.058	0.105
	CC/GG	0.820	0.538-1.233	0.353
HLA-G rs1233334				
	GC/GG	0.880	0.631-1.227	0.459
	CC/GG	n/a	n/a	n/a
HLA-DRB1 rs6910071				
	AG/AA	0.951	0.688-1.315	0.774
	GG/AA	0.825	0.165-3.485	0.816
HLA-DRB1 rs660895				
	AG/AA	**1.654**	**1.184-2.288**	**0.003**
	GG/AA	2.103	0.522-9.479	0.320
HLA-DQA2 rs9275595				
	TC/TT	**1.981**	**1.463-2.682**	**1E-05**
	CC/TT	**3.672**	**1.994-7.748**	**2E-04**
HLA-DQB2 rs10807113				
	AC/AA	0.956	0.706-1.295	0.784
	CC/AA	1.468	0.972-2.245	0.072
PADI4 rs2240340				
	CT/CC	**1.600**	**1.153-2.221**	**0.005**
	TT/CC	**1.813**	**1.193-2.818**	**0.007**
TRAF1 rs3761847				
	AG/AA	1.018	0.745-1.393	0.916
	GG/AA	1.267	0.839-1.921	0.265
PTPN22 rs2476601				
	GA/GG	**1.799**	**1.161-2.878**	**0.009**
	AA/GG	1.446	0.203-10.327	0.726
STAT4 rs7574865				
	GT/GG	**1.558**	**1.153-2.105**	**0.004**
	TT/GG	1.811	0.955-3.510	0.073

[a] Odds Ratio
[b] Confidence Interval 95%

Table 2.2 *Case-Control Association study: in the totality of RA Italian patients.*

Concerning the 7 HLA variants, the strongest RA occurrence risk has been reported for HLA-DQA2 rs9275595: both T/C heterozygotes and C/C homozygotes showed significant higher risk to develop RA than T/T wild type homozygotes, respectively with p value 0.00005 and 0.0002. This evidence in the codominant model did not make it possible to determine whether the variant follows a dominant or recessive pattern; this is an intermediate dominant effect, in addition manifesting allele

dose dependency: in fact, having only one C allele gave 1.99-fold higher risk, whereas carrying both variant alleles increased nearly twice the risk of RA development.

Among the others HLA variants, HLA-DRB1 rs660895 noticed significant higher risk to develop RA for subjects heterozygous A/G respect A/A wild type homozygous. The variant followed the dominant genetic model, showing for G-/AA patients statistical significant occurrence risk (OR= 1.660 95%CI 1.199-2.298, p value=0.002). Concerning HLA-G 14bp variant, the results reported in Table 2.2 show a trend towards disease association: I/I homozygous patients showed 1.5-fold higher risk of RA development, respect D/D genotype. The observed data has been verified considering the recessive genetic model II/D- in which patients I/I, respect to those heterozygous or homozygotes for the reference alleles, reported evident RA occurrence risk (OR=1.637 95%CI 1.142-2.347, p-value=0.007), without reaching the statistical significant after Bonferroni correction.

HLA-DQB2 rs10807113 did not reveal association in the codominant model, but taking the recessive genetic model into account, C/C homozygous patients showed a trend of association with a 1.5-fold increased risk of RA (95% CI 1.007-2.235; p-value=0.046), respect to whom had at least one copy of wild type allele.

The others HLA variants analyzed did not find association with RA occurrence. However, HLA-DRB1 rs6910071 and HLA-G rs1063320 reported a slight pattern toward protection from RA onset in subjects with allele variant, respect the wild type genotype. The effect was similar for whom carried one copy of the allele variant or homozygotes, both variant analyzed followed the codominant genetic model. HLA-G rs1233334 variant failed to reveal any association and MAF of C determined the inability to define the genetic model.

With regard to 4 non-HLA candidate-gene variant, in the Table 2.2 PADI4 rs2240340 noticed a strong and remarkable trend toward association, almost statistically significant. The variants followed the dominant genetic model: in fact, taking C/C homozygotes as the reference, subjects with at least one copy of variant allele T showed more than 1.6-fold higher risk of RA developing (T-/CC: OR=1.646 95%CI 1.201-2.256, p-value=0.002), reaching the significant value.

Secondly, PTPN22 rs2476601 variant showed RA occurrence trend. Observing the results reported in the codominant model, the dominant genetic effect has been studied: patients carried one or two copies of A allele variant, reported higher susceptibility to RA onset respect G/G homozygotes (A-/GG: OR=1.783 95%CI 1.160-2.739, p-value=0.0083).

Genetic association with STAT4 rs7574865 and RA occurrence risk revealed significant result concerning heterozygotes patients. SNP rs7574865 acted under a dominant genetic model: subjects carrying at least one copy of the allele variant T showed a significant association with RA development, reporting more than 1.5-fold higher RA risk (95% CI 1.189-2.119, p-value=0.0018), respect homozygotes patients for the wild type genotype GG. Lastly, the variant rs3761847 near TRAF1 gene did not report RA occurrence risk and it followed the codominant model.

2.4.2 Case-Control Genetic Association Study on sex comparison

Furthermore, genetic association analyses have been performed stratified RA patients and the matched control group by sex, in order to evaluate if the investigated candidate-gene variants might be associated in males and females, following different patterns.

OR, 95%CI and the correspondent p-value have been reported in the Table 2.3 for the codominant genetic model. The sex-compared p-value has been calculated only for variant significant associated in males or females or both.

Candidate Gene Variant	Codominant Genetic Model	MALE Test of association			FEMALE Test of association			Sex compared P value
		OR [a]	95% CI [b]	P value	OR [a]	95% CI [b]	P value	
HLA-G 14bp								
rs66554220	*DI/DD*	1.090	0.614-1.936	0.781	0.765	0.518-1.132	0.181	-
	II/DD	1.330	0.609-2.817	0.475	1.508	0.940-2.513	0.101	-
HLA-G								
rs1063320	*GC/GG*	0.804	0.434-1.489	0.498	0.747	0.500-1.118	0.157	-
	CC/GG	0.943	0.443-1.966	0.886	0.856	0.509-1.431	0.568	-
HLA-G								
rs1233334	*GC/GG*	1.263	0.694-2.300	0.453	0.804	0.531-1.217	0.306	-
	CC/GG	n/a	n/a	n/a	n/a	n/a	n/a	-
HLA-DRB1								
rs6910071	*AG/AA*	1.091	0.603-1.973	0.786	0.958	0.640-1.435	0.848	-
	GG/AA	2.649	0.164-43.968	0.503	0.495	0.069-2.734	0.462	-
HLA-DRB1								
rs660895	*AG/AA*	1.726	0.940-3.169	0.078	**1.600**	**1.064-2.407**	**0.024**	0.753
	GG/AA	2.982	0.415-21.676	0.283	2.249	0.314-25.01	0.478	-
HLA-DQA2								
rs9275595	*TC/TT*	**2.888**	**1.633-5.105**	**3E-04**	**1.517**	**1.048-2.194**	**0.027**	**0.011**
	CC/TT	**7.700**	**2.430-32.141**	**0.002**	**2.409**	**1.166-5.798**	**0.031**	**5E-06**
HLA-DQB2								
rs10807113	*AC/AA*	**1.860**	**1.054-3.284**	**0.032**	0.748	0.513-1.092	0.133	**2E-04**
	CC/AA	1.736	0.719-4.002	0.210	1.352	0.832-2.281	0.244	-
PADI4								
rs2240340	*CT/CC*	1.740	0.924-3.277	0.086	**1.678**	**1.125-2.501**	**0.011**	0.885
	TT/CC	1.631	0.702-3.788	0.258	**2.036**	**1.242-3.514**	**0.007**	0.382
TRAF1								
rs3761847	*AG/AA*	1.255	0.695-2.264	0.460	0.889	0.604-1.307	0.561	-
	GG/AA	1.768	0.842-3.729	0.134	1.229	0.739-2.090	0.445	-
PTPN22								
rs2476601	*GA/GG*	**3.180**	**1.420-7.123**	**0.005**	1.423	0.834-2.429	0.197	**0.002**
	AA/GG	n/a	n/a	n/a	0.500	0.031-5.554	0.613	-
STAT4								
rs7574865	*GT/GG*	**2.153**	**1.245-3.726**	**0.006**	1.421	0.973-2.074	0.068	0.085
	TT/GG	2.619	0.741-8.742	0.126	1.561	0.718-3.402	0.298	-

[a] Odds Ratio
[b] Confidence Interval 95%

Table 2.3 *Case-Control Association study sex-related in RA patients and in matched control group.*

Both RA and control cohorts were in Hardy-Weinberg equilibrium (data not shown). Genetic association between candidate genes variants and RA occurrence risk referred to HLA complex, confirmed the strong association for HLA-DQA2 rs9275595, previous described in the totality of

patients. Both male and female cohorts showed significant higher risk to RA develop, underlined the allele dose dependency: taking T/T as the reference, C/C homozygotes manifested double occurrence risk respect T/C heterozygotes. More precisely, the effect was more evident in males, showing remarkable significant sex-compared p-value, regarding homozygotes patients for the variant (5E-06). Concerning HLA-DQB2 rs10807113, it was possible to notice the different pattern effect among sex: male heterozygotes reported 1.86-fold higher risk to RA susceptibility (95%CI 1.054-3.284, p-value=0.032), on the contrary female heterozygotes revealed 1.30-fold lower risk for RA developing (95%CI 0.915-1.951, p-value=0.133). The sex-compared p-value was significant (2E-04), underlying that rs10807113 acted in opposite direction between sex.

HLA-DRB1 rs660895 did not revealed significant sex difference, due to the association with RA risk alike in both cohorts. The genetic model followed was dominant: taking A/A as the reference, male or female subjects carrying at least one copy of the allele variant G, manifested higher probability to RA occurrence. More precisely males reported a slight trend toward risk: OR=1.789 (95%CI 0.991-3.231, p-value=0.053), whereas in females the effect was stronger (OR=1.612 95%CI 1.075-2.417, p-value=0.021).

The other HLA variant with a trend of association following a specific genetic model is HLA-G 14 bp: in female group, it acted under recessive model showing I/I homozygotes with 1.77-fold higher RA risk (95%CI 1.129-2.788, p-value=0.013) respect subjects with at least one copy of D allele. HLA-G 14bp variant association was only ascribable in female patients, whereas in males was completely irrelevant. HLA candidate-gene variants concerning HLA-DRB1 rs6910071, HLA-G rs1063320 and HLA-G rs1233334 failed to reveal any association sex-related, confirming what has previous reported in the totality of patients.

Considering non-HLA gene variants, PADI4 rs2240340 showed stronger association in female group respect to males. The genetic model followed was the dominant, reporting women with at least one copy of the allele variant T had significant 1.7-fold higher risk to develop RA (95%CI 1.197-2.572, p-value=0.004), compared to C/C wild type homozygotes. The p value of the sex comparison was 0.931, cause the same trend noticed in male group.

On the other side, the variant PTPN22 rs2476601, which has previously manifested strong association in the totality of patients, showed more evident effect in males than female cohort, acting under a dominant genetic model. Comparing the p-value of the dominant model between sex, it came out significant (3E-04), showing men with at least one copy of the variant allele A with significant increased risk to RA occurrence (OR=3.407 95%CI 1.539-7.545, p-value=0.003), than G/G homozygotes. The other variant showing significant stronger associated in male cohort was STAT4 rs7574865. The SNP followed the dominant genetic model reporting men with at least on the allele variant T with a significant 2.2-fold higher risk to RA develop (95%CI 1.1293-3.742, p-value=0.0037), respect subjects with homozygotes for the wild type genotype GG. The pattern was

similar in females, manifesting borderline significant values concerning the dominant genetic model (p value=0.05). Lastly, rs3761847 variant, near TRAF1 gene, did not reveal any association in male or female cohorts, confirming what has previous reported in the totality of patients.
To summarize, candidate-gene variants analyzed which found significant differences in RA occurrence risk between sexes were HLA-DQA2 rs9275595, HLA-DQB2 rs10807113 and PTPN22 rs2476601. Moreover, the stratification allowed identifying SNPs effect according to sex: in fact, RA significant higher risk occurrence awarded by HLA-G 14bp, HLA-DRB1 rs660895, PADI4 rs2240340 was ascribable only in women cohort, whereas STAT4 rs7574865 was associated to RA risk only in men.

2.4.3 Multifactorial Interaction

Total Cohorts: Case-Control Genetic interaction

MDR analysis confirmed SNP rs9275595 T>C near HLA-DQA2 gene as the variant most associated with evolution to RA. Following the dominant model, patients carrying at least one copy of the allele variant C showed a significant association with a 2.19-fold increased risk of RA (OR=2.187 95%CI (1.616-2.960) p-value=6E-07), respect wild type homozygotes.

The best SNP-SNP interaction resulted between HLA-DQA2 rs9275595 and HLA-DRB1 rs660895 G>A: HLA-DQA2 T/T homozygotes and HLA-DRB1 A/A homozygote vs the other combinations turned out into a significant 2.45-fold decreased risk of RA (OR=0.409 95%CI (0.304-0.550) p-value=7E-09). The difference between the effect of HLA-DQA2 rs9275595 variant and the interaction was not significant (p-value=0.641). In fact, the evaluation of the type of interaction between the two variants came out redundant, showing -32.6% effect respect to the addition of the individual variant outcomes.

Moreover, the MDR dendrogram output reported strong gene-gene interaction between HLA-DRB1 rs660895 G>A and HLA-G rs1233334 G>C (Figure 2.1 A). A/G heterozygotes for rs660895 and G/G homozygotes for rs1233334 or G/G homozygotes for rs660895 and G/C heterozygotes for rs1233334 vs other combination noticed 2.14-fold increased risk of RA (OR=2.139 95%CI (1.482-3.086) p-value=6E-05). The difference between interaction and HLA-G rs1233334 variant effect was significant (p value=2E-04) (Figure 2.1 B), whereas with HLA-DRB1 rs660895 variant was not (p value= 0.298). Nonetheless, the interaction showed synergy, exceeding 46.3% of the result that would has obtained if the interaction had been additive (OR = 1.46 (95% CI = 1.014-2.110)).

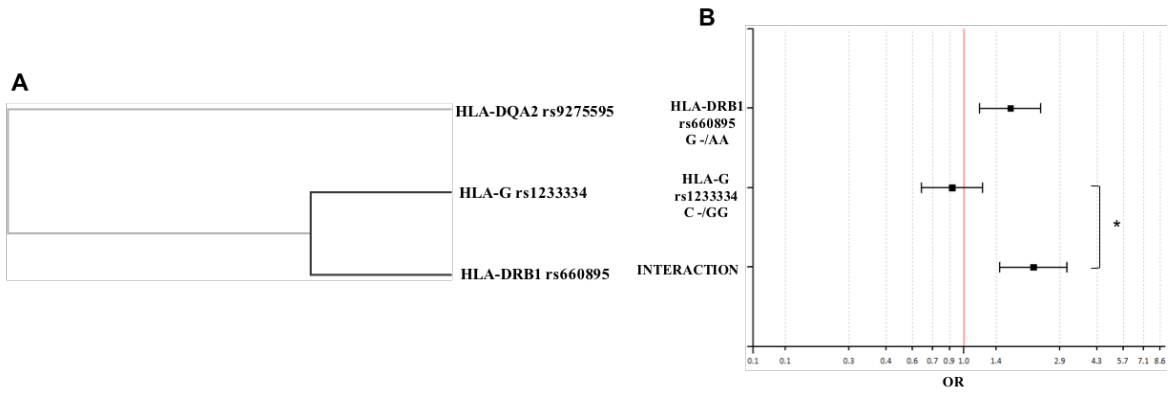


Figure 2.1 *Gene-gene interaction.* ***A****. Dendrogram of MDR software output. Darker and closer lines were referred to the stronger association: HLA-DRB1 rs660895 and HLA-G rs1233334.* ***B.*** *Forests plot with association results of single variants and interaction.*

Furthermore, considering the very significant HLA-DQA2 rs9275595 effect in RA risk development which could overhang the individuation of others gene-gene interactions, the analysis has been performed excluding that variant. Firstly, MDR analysis identified SNP rs7574865 G>T near STAT4 gene as the main associated to RA occurrence risk. Subjects with at least one copy of allele variant T, showed a significant 1.6-fold increased risk of RA (OR=1.651 95%CI (1.226-2.224) p-value=0.001). The SNP-SNP interaction revealed was between STAT4 rs7574865 and HLA-DRB1 rs660895: STAT4 G/G homozygotes and HLA-DRB1 A/A homozygote vs the other combinations showed significant 1.82-fold decreased risk of RA (OR=0.551 95%CI (0.411-0.739) p-value=8E-05). However, the difference between single variants effect and the interaction was not significant (p value=0.696), and it showed redundancy: -33.8% effect respect to the addition of the individual variant outcomes. No evident interactions were detectable among multiple SNPs.

Male Subjects: Case-Control Genetic interaction

Introducing the sex stratification, MDR gene-gene interaction in male patients followed the same pattern described in the totality of cases and controls. The most variant associated to RA occurrence risk was HLA-DQA2 rs9275595: men with at least one copy of the allele variant C manifested significant association with a 3.25-fold increased risk of RA (OR=3.248 95%CI (1.860-5.671) p-value=4E-05), respect T/T homozygotes. The best SNP-SNP interaction was confirmed between HLA-DQA2 rs9275595 and HLA-DRB1 rs660895 G>A: HLA-DQA2 T/T homozygotes and HLA-DRB1 A/A homozygote vs the other combinations showed significant 3.36-fold decreased risk of RA (OR=0.298 95%CI (0.172-0.515) p-value=2E-05). The interaction was redundant, reporting -24.8% effect respect if the interaction had been additive (OR = 4.46; IC 95% = 2.583-7.734). Concerning MDR results excluding HLA-DQA2 rs9275595 variant into analysis, SNP rs7574865 G>T near STAT4 gene was found to be associated, corroborating what has been previously described. Males patients with at least one copy of allele variant T, showed significant 2.3-fold higher

risk of RA (OR=2.345 95%CI (1.370-4.016) p-value=0.002). SNP-SNP interaction was individuated between STAT4 rs574865 and PTPN22 rs2476601 variants. STAT4 G/G homozygotes and PTPN22 G/G homozygotes vs the other combinations showed significant 3.1-fold decreased risk of RA (OR=0.323 95%CI (0.186-0.560) p-value=6E-05). The difference between single variants effect and the interaction was not significant (p value=0.251) and it indicated redundancy: -61.3% effect respect to the addition of the individual variant outcomes.

Female Subjects: Case-Control Genetic interaction

MDR gene-gene interaction in female patients confirmed HLA-DQA2 rs9275595 as the top variant associated to RA risk. Women with at least one copy of the allele variant C manifested significant association with a 1.7-fold increased risk of RA (OR=1.702 95%CI (1.176-2.461) p-value=0.0048), respect wild type homozygotes. The best SNP-SNP interaction found followed what has previously noticed in males, but with less strength: HLA-DQA2 T/T homozygotes and HLA-DRB1 A/A homozygote vs the other combinations showed significant 1.96-fold decreased risk of RA (OR=0.519 95%CI (0.355-0.734) p-value=3E-04). The interaction was redundant, reporting -28.6% effect respect if the interaction had been additive (OR = 2.74; IC 95% = 1.908-3.944).

Moreover, the MDR dendrogram output reported strong gene-gene interaction between HLA-G rs1063320 G>C and HLA-DQB2 rs10807113 A>C (Figure 2.2 A), with the latter showing an epistatic effect over HLA-G variant. Females A/C heterozygotes for rs10807113 alone associated with 1.3-fold decreased risk of RA occurrence (OR = 0.748; 95% CI (0.513-1.092), p value=0.133). MDR analysis indicated that rs1063320 variant acted as protection against RA development only in cases of heterozygous genotype combined with A/A homozygotes for rs10807113, and C/C genotype only in presence of C/C homozygotes for rs10807113 variant, with significant interaction value (OR = 0.543; 95% CI (0.370-0.796), p= 0.0018) (Figure 2.2 B). The difference between the effect of HLA-DQB2 rs10807113 variant and the interaction showed a trend toward association (p-value=0.061). The evaluation of the type of interaction between the two variants was synergic to protection, showing + 21.9 % effect respect to the addition of the individual variant outcomes.

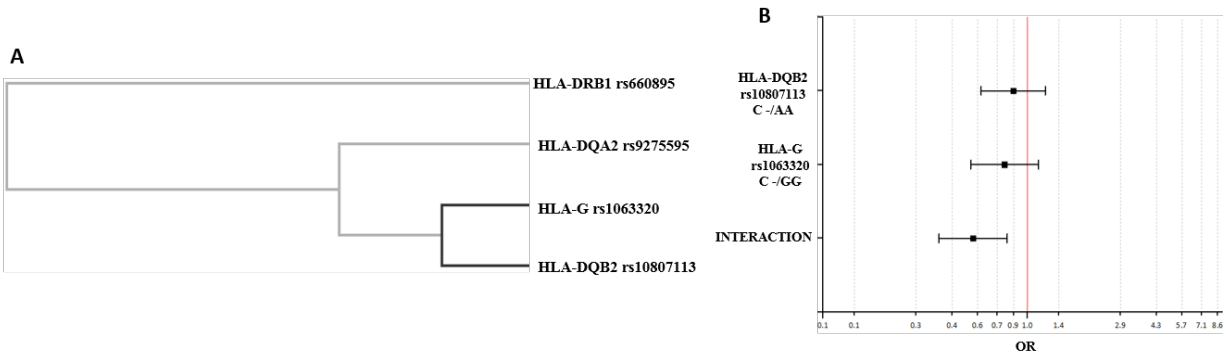


Figure 2.2 *Gene-gene interaction. **A**. Dendrogram of MDR software output. Darker and closer lines were referred to the stronger association. **B.** Forests plot with association results of single variants and interaction.*

Excluding HLA-DQA2 rs9275595, top SNP turned out PADI4 rs2240340, showing females with at least one copy on T variant allele with higher RA risk (OR = 1.578; 95% CI (1.068-2.333), p= 0.022)), respect C/C genotype.

The best interaction revealed by MDR dendrogram output was between HLA-DQB2 rs10807113 and PADI4 rs2240340 (Figure 2.3 A), with the latter showing an epistatic effect over HLA-DQB2 variant. In fact, women C/C homozygotes for rs2240340 alone associated decreased risk of RA occurrence (OR = 0.634; 95% CI (0.429-0.936), p value=0.022). The rs10807113 variant acted as protection against RA development only in case of A/C heterozygous genotype combined with C/T homozygotes for rs2240340, with significant interaction value (OR = 0.525; 95% CI (0.365-0.755), p= 5E-04) (Figure 2.3 B).

The difference between interaction and HLA-DQB2 rs10807113 variant effect was significant (p value=9E-04) (Figure 2.3 C), whereas with PADI4 rs2240340 variant was not (p value= 0.435). The interaction was synergic to RA risk, exceeding 41.4% of the result that would has obtained if the interaction had been additive (OR = 1.34; 95% CI (1.014-2.110)).

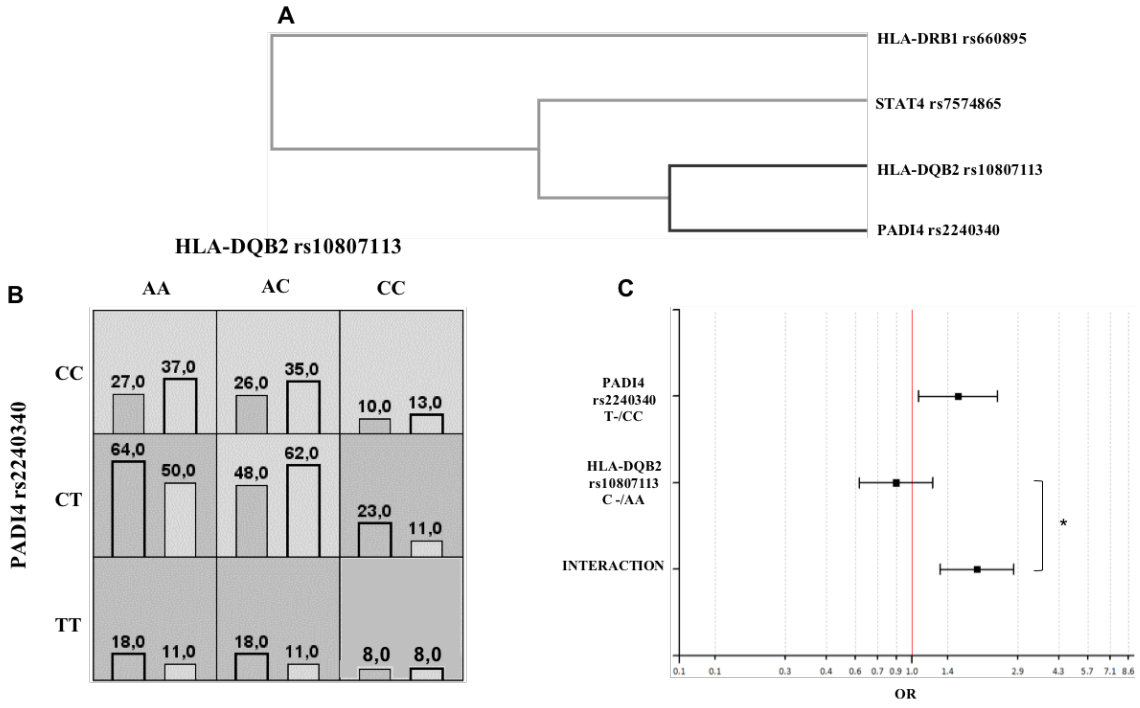


Figure 2.3 *Gene-gene interaction.* ***A****. Dendrogram of MDR software output. Darker and closer lines were referred to the stronger association: PADI rs2240340 and HLA-DQB2 rs10807113* ***B****. Histogram of MDR software output. Each cell shows counts of RA on left and control on right.* ***C.*** *Forests plot with association results of single variants and interaction.*

2.5 Discussion and Conclusion

Rheumatoid Arthritis (RA) is a multifactorial disease, in which the combination of genetic, environmental and constitutional factors concurs to its susceptibility and onset. It has been widely demonstrated the genetic predisposition to the basis of the RA development and progression, due to the occurrence risk contribution of genetic factors around 60% (Chung et al., 2016; Kurkò J et al., 2013). Several studies have been found and confirmed the involvement of many single-nucleotide polymorphisms (SNPs) within the HLA region (Barton A et al., 2008) such as HLA-DRB1 gene strongly established (WTCCC, 2007), HLA-G (Donadi EA et al., 2010), supporting its role as a tolerogenic molecule fundamental in the suppression of immune response in several autoimmune diseases (Carosella ED et al., 2008) and HLA-DQA2, included in the top three gene modules identified for RA peripheral blood mononuclear cells (Xiao X et al., 2016). In addition, Genome-Wide Association Studies (GWASs) and candidate-gene studies have identified more than 30 non-HLA genetic loci associated to RA occurrence (Stahl EA et al., 2010), validating the majority SNPs in patients whom were seropositive for either anti-citrullinated protein antibodies (ACPAs) or rheumatoid factor (RF).

In this view, the case-control observational study has been performed, in order to evaluate the genetic profile concerning the RA occurrence risk in a complete Italian case-series. Single variant association has been carried out firstly, then has been performed the gene-gene interaction study, to assess any eventual epistatic phenomenon. A total of 11 candidate gene variants were been analyzed, 7 belonging to HLA complex genes, while 4 were not.

Our results highlighted the predominant and remarkable RA association risk with HLA-DQA2 rs9275595 variant, showing statistically significant higher occurrence risk for patients carrying at least one copy of the allele variant C, respect T/T wild type homozygotes. The variant manifest significant allele-dose dependency: heterozygotes subjects noticed 1.98-fold increased risk, whereas homozygotes C/C 3.67-fold greater value, p were respectively 1E-05 and 2E-04. Stratifying by sex, the effect was widespread both in male and female cohorts following the same risk pattern; the difference between sex was significant for C/C genotype, showing greater effect in males, probably due to the lower men sample size. Up to now, whether HLA-DQA2 is associated with RA has not been identified, but it has been reported that it encoded for MHC-II proteins expressed on the APC surface, involved in the recognition and presentation of different antigens to T-cells (Li H et al., 2013). Highly variable MHC-II DQA2 molecules, carrying allele variant C for the rs9275595, could play an important role in selecting the T-cell repertoire, leading to unusual T-cell clone and exacerbating the arthritis inflammation.

Since it has been reported the heterodimer complex formed by glycoproteins encoded by HLA-DQA2 and HLA-DQB2 (Lenormand C et al., 2012), it has been evaluated the RA association with

rs10807113 within HLA-DQB2 gene. Results of this SNP were inconsistent compared to rs9275595, noticing just a trend toward RA risk. Our data were coherent with what has been observed in several studies, which identified no statistically significant association between HLA-DQB2 variants and RA occurrence (Takeuchi F et al., 1997; Moxley G, Han J, 2001; Vejbaesya s et al., 2000). However, stratifying HLA-DQB2 rs10807113 by gender, results revealed the opposite effect especially concerning heterozygotes patients: males showed higher RA risk, whereas female did not; the difference between gender was significant (2E-04). Studies have observed the DQ loci association with RA independently from SE, probably playing a role in different pathways (Kochi Y et al., 2004), which could be specific for males. The involvement in sex hormones mechanisms has been hypothesized connected to HLA-DQB2 even for others pathologies, such as HBV infection with HBsAg higher prevalence in men (Chang SW et al., 2014; Tsay PK et al., 2009).

Two variants, belonging to HLA class II DRB1 gene, rs6910071 (A>A/G) and rs660895 (A>A/G) were studied, due to their link with Shared Epitope (SE) sequence in position 70-74 of the third hypervariable region of HLA-DR β chain. SE is known to be implicated in higher susceptibility for RA risk (Scally SV et al., 2013). For the rs6910071 variant, no association has been revealed; while rs660895 variant reported a different RA occurrence risk on the basis of the genotype. The dominant genetic model showed significant 1.6-fold higher RA development (OR=1.660 95%CI 1.99-2.298, p value=0.002), respect to homozygotes wild type; the effect was similar in both sex, although stronger in female patients (p value= 0.024). This finding is confirmed what has previously reported, identifying rs660895 as one of the SNPs included in the most significant associated clusters with RA disease, considering case-control model (Black MH, Watanabe RM, 2009).

Concerning the non-HLA genes, our results reported significant RA susceptibility with 3 out of 4 variants analyzed, all following the dominant genetic model. More precisely, PADI4 rs2240340 and STAT4 rs7574865 showed both significant higher risk (p values respectively 0.002 and 0.0018); whereas PTPN22 rs2476601 revealed a trend toward association for patients carrying at least one copy of the allele variant (p value= 0.0083). Our data are reliable with other previous studies, showing these genes associated with RA occurrence through different pathways: PADI4 is crucial in the citrullation mechanism, PTPN22 plays a role in protein amino acid dephosphorilation and STAT4 exerts a function in the JAK-STAT cascade (Manning AK et al., 2009). Additional interesting findings on RA susceptibility included the effect of these non-HLA variants in a sex-specific manner. PADI4 rs2240340 occurrence risk was ascribable only in women cohort, whereas PTPN22 rs2476601 and STAT4 rs7475865 were significant associated to RA risk only in men. Although the difference between sex is significant only for rs2476601 (p value= 0.002), data indicated particular variants outcomes according the RA patients sex and suggested the different grading of pathways involvement in men and women. In fact, several studies observed that direct genetic differences could be the cause of diversity in susceptibility to several autoimmune diseases, especially related to

rheumatic affection, such as RA, Sjögren's syndrome or systemic lupus erythematosus (SLE) (Sharma S et al., 2014; Arnold AP, 2004). Other studies proposed gender as one of the factors causing differences in terms of RA symptoms, disease duration, treatment response, circulating serotonin level and bone loss mass, these lasts traits were found especially related to women in postmenopausal state (Bernardes M et al., 2017).

The analysis of genetic interactions provided remarkable findings on the epistasis phenomenon, showing the effect of an allelic gene variant on the susceptibility only if it is combined with others allelic variants of other genes. The nature and effects of these interactions are far from being fulfilled defined and, to a large extent, remain unpredictable; however the identification of specific combinations might give greatest knowledge of patients genetic profile associated to RA risk developing or even protection. We found significant SNP × SNP interaction among the SNPs with significant main effects HLA-DQA2 rs9275595 and HLA-DRB1 rs660895: subjects T/T for rs9275595 and A/A for rs660895 vs the others combinations showed significant 2.45-fold decreased RA risk (p value=7E-09). Both genes belong to MHC class II, which are similar in conformation and usually accommodate peptides of 13–25 residues in length in their open binding groove (Chicz RM et al., 1992). The allelic variation occurring in these genes mainly affects the nature and composition of the peptide-binging groove, thus it might regulate the peptide repertoire that is presented on the surface of MHC class II proteins for CD4+T cell recognition, modulating the immune activation (Wieczorek M et al., 2017).

Excluding HLA-DQA2 rs9275595 into the account, the SNP-based method revealed synergy interaction toward RA risk between HLA-DRB1 rs660895 and HLA-G rs1233334, exceeding 46.3% of the result that would have been achieved if the interaction had been additive. The interaction between the 5 'UTR of the HLA-G and HLA-DRB1 rs660895 has never been studied before and it seems interesting. Jassem and colleagues reported the presence of the reference allele G for rs1233334 was associated with an increased expression of the promoter, leading to higher HLA-G and sHLA-G presence; conversely, the presence of homozygosity for the variant C allele contributes to a lower promoter expression causing lower levels of sHLA-G (Jassem et al., 2013). The expression of the promoter, based on the presence of the reference allele G, is due to the methylation of this site which then interferes with the binding of IRF-1 (Interferon Factor-1). IRF-1 not only acts as a transcription factor, but also plays an important role in the immune response. Indeed, several SNPs are associated with this gene, leading to a decrease in NK cells and an increase in T cells (Govind N, Choudhury A, 2014). It would be interesting to study the possible interaction between the SNPs of the IRF-1 region and HLA-DRB1 rs660895.

With regard to only female cohort and excluding the preponderant effect of HLA-DQA2 rs9275595, the main SNP × SNP interaction has been revealed between HLA-DQB2 rs10807113 and HLA-G

rs1063320, showing a synergy toward protection effect. This finding could be explained through the identified HLA-DQB2 role in delivering peptides to the endoplasmic reticulum for the assembly of MHC class I molecules (such as HLA-G), suggesting the concerted action between the two molecules in immune activation (Kochi Y et al., 2004). In addition, it has been reported that the interactions at the pocket region in MHC class I and the region in MHC class II appear to have a dominant effect on the presentation of stable MHC complexes and on the immune-dominance of certain peptide epitopes (Natarajan SK et al., 1999). Whether the pathway of final effect remain uncertain, it should be hypothesized the particular presence of allelic variant belonging to different genes could jointly act, directing the immune response.

In conclusion, gene-gene interaction study highlighted the remarkable and primarily value of HLA candidate-gene variants combined together, whereas the role of non-HLA variants emerged secondarily. In addition, our findings demonstrated substantial variation across sex, in both single SNP analysis and SNP-SNP combination, suggesting to investigated deeper specific characteristics of male and female RA patients. Our study identifies HLA-DQA2 rs9275595 as the main variant associated to RA occurrence risk in the Italian case-series; although this conclusion may not generalize to studies in other locations, this finding reinforces the particular value of the population-based study and put forward the individuation of others allele variants, respect the HLA-DRB1 SE, already known and investigated. In order to confirm our findings and to remark the value of predicted biomarkers genetic variant in RA occurrence risk, further studies could be performed on Italian patients affected by Early Rheumatoid Arthritis (ERA).

References

Aletaha D et al., 2010. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum;2010. 62:256981.

Arnold A.P. Sex chromosomes and brain gender. Nat. Rev. Neurosci. 2004;5:701–708.

Bahri R, Hirsch F, Josse A, Rouas-Freiss N, Bidere N, Vasquez A, Carosella ED, Charpentier B, Durrbach A. Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. J Immunol. 2006 Feb 1;176(3):1331-9.

Bang SY, Han TU, Choi CB, Sung YK, Bae SC, Kang C. Peptidyl arginine deiminase type IV (PADI4) haplotypes interact with shared epitope regardless of anti-cyclic citrullinated peptide antibody or erosive joint status in rheumatoid arthritis: a case control study. Arthritis Res Ther. 2010;12(3):R115.

Baricordi OR, Stignani M, Melchiorri L, Rizzo R, HLA-G and inflammatory diseases. Inflamm Allergy Drug Targets. 2008 Jun;7(2):67-74.

Barton A et al. Rheumatoid arthritis susceptibility loci at chromosomes 10p15, 12q13 and 22q13. Nat Genet. 2008;40:1156–9.

Begovich AB et al. 2004. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am. J. Hum. Genet*. 75:330–337.

Beri R, Peterlana D, Puccetti A, et al. Alleli HLA-DRB1 e artrite reumatoide nel nord Italia : assenza di correlazione con la gravità della malattia e con manifestazioni extra-articolari. 2005;96:139-143.

Bernardes M, Vieira T, Lucas R, Pereira J, Costa L, Simões-ventura F, Martins MJ. Serum serotonin levels and bone in rheumatoid arthritis patients. Rheumatol Int. 2017 Oct 9.

Black MH, Watanabe RM. A principal-components-based clustering method to identify multiple variants associated with rheumatoid arthritis and arthritis-related autoantibodies. BMC Proc. 2009; 3(Suppl 7): S129.

Carosella ED, Favier B, Rouas-Freiss N, Moreau P, Lemaoult J. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. Blood. 2008;111:4862–4870.

Castelli EC, Ramalho J, Porto IO, Lima TH, Felicio LP, Sabbagh A, et al. Insights into HLA-G Genetics provided by worldwide haplotype diversity. Front Immunol. 2014 Oct6;5:476.

Chang SW, Fann CS, Su WH, Wang YC, Weng CC, Yu CJ, Hsieh AR et al. A genome-wide association study on chronic HBV infection and its clinical progression in male Han-Taiwanese. PLoS One. 2014 Jun 18;9(6):e99724.

Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, Strominger JL. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature. 1992 Aug 27; 358(6389):764-8.

Chung I-M, Ketharnathan S, Thiruvengadam M, Rajakumar G. Rheumatoid Arthritis: The Stride from Research to Clinical Practice. Int J Mol Sci. 2016;17(6).

Di Giacinto G. Artrite reumatoide: diagnosi e terapia nella medicina generale. M.D. Medicinae Doctor; Anno XXI 2014; 2: 41-6.

Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, Rey D, Moreau P. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association. Cell Mol Life Sci. 2011 Feb; 68(3): 369–395.

Ferraz-Amaro I, González-Juanatey C, López-Mejias R, Riancho-Zarrabeitia L, González-Gay MA. Metabolic Syndrome in Rheumatoid Arthritis. Mediators of Inflammation 2013; 710928, Hindawi Publishing Corporation ed., DOI 10.1155/2013/710928.

Franceschi PL, Mannoni A. L'artrite reumatoide in medicina generale. Collana Disease Management 2008; 24: 7-27.

Gianchecchi E, Palombi M, Fierabracci A. The putative role of the C1858T polymorphism of protein tyrosine phosphatase PTPN22 gene in autoimmunity. Autoimmun Rev. 2013;12(7):717-725.

Gonzalez-Gay MA, Gonzalez-Juanatey C, Martin J. The increased risk of ventricular diastolic dysfunction and congestive heart failure in patients with rheumatoid arthritis is independent of the duration of the disease. Semin Arthritis Rheum. 2005 Oct;35(2):132-3.

Gough SCL, Simmonds MJ. The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. Curr Genomics. 2007;8(7):453-465.

Govind N, Choudhury A. Immunochip identifies novel, and replicates known,genetic risk loci for rheumatoid arthritis in black South Africans. Mol Med. 2014; 20(1):341-9.

Horuzsko A, Lenfant F, Munn DH, Mellor AL. Maturation of antigen-presenting cells is compromised in HLA-G transgenic mice. Int Immunol. 2001;13(3):385-394.

Hua Li, Yonghe Hu, Tao Zhang, Yang Liu, Yantang Wang, Tai Yang, Minhui Li, Qiaoli Luo, Yu Cheng, Qiang Zou. Replication of British Rheumatoid Arthritis Susceptibility Loci in Two Unrelated Chinese population Groups. Clin Dev Immunol. 2013; 2013: 891306.

Iebba F, Di Sora F, Tarasi A, Leti W, Montella F. L'artrite reumatoide come modello di malattia genetica multifattoriale: una rassegna della letteratura. Recenti Prog Med 2011; 102: 175-82.

Jansen LM, van Schaardengurg D, van der Horst-Bruinsma I, van der Stadt RJ, de Koning MH, Dijkmans BA. The predictive value of anti-cyclic citrullinated peptide antibodies in early arthritis. J Rheumatol 2003; 30: 1691-5.

Jassem RM, Shani WS, Loisel DA, Sharief M. HLA-G polymorphisms and soluble HLA-G protein levels in women with recurrent pregnancy loss from Basrah province in Iraq. Hum Immunol. 2013;73(8):811-817.

Klareskog L, Stolt P, Lundberg K, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope) - restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum 2006; 54: 38-46.

Kochi Y, Yamada R, Kobayashi K, Takahashi A, Suzuki A, Sekine A, Mabuchi A, Akiyama F, Tsunoda T, Nakamura Y and Yamamoto K: Analysis of single-nucleotide polymorphisms in Japanese rheumatoid arthritis patients shows additional susceptibility markers besides the classic shared epitope susceptibility sequences. Arthritis Rheum2004,50:63–71.

Konda Mohan V, Ganesan N, Gopalakrishnan R. Association of susceptible genetic markers and autoantibodies in rheumatoid arthritis. J Genet. 2014;93(2):597-605.

Koopman WJ, Schrohenberg RE. Rheumatoid factor. Rheumatoid arthritis: etiology, diagnosis and therapy 1985; Utsinger PD, Zvaifler NJ, Ehrlich GE, ed., JB Lippincott.

Korczowska I. Rheumatoid arthritis susceptibility genes: An overview. World J Orthop. 2014;5(4):544-549.

Kurkò J, Besenyei T, Laki J, Glant TT, Mikecz K, Szekanecz Z. Genetics of rheumatoid arthritis - A comprehensive review. Clin Rev Allergy Immunol. 2013;45(2):170-179.

Lee YH, Rho YH, Choi SJ, et al. The PTPN22 C1858T functional polymorphism and autoimmune diseases - A meta-analysis. Rheumatology. 2007;46(1):49-56.

Lenormand C, Bausinger H, Gross F, Signorino-Gelo F, Koich S, Peressin M, Fricker D, Cazenave JP, Bieber T, Hanau D, de la Salle H, Tourne S. HLA-DQA2 and HLA-DQB2 genes are specifically expressed in human Langherans cells and encode a new HLA class II molecule. J Immunol. 2012, 188:3903-11.

Levisky A, Brismar k; Hafström I, Hambardzumyan K, Lourdudoss C, van Vollenhoven RF, Saevarsdottir S. Obesity is a strong predictor of worse clinical outcomes and treatment responses in early rheumatoid arthritis: results from the SWEFOT trial. RMD Open. 2017 Aug 9;3(2):e000458.

Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. Ann Rheum Dis 2006; 65: 366-71.

Liu C, Ackerman HH, Carulli JP. A genome-wide screen of gene-gene interactions for rheumatoid arthritis susceptibility. Hum Genet. 2011;129(5):473-485.

Manning AK, Ngwa JS, Hendricks AE, Liu CT, Johnson AD, Dupuis J, Cupples LA, Incorporating biological knowledge in the search for gene × gene interaction in genome-wide association study. BMC Proc. 2009; 3(Suppl 7): S81.

Mariaselvam CM, Chaaben AB, Salah S, Charron D, Krishnamoorthy R, Tamouza R, et al. Human leukocyte antigen-G polymorphism influences the age of onset and autoantibody status in rheumatoid arthritis. Tissue Antigens. 2015 Mar;85(3):182-9.

Meron MK, Amital H, Shepshelovich D, Barzilai O, Ram M, Anaya JM. Infectious aspects and the etiopathogenesis of rheumatoid arthritis. Clin Rev Allergy Immunol 2010:38:287-91.

Mewar D, Marinou I, Coote AL, Moore DJ, Akil M, Smillie D, et al. Association between radiographic severity of rheumatoid arthritis and shared epitope alleles: differing mechanisms of susceptibility and protection. Ann Rheum Dis 2008;67:980–3.

Moore JH et al., 2006. A flexible computational framework for detecting, characterizing, and interpreting statistical patterns of epistasis in genetic studies of human disease susceptibility. J Theor Biol.,241:252–261.

Mosley JD, Witte JS,Larkin EK, Bastarache L, Shaffer CM,Karnes JH, Stein CM et al. Identifying genetically driven clinical phenotypes using linear mixed models. Nat Commun. 2016; 7:11433

Moxley G, Han J. HLA DMA and DMB show no association with rheumatoid arthritis in US Caucasians. Eur J Immunogenet 2001; 28: 539–43.

Mueller RB, Kaegi T, Finckh A, Haile SR, Schulze-Koops H, von Kempis J, et al. Is radiographic progression of late-onset rheumatoid arthritis different from young-onset rheumatoid arthritis? Result from the Swiss prospective observational cohort. Rheumatology (Oxford) 2014 Apr;53(4):671-7.

Murdaca G, Contini P, Negrini S, Ciprandi G, Puppo F. Immunoregulatory Role of HLA-G in Allergic Diseases. J Immunol Res. 2016:6865758.

Natarajan SK, Stern LJ, Sadegh-Nasseri S. Sodium dodecyl sulfate stability of HLA-DR1 complexes correlates with burial of hydrophobic residues in pocket 1. J Immunol (1999) 162:3463–70.

Nilsson LL, Djurisic S, Hviid TVF. Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction. Front Immunol. 2014;5(MAY):1-10.

Ober C, Aldrich CL, Chervoneva I, et al. Variation in the HLA-G promoter region influences miscarriage rates. Am J Hum Genet. 2003;72(6):1425-1435.

Oliver JE, Silman AJ. Why are women predisposed to autoimmune rheumatic diseases? Arthritis ResTher 2009;11:252

Pedersen M, Jacobsen S, Klarlund M, et al. Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides. Arthritis Res Ther 2006; 8: R133.

Pera C, Delfino L, Longo A, Pistillo MP, Ferrara GB. Novel associations among HLA-DQA1 and -DQB1 alleles, revealed by high-resolution sequence-based typing (SBT). Tissue Antigens. 2000 Mar;55(3):275-9.

Pratesi F et al., 2013.HLA shared epitope and ACPA: just a marker or an active player?.Autoimmun Rev.,12(12):1182-7.

Raffaele G. Enciclopedia Medica Italina, Volume 8; 2nd ed. (Uses);2014.

Rebmann V, da Silva Nardi F, Wagner B, Horn PA. HLA-G as a Tolerogenic Molecule in Transplantation and Pregnancy. J Immunol Res., 2014; 2014:297073.

Rodrìguez-Elìas AK et al., Genetics and Genomics in Rheumatoid Arthritis (RA): an update. Gac Med Mex. 2016;152:194-203.

Roman MJ, Moeller E, Davis A, Paget SA, Crow MK, Lockshin MD, et al. Preclinical carotid atherosclerosis in patients with rheumatoid arthritis. Ann Intern Med 2006;144:249-56.

Scally SW, Petersen J, Law SC, Dudek NL, Nel HJ, Loh KL, Wijeyewickrema LC et al., A molecular basis for the association of the HLA-DRB1 locus, citrullination and rheumatoid arthritis. J Exp Med. 2013 Nov 18; 210(12): 2569–2582.

Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid Arthritis. Lancet 2010;376: 1094-1108.

Sharma S., Eghbali M. Influence of sex differences on microRNA gene regulation in disease. Biol. Sex Differ. 2014;5:3.

Shoda H, Nagafuchi Y, Tsuchida Y, Sakurai K, Sumitomo S, Fujio K, Yamamoto K. Increased serum concentrations of IL-1 beta, IL-21 and Th17 cells in overweight patients with rheumatoid arthritis. Arthritis Res Ther. 2017 May 31;19(1):111.

Snir O, Gomez-Cabrero D, Montes A, et al. Non-HLA genes PTPN22, CDK6 and PADI4 are associated with specific autoantibodies in HLA-defined subgroups of rheumatoid arthritis. Arthritis Res Ther. 2014;16(4):414.

Speiser DE et al. 1997. A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. J. *Exp. Med.* 185:1777–1783.

Stack RJ, Stoffer M, Englbrecht M, et al. Perceptions of risk and predictive testing held by the first-degree relatives of patients with rheumatoid arthritis in England, Austria and Germany: a qualitative study. BMJ Open. 2016;6(6):e010555.

Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, Li Y, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet. 2010;14:508–514.

Suzuki A et al., 2003 Functional haplotypes of PADI4, encoding citrulli- nating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet*. 34:395–402.

Tan Z, Randall G, Fan J, Camoretti-Mercado D, Brockman-Schneider R, Pan L, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. Am J Hum Genet. 2007 Oct;81(4):829-34.

Takeuchi F, Nakano K, Matsuta K, Takizawa K, Nabeta H, Kuwata S, et al. Polymorphism of TAP1 and TAP2 in Japanese patients with rheumatoid arthritis. Tissue Antigens1997; 49: 280–2.

Tsay PK, Tai DI, Chen YM, Yu CP, Wan SY, Shen YJ, Lin DY. Impact of gender, viral transmission and aging in the prevalence of hepatitis B surface antigen. Chang Gung Med J. 2009 Mar-Apr;32(2):155-64.

Turkiewicz AM, Moreland LW. Rheumatoid Arthritis. Clinical Care in the Rheumatic Diseases 3rd ed. 2006; 157-66.

van der Horst-Bruinsma IE, Hazes JM, Schreuder GM, et al. Influence of non-inherited maternal HLA-DR antigens on susceptibility to rheumatoid arthritis. Ann Rheum Dis 1998; 57: 672-5.

Uhlig T, Hagen KB, Kvien TK. Current tobacco smoking, formal education, and the risk of rheumatoid arthritis. J Rheumatol 1999; 26: 47–54.

Vejbaesya S, Luangtrakool P, Luangtrakool K, Sermduangprateep C, Parivisutt L. Analysis of TAP and HLA-DM polymorphism in Thai rheumatoid arthritis. Hum Immunol 2000; 61: 309–13.

Veit TD, De Lima CPS, Cavalheiro LC, et al. HLA-G +3142 polymorphism as a susceptibility marker in two rheumatoid arthritis populations in Brazil. Tissue Antigens. 2014;83(4):260-266.

Verbruggen LA, Rebmann V, Demanet C, De Cock S, Grosse-Wilde H. Soluble HLA-G in Rheumatoid Arthritis. Hum Immunol. 2006;67(8):561-567.

Vidal C, Barnetche T, Morel J, Combe B, Daïen C. Association of Body Mass Index Categories with Disease Activity and Radiographic Joint Damage in Rheumatoid Arthritis: A Systematic Review and Metaanalysis. J Rheumatol. 2015 Dec; 42(12):2261-9.

Wasserman AM. 2011. Diagnosis and management of rheumatoid arthritis. Am Fam Physician. 2011 Dec1;84(11):1245-52.

Watford WT et al., 2004. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.* 202:139–156.

Wieczorek M, Abualrous ET, Stich J, Àlvaro-Benito M, Stolzenberg S, Noé F, Freund C. Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. Front Immunol. 2017 Mar 17;8:292.

Williams TM. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. J Mol Diagn. 2001 Aug;3(3):98-104.

Wisniewski A, Kowal A, Wyrodek E, Nowak I, Majorczyk E, Wagner M, et al. Genetic polymorphisms and expression of HLA-G and its receptor, KIR2DL4 and LILRB1, in non-small cell lung cancer. Tissue Antigens 2015 Jun;85(6):466-75.

Weng CH, Gupta S, Geraghty P, Foronjy R, Pernis AB. Cigarette smoke inhibits ROCK2 activation in T cells and modulates IL-22 production. Mol Immunol. 2016;71:115-122.

WTCCC, The Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447:661–678.

X. Xiao, J. Hao, Y. Wen, W. Wang, X. Guo, F. Zhang. Genome-wide association studies and gene expression profiles of rheumatoid arthritis: An analysis. Bone Joint Res. 2016 Jul; 5(7): 314–319.

Yie SM, Xiao R, Librach CL, Progesterone regulates HLA-G gene expression through a novel progesterone response element. Hum Reprod, 2006.

Zambra FM, Biolchi V, de Cerqueira CC, Brum IS, Castelli EC, Chies JA, Immunogenetics of prostate cancer and bening hyperplasia-the potential use of an HLA-G variant as a tag SNP for prostate cancer risk. HLA. 2016 Feb;87(2):79-88.

WHO, Global database on body mass index (http://apps.who.int/bmi/index)