CHAPTER 3

IDENTIFICATION OF PREDICTIVE BIOMARKERS PANEL IN EARLY ARTRHITIS PATIENTS

FROM SYMPTOMS TO DISEASE

"Treatment without prevention

is simply unsustainable"

Bill Gates

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Abstract

Background: Rheumatoid arthritis (RA) is a long-term inflammatory disorder causing pain, deformity and disability. The etiology is multifactorial and the identification of early symptoms and diagnosis leads to better prevention of articular damage and it allows to pharmacoeconomic costs saving.

Objective: To find out a combined biomarkers panel of both genetics and soluble parameters, in order to evaluate symptoms (UA) and early onset of arthritis (ERA) and to predict the evolution and progression to RA. Subsequently, to assess if our genetic association results in independent RA case-series should be linked to early stage of the disease.

Materials and methods: A total of 501 UA/ERA patients have been enrolled. From each subject, whole blood and serum/plasma samples were obtained. From the whole blood, genomic DNA was extracted and identified genetic variants in HLA and non-HLA regions were assessed by allelic discrimination. Soluble parameters have been analyzed through ELISA and Multiplex techniques. Statistical analyses of genetic association and soluble comparison were carried out stratifying patients accordingly to disease evolution (from UA to RA) and to disease progression (from ERA to RA) after 6 months from the first visit (follow-up T6). Gene-gene interaction and predict combined model elaboration have been identified using Multifactorial Dimensionality Reduction (MDR) software.

Results: Concerning the evolution from UA to RA disease, the main genetic association result was between sex and TRAF1rs3761847 variant: female T/T subjects showed 2-fold increased risk to develop RA, respect to males, reaching the statistically significant p-value of the difference (0.0037). TNFR1 was the only soluble parameter significant associated to RA evolution and the combined MDR output confirmed the association between TRAF1 and sex. Regarding the disease progression, T/T homozygous for PADI4 rs2240340 were significant associated to more than 2-fold higher responsiveness, respect to C/C genotype. MDR output for the combined model found out a remarkable interaction in which Rheumatoid Factor (RF), resistin and HLA-DRB1 rs6910071 played a role together to predict "*best responders"*: in compresence of high levels of RF and resistin, subjects showed 25-fold increased probability to responsiveness (OR=25.714 95%CI (6.70-98.66) p-value=$1.55x10^{-7}$), whereas, with regular concentration of resistin, *best responders* have been detected by the interaction between RF and HLA-DRB1 rs6910071. The algorithm was able to identify *best responders* with the ratio 4:1.

Conclusion: Beyond single parameter association, results showed a powerful additional value of the combined model. The algorithm formulation should be used into clinical practice allowing to promptly overcome diagnosis issues and to direct personalized therapeutic choices in advance.

3.1 Introduction

The capability to optimize the patient's treatment represents one of the major and most challenging objective for the National Health System in terms of personalized medicine. This concept results more remarkable and useful in the context of diseases with early symptoms, difficult to assess with diagnostic criteria. In particularly, for patients with early onset of Undifferentiated Arthritis (UA) or with Early onset of Rheumatoid Arthritis (ERA), it might be crucial predicting the disease progression to Rheumatoid Arthritis (RA), in order to identify patients with higher risk of bad prognosis and to direct them immediately to the best treatment. The early differential diagnosis permits to evaluate the initial status of patients and promptly and effectively to decide the strategy care, in order to avoid non-responsiveness treatments and finally bone erosion, articular damage and disability. Several evidences have demonstrated the capability to reach this aim due to early diagnosis and prompt therapeutic intervention in the "window of opportunity" within 3/6 months from symptoms onset (Cush JJ. 2007). This strategy should be linked to modify the disease progression, to improve the inflammatory status and to lead to remission, preventing radiological damage and preserving physical functioning and patients work ability (Hazes JM. 2011).

RA is an important autoimmune long-term disease, characterized by persistent inflammation and synovitis (Scott DL et al., 2010). Early diagnosis of RA or even the evaluation of initial symptomatic phase lead to better prevention of joint erosion and resulting deformity (Simmons DP et al., 1998). UA or *causa ignota* is defined as any arthritis of recent onset that poses the potential for a persistent path without fulfilling the rheumatologic classification criteria for specific conditions (Ghosh K et al., 2016), whereas the first 2 years after the onset of RA is considered as Early RA (ERA). UA prevalence has been evaluated around 30%-50% of total patients referred to rheumatic surgeries (Van der Horst-Bruinsma I et al., 1998; Wolfe F et al., 1993; El Miedany Y et al., 2008). The early differential diagnosis should be arduous due to multiple and nonspecific symptoms not directly related and not satisfying diagnostic criteria. UA should evolve into RA (30% of cases) or others rheumatic disease, it might remain undifferentiated or it could spontaneously remit (around 40%-50% of cases) (Van Aken J et al., 2006; Tunn EJ et al., 1993; Harrison BJ et al., 1996; van der Helm-van Mil AH et al., 2008).

Several single genetic variants are known as associated to risk occurrence of RA and considered together they might be useful to create predictive algorithm in terms of disease susceptibility, evolution from UA or ERA and disease progression. The etiology of RA is multifactorial and the hereditability is about 60% in which Human Leukocyte Antigens (HLA) gene variants play a major role in RA susceptibility (MacGregor AJ et al., 2000). HLA represents a group of polymorphic genes, localized in the region 6p21.3 and long 3500 Kb. Around 30% of the genetic component of susceptibility of RA is referred to haplotypes HLA II class, (Ferraù V et al., 2009) comprehensive of 3 isotypes: HLA-DR, HLA-DQ and HLA-DP.

HLA-DRB1 is the locus mostly involved in RA etiology. DRα and DRβ chains compose the heterodimer and the presence of *Shared Epitope* (SE) amino acidic sequence in the position 70-74 of the third hypervariable region of HLA-DRβ leads to high risk to develop RA. Several studies reported SE susceptibility sequences: ^{70}QRRAA^{74}, ^{70}QKRAA^{74}, (Bang et al., 2010; Konda Mohan et al., 2014) with specificity respectively DRB1*0101, DRB1*0102, DRB1*0404, DRB1*0405, DRB1*1402, and DRB1*0401 (Pratesi F et al., 2013, Table 1). Besides, the presence of DEERA sequence (DRB1*0103, DRB1*0402, DRB1*1301, DRB1*1302, DRB1*1323) is associated to protection in developing RA (Carrier et al., 2009). Du Montcel et al. (Du Montcel et al., 2005) proposed a method to classify RA risk: positions 70-71 modulate the RAA sequence in position 72-74. The presence of Lys (K) or Arg (R) in position 71 allows higher risk, whereas Ala (A) or Glu (E) are associated to lower risk or neutrality. On the other side, the presence of Gln (Q) or Arg (R) in position 70 gives higher risk respect Asp (D) (Kurkò et al., 2013). Mechanisms through the predisposing risk of the amino-acidic sequence SE are still controversial. Hypotheses propose that autoimmunity induction is carried out by molecular mimicry or presenting particular peptides and citrullinated proteins to lymphocyte T receptor (TCR), causing a response modification of T CD4$^+$ cells (Beri et al., 2005), besides there is a stimulation of ACPA release from B-lymphocytes (Van der Helm-van Mil et al., 2007; Feitsma et al., 2010). Studies reported SE alleles associated to high disease activity, increase risk to develop erosive synovitis and greater frequency of systemic events (Iebba et al., 2011). However, those hypotheses do not entirely explain all HLA contribution to disease susceptibility (Mariaselvam CM et al., 2016). The evaluation of epistatic interaction between HLA-DRB1 SE loci and non-HLA loci (Briggs FB et al., 2010) and between genes variants in the region HLA-DQ (Liu C et al., 2011) could be further maximize the predictive capability to determinate the occurrence risk and to identify patients with bad prognosis. Both ***HLA-DQA2*** and ***HLA-DQB2*** genes are expressed in dendritic cells with the role of presenting antigens to T CD4$^+$ cells (Lenormand et al., 2012) and between DR and DQ has been underlined a strong linkage disequilibrium, suggesting RA susceptibility in independent manner (Miyadera et al., 2015).
Moreover, several studies reported that RA patients showed lower levels of HLA-G soluble (sHLA-G) form respect to controls (Verbruggen et al., 2006). It should be explained through HLA-G physiological function to inhibit T and NK cells: the presence of lower level of HLA-G in RA might cause the inefficient inhibition of T cells (Baricordi et al., 2008).

Besides, some studies have been identified non-HLA genes related to RA etiology (Viatte S et al., 2013): ***STAT4,*** it transmits signals induced by several cytokines, as IL-12, IL-23 and type I interferons (IFNs) performing a crucial role in rheumatoid synovial inflammation (Watford et al., 2004); ***TRAF1,*** involved in mechanisms implicated in cell proliferation and differentiation, apoptosis, bone remodeling and cytokines signaling pathways (Speiser et al., 1997); ***PTPN22,*** implicated in autoimmune disease engrossment, it encodes the intracellular protein tyrosine

phosphatase. It plays a role in signal transduction and integrates the T-cell antigen receptor signaling pathway (Begovich AB et al., 2004); ***PADI4,*** expressed in hematological and RA synovial tissue, it is associated to increased production of citrullinated peptides acting as autoantigens (Suzuki A et al., 2003).

Furthermore, considering the higher percentage of patients with good prognosis and benign progression, the early differential diagnosis should be remarkable in order to avoid expensive, useless and inappropriate treatment. Hence, in order to overcome this issue, in 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) have defined the new classification criteria for RA (2010 ACR/EULAR criteria) including the evaluation of several soluble serological biomarkers, such as rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) and anti-citrullinated peptide antibodies (ACPA) (Aletaha D et al., 2010). Several studies have been reported the association between higher circulating levels of some soluble biomarkers and early phase of RA disease (Kokkenen H et al., 2010; Hueber W et al., 2007; Chandra PE et al., 2011). Overall, a huge protein screening has been evaluated 11 soluble parameters, which validated the model called "Multi Biomarkers Disease Activity Score/MBDA Score" related to the algorithm for the prediction of the disease activity score in RA patients. (Eastman PS et al., 2012; Curtis JR et al., 2012; Centola M et al., 2013).

Proteins are involved in inflammatory response, innate and adaptive immunity, leucocyte recruitment and angiogenesis, hyperplasia and bone and chondrocytes damage mechanisms:

- ***Epidermal Growth Factor (EGF)***: it is secreted by macrophages, fibroblasts and endothelial cells in the RA affected joint. It is associated to proliferation and differentiation of stromal cells and it induces the inflammatory mediators' productions.
- ***Vascular Endothelial Growth Factor (VEGF)***: it stimulates angiogenesis and it is expressed by fibroblasts and synovial macrophages; it promotes inflammation, swollen joints and bone erosion.
- ***Leptin****:* hormone secreted by adipose tissue. It is associated to obesity, to leucocyte activation and bone remodeling.
- ***Resistin****:* as leptin, this hormone is secreted by adipose tissue and it is linked to obesity, diabetes and cardiovascular diseases. It promotes inflammation and endothelial dysfunction.
- ***Vascular Cell Adhesion protein 1 (VCAM-1)***: expressed by endothelial and synovial cells, it might contributes to cells recall in the synovial tissue, to invasion and cartilage destruction by fibroblasts.
- ***Interleukin 6 (IL-6)****:* cytokine produced by several types of cells during RA disease. It mainly contributes to inflammation, cartilage degradation, bone erosion and liver involvement in the acute phase of RA.
- ***Tumor Necrosis Factor receptor 1(TNF receptor-1)***: signaling modulates by this receptor is involved in the pathway of TNF-α. The soluble form bonds to TNF-α.

- ***Matrix Metaloproteinase-1 (MMP-1)**:* enzyme implicated in the collagen degradation, it contributes to cartilage damage and lymphocytes invasion in synovial tissue.
- ***Matrix Metaloproteinase-3 (MMP-3)***: enzyme able to degrade glycosaminoglycan cartilage matrix and to activate MMP-1.
- ***Serum Amyloid A (SAA)**:* one of the major acute phase mediators liver-secreted. It is produced even by synovial fibroblasts and chondrocytes, inducing pro inflammatory activation of macrophages and T cells.
- ***Chitinase-3-like protein 1 (YKL40)**:* called "human cartilage glycoprotein 39", it promotes the chondrocytes and fibroblasts proliferation, counteracting cartilage's disruption.

Moreover, others 5 soluble candidate biomarkers have been included in the panel of analyses, listed below:

- ***Angiopoietin-2**:* involved in the angiogenesis, endothelial integrity and inflammation. In RA, it is associated with atherosclerosis and higher cardiovascular risk (López-Mejías R et al., 2013).
- ***Interleukin 15 (IL-15)**:* involved in the cellular interactions promoting TNF production, Th17 lymphocytes activation and functional maturation of dendritic cells. In RA synovial joints it contributes to neutrophils, B cells and NK cells survival (González-Álvaro l et al., 2011; González-Álvaro I et al., 2015).
- ***Macrophage inflammatory protein-3-beta (MIP3beta/CCL19)**:* secreted by B cells, it has a crucial role in adaptive immunity, promoting immune system cells migration and perivascular inflammation (Sellam J et al., 2013).
- ***14-3-3η polypeptide**:* belonged to chaperonins family, it contributes through different pathways to pro inflammatory cytokines production and articular damage (Maksymowych WP et al., 2014, Maksymowych WP et al., 2014).
- ***Calprotectin (S100A8/A99)**:* expressed in monocytes and granulocytes, its higher level is present in activated cells adjacent to the cartilage-pannus junction (Abildtrup M et al., 2015; Inciarte-Mundo J et al., 2015).

Nowadays, despite the individuation of some serological parameters and several genetic factors, the single assessment of those biomarkers is not able to provide a great diagnostic value helpful to clinical decisional process, whereas their combination should be strengthened to develop a predictive and powerful model (Feitsma AL et al. 2007; Silva Fernandez L et al. 2011).
In this view, the integrated model including anamnesis, genetic and serological data should be used into clinical practice as a predictive, additional and great instrument to provide early diagnosis and to personalize deeper the pharmaceutical treatment.

3.2 Rationale and aims

The main purpose of this study is to verify the applicability and reliability of a biomarkers panel including both genetics and soluble factors, in order to predict the outcome of early onset arthritis. This is directly linked to the aim of developing and potentiating the accurate prognosis level of the model applicable to the clinical practice.

Specific objectives of the study are following:

- Identification of a genotypic and serological profile associated to RA susceptibility in patients with symptoms, which do not fulfill completely RA classification criteria.
- Investigation of a genotypic and serological profile in patients with early onset of RA disease, individuating the association between these parameters and clinical outcome, in terms of disease evolution and response to treatment.
- Estimation of diagnostic potentiality of the combined panel of both genetic and serological biomarkers.
- Assessment if our genetic association result in independent RA case-series (CHAPTER 2) should be linked to early stage of the disease.

3.3 Materials and Methods

Subjects

The multicentric study included 501 patients recruited in the context of the Project "*Prognostic value of a combined panel of soluble and genetics biomarkers in patients with Early Arthritis*" (Project Code: RF-2010-2317168). The study was reviewed and approved by the committee for Medical Ethics in Research of Bologna, Ferrara, Reggio Emilia and Rome and the written informed consent was taken from all patients recruited. Enrollment took place in the units:

- Bologna: Orthopedic Institute Rizzoli, Immunorheumatology and Tissue Regeneration Lab, *Prof. R. Meliconi*.
- Ferrara: Sant'Anna University Hospital, Rheumatology Unit, *Prof. M. Govoni*.
- Reggio Emilia: Hospital S. Maria Nuova, Rheumatology Unit, *Prof. C. Salvarani*.
- Roma: Catholic University of Sacred Heart, Rheumatology Unit, *Prof. G Ferraccioli*.

Inclusion parameters gathered patients with Undifferentiated Arthritis (UA) or Early Rheumatoid Arthritis (ERA) satisfying ACR/EULAR criteria (Aletaha D et al. 2010). The length disease was ≤ 6 months. Clinical data (Table 3.1) were evaluated during the first visit (T0) and follow-up after 6 (T6) months, along with the collection of biological samples, divided in plasma, serum and whole blood. All aliquots were storage at -20°C until processed for serological analyses and genomic DNA extraction.

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

Table 3.1 *Clinical parameters to evaluate patients' status.*

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 manufacturer'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/µl or 1ng/µl, 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*
- *HLA-DRB1 Shared Epitope*

Using Genome Browser (http://genome.ucsc.edu/), has been obtained the nucleotide 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 (apart HLA-G 14bp indel and HLA-DRB1 Shared Epitope) 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 224bp for Ins/Ins (I/I) and 210bp for Del/Del (D/D) and both 224bp and 210bp for Del/Ins (D/I) genotypes. The PCR products were visualized using silver staining.

Determination of Shared Epitope Status

HLA-DRB1 alleles sharing a consensus sequence, known as "Shared Epitope" (SE), has been detected through Sanger Sequencing.

Firstly, the region of interest (codons 71-74) was amplified using forward primer: 5'-CGCTGCACTGTGAAGCTCTC-3' and reverse primer: 5'-CGGGTCGAGGCAGTG-3' manually generated and optimized primers designed using Primer3 (Untergasser A et al., 2012; Koressaar T et al., 2007). 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.

Subsequently, the purification of PCR product has been executed using 96-wells cleanup filter plate (Merck-Millipore) and vacuum pump. The purified PCR product size were checked using an 8% polyacrylamide gel. Sequencing Reaction has been carried out using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and forward extension primer. After that, sequencing products were firstly purified using Montage SEQ96 Cleanup (Merck-Millipore), then denatured using HI-DI Formamide (Life Technologies) and lastly run on the instrument Sanger Sequencer 3500DX (Thermo Fisher Scientific).

Results and chromatograms were analyzed using a chromatogram viewer program, such as Sequencing Analyses 5.4, Chromas (Figure 3.1) and *FinchTV*®. Reads generated by sanger sequencing were aligned to genome build hg38 using Blastn (NCBI) and has been done the evaluation of HLA-DRB1 alleles according to the third hypervariable region of DRβ chain, as previous described (Pratesi F et al., 2013).

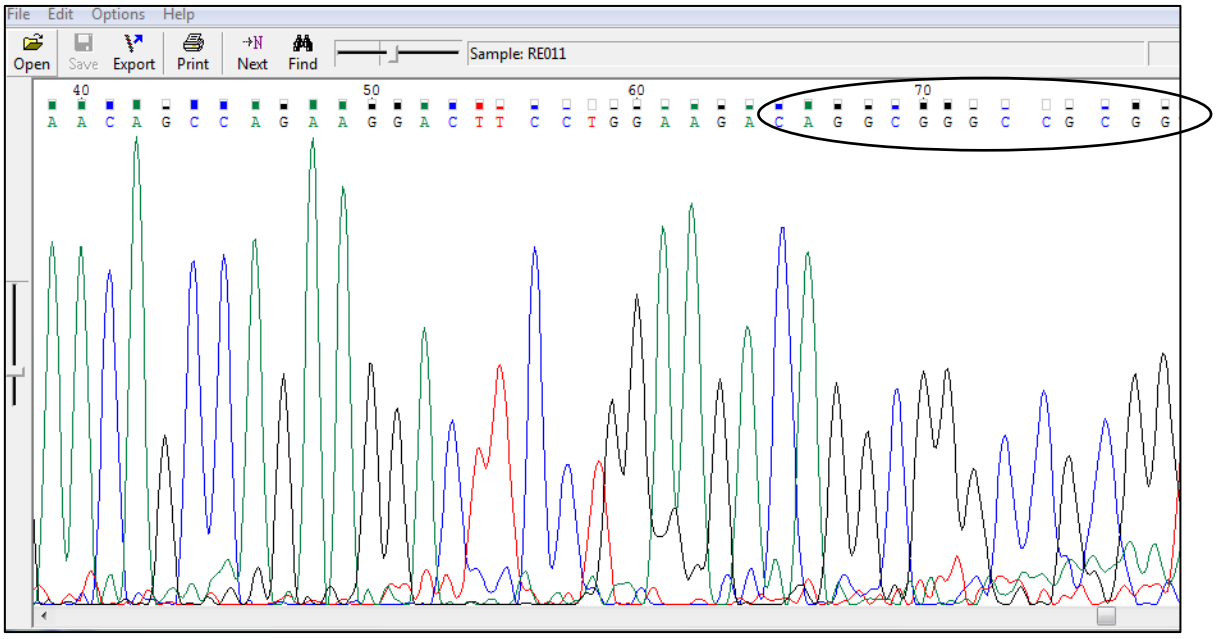


Figure 3.1 *Chromatogram obtained by Sanger Sequencing. The region of interest (SE) was underlined.*

Multiplex

Serological analyses of *EGF, VEGF, Leptin, Resistin, VCAM-1, IL-6, TNF receptor-1, MMP-1, MMP-3, SAA, YKL40, Angiopoietin -2, CCL19/MIP3beta, IL-15* were performed using Luminex Technology xMAP (MultiAnalyte Profiling) with magnetic beads, (R&D Systems, Luminex Bead-Based Multiplex Kit), following manufacturer's protocol.

Briefly, the sample is added to a mixture of color-coded beads, pre-coated with analyte-specific capture antibodies, which bind to the analyte of interest. Subsequently, biotinylated detection antibodies specific to the analyte of interest are added and form antibody-antigen sandwich and the added phycoerythin (PE)-conjugated streptavidin binds to the biotinylated detection antibodies. Finally, magnetic beads bind the immune complex formed and the quantification was performed using the software manager Bio-Plex version 6.0 (Bio-Rad Laboratories, Hercules, CA, USA). The Luminex system is able to delete technical problems due to the presence of factor which could interfere with proteins dosages in RA serum, as Rheumatoid Factor. Sensibility, specificity and dynamic range of calibration curve have been evaluated considering expected values of soluble factors in RA patients and healthy controls.

Enzyme-Linked Immunosorbent Assay (E.L.I.S.A.)

The serological dosages for *14-3-3 eta polypeptide, S100A8/A9* were carried out trough commercial ELISA kit. The reader Labysistem Multiskan EX and Biowash 1575 were used and results were analyzed with 4-parameters logistic curve. In order to avoid interferes with FR and heterofilic

antibodies in patients' serum, the quality check in the analytical phase has been done using "spike/recovery" protocols and linearity test. Briefly, a known amount of standard (spike) was added to a serum sample. The resultant concentration (recovery) compared to the sample clear-cut concentration showed if a component inside the sample is interfering in E.L.I.S.A. assay. The linearity test has been performed with serial dilutions. In case of interference, serum samples have been previously treated using specific block (Heteroblock, Omega Biologicals) (Todd DJ et al. 2011).

Statistical Analyses

- ***Disease evolution:*** occurrence analyses were performed considering the cohort of T0 UA patients. Subjects were stratified accordingly to Rheumatoid Arthritis diagnosis or not after 6 months (follow-up T6), subdividing the group in cases (whom have developed the disease) and controls (whom have symptoms but no RA diagnosis).

- ***Disease progression:*** each patient included in the study was under pharmacological treatment of steroid (Medrol®). To this, has been added csDMARD (Clorochin/Sulfasalazin/Methotrexate) depending on clinical evaluation. Disease progression was evaluated accordingly to DAS28 variation follow-up T6 months point as table down describes (Table 3.2). EULAR Improvement Criteria identifies three groups of patients: Good or High Responders, Moderate Responders, Non-Responders. The comparison was performed through models of "*Responders*" (the group of non-responders versus all others) and "*Best Responder*" (the group of high responders versus all others).

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

Table 3.2 *EULAR Response Criteria considering DAS28 variation. Modified from Bentley MJ et al., 2010*

- ***Demographics, clinics and soluble biomarkers levels***

The distribution of continue variables has been verified using Kolmogorov-Smirnov: all variables resulted non-normal distributed.

Univariate analysis has been performed comparing continued variable between the two groups (as described in the part of Statistical Analyses: Disease evolution) using non-parametric Mann-Whitney test and among three groups (as described in the part of Statistical Analyses: Disease progression)

using Kruskal-Wallis test followed by post-hoc double comparison with Dunn test.

Comparison between dichotomous variables has been carried out using Fisher's chi-square test, whereas comparison between categorical variables using Pearson's chi-square test. Correlation among continued variables has been executed through Spearman's correction, reporting Rho coefficient.

A p-value <0.05 was considered significant and p-value included between 0.05 and 1 was signaled.

The probability calculated with the model of logistic regression has been used to build Receiver Operating Curve (ROC) in order to evaluate the discrimination model capability (Swets JA. 1979) (Table 3.3).

Results were obtained using following software: SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 (CA, USA).

AUC	0.5	Not informative
AUC	0.5-0.7	Low accuracy
AUC	0.7-0-9	Moderate accuracy
AUC	0.9-1	High accuracy

Table 3.3 *Swets' method for ROC curve accuracy.*

- ***Genetic Variants and Integrated Model***

For each SNP, in each cohort, were assessed genotypic frequencies, allelic frequencies and Hardy Weinberg equilibrium. Chi-squared test χ^2 with Yates continuity correction were 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 package. 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, epistasis phenomenon and to integrate genetic, serology and clinical results it was used a genetic model-free method called multifactor dimensionality reduction (MDR) (Moore JH et al., 2006). With this purpose, continued variables referred to serological biomarkers were dichotomized with cut-off of the average ± 2 standard deviation respect to healthy controls. MDR outputs were in form of histograms with main interactions and dendrograms, where darker and closer lines were referred to the strongest interaction.

3.4 Results

For the multicentric study have been enrolled 501 patients, comprehensive of 187 Undifferentiated Arthritis (UA) and 314 Early Rheumatoid Arthritis (ERA) subjects. After 6 months (follow-up T6), from the first visit (T0), 62 patients have been lost, equal to 12% of the total cohort: 17% in UA group, 10% in ERA subset.

During the first visit have been evaluated anamnesis, demographics, lifestyle characteristics, clinical data and specific rheumatology tests, as reported in the Table 3.4. Besides, a group of healthy controls has been considered, in order to evaluate some characteristics independent from the disease.

	UA (N=187)	ERA (N=314)	CTRL (N=98)	p
Age	59 (46-69)	61 (50-72)	58 (52-66)	NS
Gender, n° females (%)	129 (69)	241 (77)	89 (91)	< 0.005
BMI Kg/m^2	24.73 (21.90-28.24)	25.33 (22.47-28.92)	24.61 (22.04-27.22)	NS
Smokers (%)	34	46	50	0.0021
Lenght Disease (weeks)	14 (8-24)	14 (8-24)	NA	NS
RF + (%)	23	54	NA	< 0.0001
ACPA + (%)	16	54	NA	< 0.0001
TJC/28	3 (1-4)	7 (3-12)	NA	< 0.0001
TJC/44	3 (2-6)	10 (4-16)	NA	< 0.0001
SJC/28	2 (1-3)	5.5 (2-9)	NA	< 0.0001
SJC/44	2 (1-4)	7 (3-12.5)	NA	< 0.0001
GH	50 (25-70)	50 (40-80)	NA	< 0.0005
HAQ	0.62 (0.25-1)	1.12 (0.62-1.75)	NA	< 0.0001
CDAI	14 (9-18.75)	25 (16-33.25)	NA	< 0.0001
SDAI	14.8 (10-20.97)	26 (16.97-36.35)	NA	< 0.0001
VAS	50 (20-70)	65 (45.5-80)	NA	< 0.0001
DAS28	3.96 (3.17-4.63)	5.31 (4.39-6.22)	NA	< 0.0001
ESR (mm/h)	21 (11-39)	37.5 (19-55.25)	NA	< 0.0001
APC (mg/dl)	0.74 (0.23-2.02)	1.38 (0.6-4.76)	NA	< 0.0001

Table 3.4 *Basal Characteristics of Undifferentiated Arthritis patients (**UA**) and Early Rheumatoid Arthritis patients (**ERA**) on first visit (T0) and healthy controls as comparison (**CTRL**). Data are expressed as median (25°-75° percentile), p<0.05 was considered significant.*
***BMI**: Body Mass Index; **RF**: Rheumatoid Factor; **ACPA**: Anti-Citrullinated Protein Antibody; **TJC 28/44**: Tender 28/44-Joints Count; **SJC 28/44**: Swollen 28/44-Joints Count; **GH:** General Health score; **HAQ**: Healthy Assessment Questionnaire; **CDAI**: Clinical Disease Activity Index; **SDAI**: Simplified Disease Activity Index; **VAS**: Visual Analogue Scale; **DAS28**: Disease Activity Score on 28 Joints; **ESR**: Erythrocyte Sedimentation Rate; **APC**: Activated Protein C.*

The difference for all the parameters concerning rheumatic test into the two groups were statistically significant. It reflected the dissimilarity between patients with some rheumatic symptoms, but no evidence of disease and subjects in the initial stage of rheumatoid affection. Age and BMI were perfectly matched in all three groups, providing the absence of these confounding factors to the further analysis. Length disease matched in both UA and ERA cohorts, it has been related to the inclusion criteria for the subjects' recruitment.

3.4.1 Evolution of Undifferentiated Arthritis (UA) to Rheumatoid Arthritis (RA)

The analysis of evolution of UA cases included in the study was firstly carried out considering as outcomes the diagnosis of RA or the stabilization or remission of symptoms.
Subject involved in the study have been stratified evaluating the end-point of disease evolution after 6 months. Among the UA group 72% of patients were steady, whereas 35% evolved to RA. 5% of subjects evolved to other diseases, as synovitis, spondyloarthritis positive HLAB-27 and enthesitis.
Characteristics of the two cohorts were showed in the Table 3.5.

	UA T0→ UA T6	UA T0→ RA T6	p
Age	60 (49-69)	59 (45-74)	NS
Gender, n° females (%)	71	67	NS
BMI Kg/m^2	25.90 (21.97-28.88)	25.31 (22.49-28.42)	NS
Smokers (%)	35	47	NS
Lenght Disease (weeks)	14 (8-24)	12 (8-24)	NS
RF + (%)	24	31	NS
ACPA + (%)	17	24	NS
TJC/28	3 (1-4)	2 (2-7)	NS
TJC/44	3 (2-5)	3 (2-7)	NS
SJC/28	2 (1-3)	2 (1-5)	NS
SJC/44	2 (1-4)	3 (1-5)	NS
GH	48 (20-56)	50 (22-70)	NS
HAQ	0.50 (0.25-1.00)	0.75 (0.25-1.00)	NS
CDAI	12.0 (8.0-16.5)	15 (10-21)	0.0045
SDAI	12.2 (8.5 -18.2)	17.1 (12.1-21.9)	0.0031
VAS	50 (20-60)	40 (20-70)	NS
DAS28	3.81 (3.08-4.44)	4.09 (3.35-4.47)	NS
ESR (mm/h)	21 (11-40)	29 (13-37)	NS
APC (mg/dl)	0.72 (0.19-1.09)	0.65 (0.40-1.79)	NS

Table 3.5 *Characteristics of steady Undifferentiated Arthritis patients (**UA**) and developed Rheumatoid Arthritis patients (**RA**) from UA at follow-up T6 healthy controls as comparison (**CTRL**). Data were expressed as median (25°-75° percentile). p<0.05 was considered significant.*
***BMI**: Body Mass Index; **RF**: Rheumatoid Factor; **ACPA**: Anti-Citrullinated Protein Antibody; **TJC 28/44**: Tender 28/44-Joints Count; **SJC 28/44**: Swollen 28/44-Joints Count; **GH:** General Health score; **HAQ**: Healthy Assessment Questionnaire; **CDAI**: Clinical Disease Activity Index; **SDAI**: Simplified Disease Activity Index; **VAS**: Visual Analogue Scale; **DAS28**: Disease Activity Score on 28 Joints; **ESR**: Erythrocyte Sedimentation Rate; **APC**: Activated Protein C.*

Only Clinical Disease Activity Index (CDAI) and Simplified Disease Activity Index (SDAI) were statistically significant: patients whom have developed RA after 6 months showed higher values comparing to the UA-T6 group. CDAI and SDAI were clinical scores composite by SJC, TJC and the evaluation of global disease activity; they associated with DAS28, the main parameter to evaluate the RA progression.

Genetic Analyses

Single variant association study

Genetic associations between 11 candidate gene-variants (7 belonged to HLA complex and 4 non-HLA) and clinical evolution have been performed considering the end point of RA diagnosis or stabilization/remission in the follow-up 6 months. OR, 95% C.I. and the p value concerning the codominant genetic model for each variant, were reported in Table 3.6.

Candidate Gene Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G 14 bp (rs66554220)				
	DI/DD	0.960	0.372-2.480	0.939
	II/DD	1.000	0.354-2.821	1.000
HLA-G rs1063320				
	GC/GG	1.002	0.415-2.417	0.997
	CC/GG	1.219	0.426-4.046	0.743
HLA-G rs1233334				
	GC/GG	1.851	0.571-5.996	0.309
	CC/GG	n/a	n/a	n/a
HLA-DRB1 rs6910071				
	AG/AA	0.851	0.340-2.134	0.774
	GG/AA	n/a	n/a	n/a
HLA-DRB1 rs660895				
	AG/AA	0.935	0.346-2.528	0.903
	GG/AA	0.818	0.107-9.405	0.870
HLA-DQA2 rs9275595				
	TC/TT	1.095	0.442-2.713	0.856
	CC/TT	1.714	0.384-16.150	0.584
HLA-DQB2 rs10807113				
	AC/AA	0.832	0.355-1.950	0.685
	CC/AA	1.707	0.576-6.992	0.409
PADI4 rs2240340				
	CT/CC	0.643	0.251-1.649	0.365
	TT/CC	0.615	0.165-2.337	0.482
TRAF1 rs3761847				
	AG/AA	1.775	0.750-4.202	0.916
	GG/AA	1.871	0.690-6.563	0.279
PTPN22 rs2476601				
	GA/GG	0.918	0.263-3.202	0.901
	AA/GG	n/a	n/a	n/a
STAT4 rs7574865				
	GT/GG	1.447	0.587-3.567	0.430
	TT/GG	**5.729**	**1.100-46.947**	**0.068**

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

Table 3.6 *Single variant association study in the totality of patients.*

Both UA and RA cohorts were in Hardy-Weinberg equilibrium (data not shown). Genetic association between candidate genes variants and RA evolution did not reveal any significant data in codominant model. However, STAT4 rs7574865 showed a remarkable trend toward association: homozygous patients T/T reported more than 5-fold decreased risk to develop RA (OR= 0.175 95%CI 0.021-0.909, p-value=0.068), respect the wild type genotype G/G.

Single variant association study on sex comparison

Subsequently, considering higher frequency of RA in female population, the effect of the sex on RA evolution has been evaluate. OR, 95% CI and the correspondent p-value have been reported in the Table 3.7, 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 14 bp								
rs66554220	*DI/DD*	0.714	0.132-3.868	0.709	1.123	0.354-3.570	0.854	-
	II/DD	1.000	0.148-6.772	1.000	1.000	0.291-3.437	1.000	-
HLA-G								
rs1063320	*GC/GG*	0.476	0.094-2.418	0.377	1.467	0.497-4.336	0.498	-
	CC/GG	0.667	0.080-5.678	0.722	1.594	0.475-6.981	0.507	-
HLA-G								
rs1233334	*GC/GG*	0.600	0.111-3.255	0.565	5.617	0.687-45.96	0.107	-
	CC/GG	n/a	n/a	n/a	n/a	n/a	n/a	-
HLA-DRB1								
rs6910071	*AG/AA*	0.295	0.061-1.423	0.128	1.570	0.458-5.384	0.483	-
	GG/AA	n/a	n/a	n/a	n/a	n/a	n/a	-
HLA-DRB1								
rs660895	*AG/AA*	0.750	0.107-5.238	0.784	0.961	0.298-3.102	0.952	-
	GG/AA	n/a	n/a	n/a	0.370	0.021-6.245	0.502	-
HLA-DQA2								
rs9275595	*TC/TT*	1.053	0.164-6.776	0.961	1.027	0.355-2.972	0.964	-
	CC/TT	n/a	n/a	n/a	1.135	0.195-11.85	0.911	-
HLA-DQB2								
rs10807113	*AC/AA*	0.727	0.161-3.281	0.692	0.886	0.315-2.493	0.831	-
	CC/AA	0.364	0.016-7.295	0.526	2.455	0.723-13.25	0.228	-
PADI4								
rs2240340	*CT/CC*	0.980	0.210-4.579	0.981	0.477	0.138-1.645	0.244	-
	TT/CC	2.857	0.445-33.903	0.348	0.263	0.041-1.441	0.142	-
TRAF1								
rs3761847	*AG/AA*	0.321	0.074-1.389	0.129	**5.526**	**1.588-19.24**	**0.007**	**0.0037**
	GG/AA	n/a	n/a	n/a	2.368	0.810-8.854	0.158	-
PTPN22								
rs2476601	*GA/GG*	2.200	0.218-22.198	0.514	0.595	0.130-2.721	0.514	-
	AA/GG	n/a	n/a	n/a	n/a	n/a	n/a	-
STAT4								
rs7574865	*GT/GG*	0.800	0.173-3.690	0.787	1.958	0.622-6.157	0.253	-
	TT/GG	n/a	n/a	n/a	4.121	1.256-35.72	0.097	-

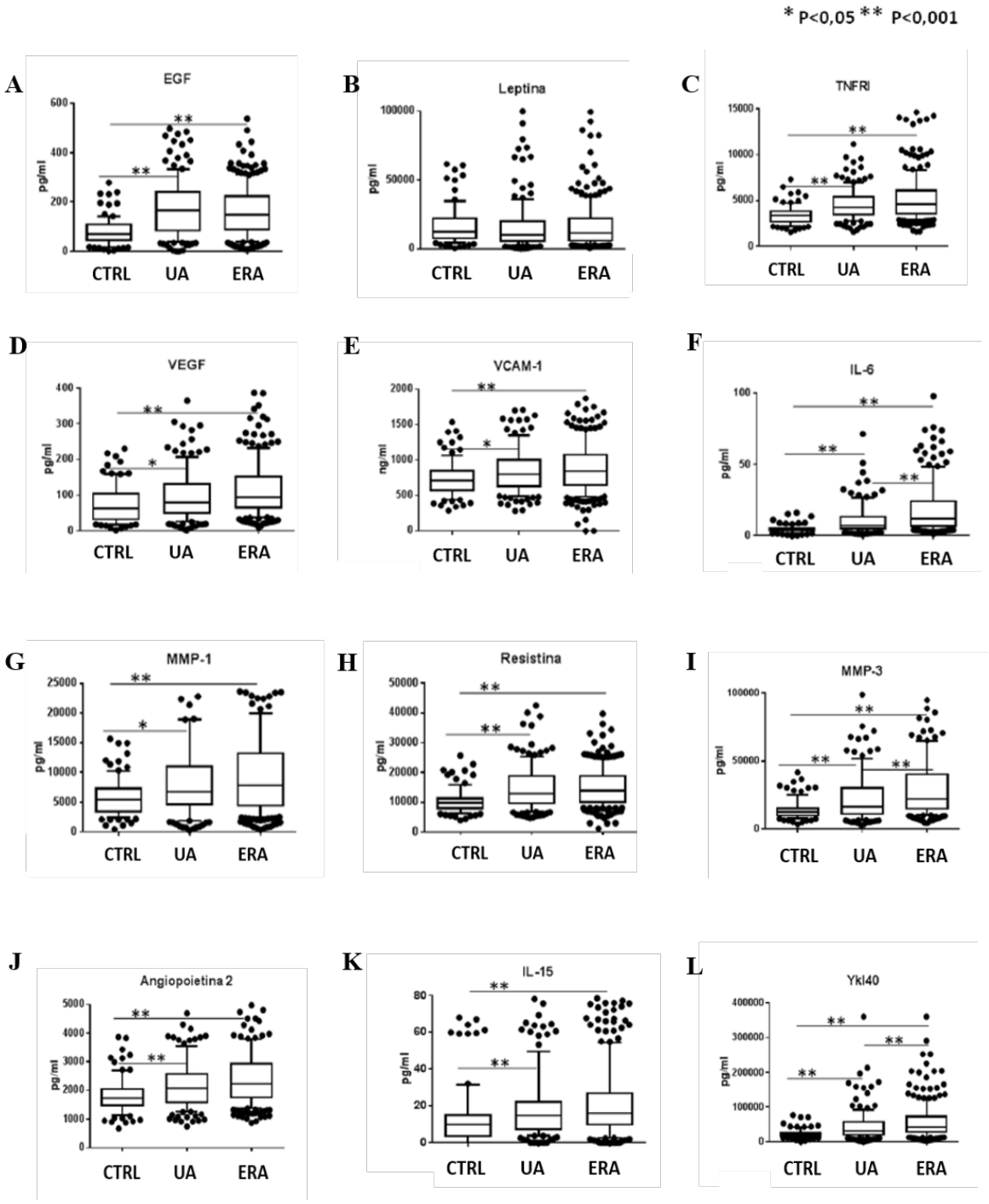
[a] Odds Ratio
[b] Confidence Interval 95%

Table 3.7 *Single variants association stratifying male/female patients.*

Both RA and control cohorts were in Hardy-Weinberg equilibrium (data not shown). Despite genetic association results were not statistically significant, sex stratification revealed opposite effect between male and female subjects in RA development concerning TRAF1 rs3761847 variant. Male heterozygotes reported 3.11-fold increased risk to RA susceptibility (95%CI 0.720-13.443, p-value=0.129), on the contrary female heterozygotes revealed a strong 5.27-fold lower risk for RA developing (OR=0.181 95%CI 0.052-0.629, p-value=0.0072). The sex-compared p-value was significant (0.0037), underlying that rs3761847 acted in opposite direction between sex.

Serological Analyses

In order to evaluate the basal level of the soluble biomarkers panel, serological analyses have been performed comparing the concentration of soluble identified parameters in UA and ERA patients and in a group of matched healthy controls.



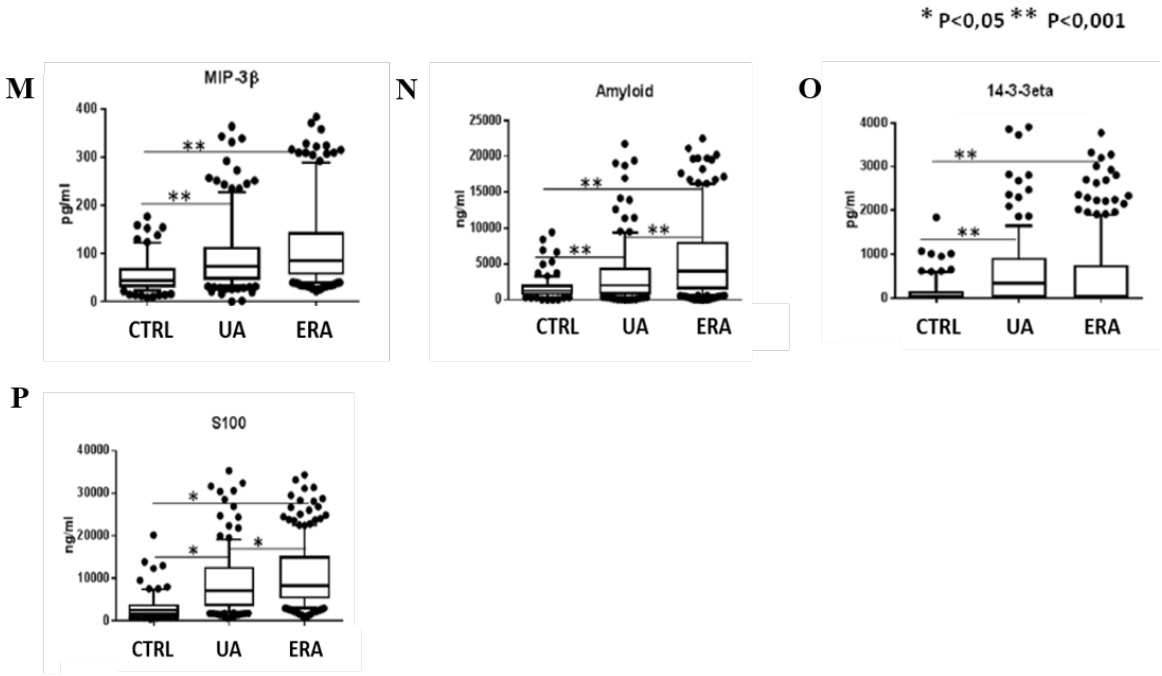


Figure 3.2 *Soluble Biomarkers Analyses in healthy subjects (CTRL), Undifferentiated Arthritis patients (UA) and Early Rheumatoid Arthritis patients (ERA)***.** *Results were expressed as median, 25°-75° percentile and 10°-90° percentile. p<0.05 was considered significant.*
A*. EGF: Epidermal Growth Factor;* ***B.*** *Leptin;* ***C.*** *TNFR-1: Tumor Necrosis Factor receptor 1;* ***D.*** *VEGF: Vascular Endothelial Growth Factor;* ***E.****VCAM-1: Vascular cell adhesion protein 1;* ***F.*** *IL-6: Interleukin 6;* ***G.*** *MMP1: Matrix Metalloproteinase-1;* ***H.*** *Resistin;* ***I.*** *MMP3: Matrix Metalloproteinase-3;* ***J.*** *Angiopoietin;* ***K.*** *IL-15: Interleukin-15;* ***L.*** *YKL-40: Chitinase-3-like protein 1;* ***M.*** *MIP-3β: Macrophage inflammatory protein-3-beta;* ***N.*** *Amyloid;* ***O.*** *14-3-3eta: 14-3-3η polypeptide;* ***P.*** *S100: Calprotectin (S100A8/A9).*

Except leptin, all soluble biomarkers levels showed significant higher concentration in UA and ERA patients comparing to healthy controls (horizontal liner upper). Between the two cohorts of patients, significant differences in soluble biomarkers concentration have been found for the following parameters: IL-6 ($p<0.001$) (Figure 3.2 F), MMP-3 ($p<0.001$) (Figure 3.2 I), YKL-40 ($p<0.001$) (Figure 3.2 L), Amyloid ($p<0.001$) (Figure 3.2 L) and S100 ($p<0.05$) (Figure 3.2 P).

Subsequently, the investigation has been performed considering the end point after 6 months of follow-up (T6). Recruited patients UA have been stratified according to whom have develop RA (UA T0→ RA T6) versus stable UA (UA T0→ UA T6).

Table 3.8 below showed the average of each soluble factor in the two cohorts of subjects.

	UA T0 → UA T6	UA T0 → RA T6	p
EGF pg/ml	152.3 (81.9-232.4)	206.5 (107.4-307.7)	NS
Leptina pg/ml	10449 (5285-23415)	11714 (4846-20754)	NS
TNFRI pg/ml	3868 (3286-5069)	4776 (3166-6771)	0.012
VEGF pg/ml	82 (46.74-139.7)	68.23 (39.32-135.0)	NS
VCAM-1 ng/ml	766.9 (558.6-977.1)	902 (673.4-1053)	NS
IL-6 pg/ml	5.97 (3.803-14.20)	7.035 (4.693-12.17)	NS
MMP-1 pg/ml	7219 (4758-11024)	7664 (4446-12097)	NS
Resistina pg/ml	12636 (8815-18360)	14378 (10167-21054)	NS
MMP-3 pg/ml	15003 (10403-28700)	12726 (9168-24121)	NS
Angiopoietina-2 pg/ml	2063 (1495-2530)	2145 (1684-2630)	NS
IL-15 pg/ml	13.84 (6.993-21.84)	12.17 (5.76-22.89)	NS
YKL-40 pg/ml	29837 (18490-63224)	32155 (16738-66930)	NS
MIP-3 beta pg/ml	70.92 (41.62-109.3)	60.82 (43.35-108.0)	NS
SAA ng/ml	1825 (595-3693)	2693 (861-5653)	NS
14-3-3Eta Polyp. pg/ml	288 (0-835)	293 (0-1285)	NS
S100 A8/A9 ng/ml	6478 (3116-12535)	7192 (4238-11459)	NS

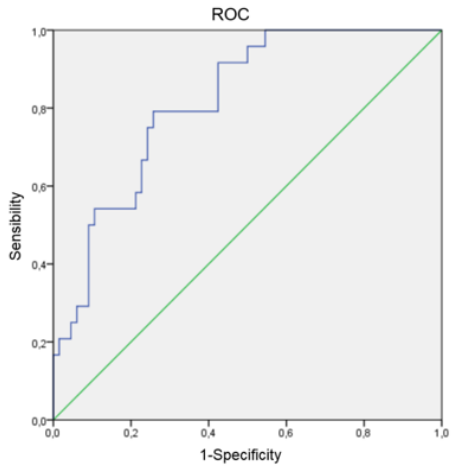
Table 3.8 *Soluble Biomarkers levels considering the follow-up T6 in stable Undifferentiated Arthritis patients (UA T0→UA T6) and in developed Rheumatoid Arthritis patients (UA T0→RA T6)***.** *Results were expressed as median (25°-75° percentile). $p<0.05$ was considered significant.*

Results noticed TNFRI as the only soluble biomarker significant statistical different in the two groups of patients with p-value=0.012. All others parameters were comparable between stable UA and developed RA.

Predictive capability of the soluble biomarkers panel in the outcome of Undifferentiated Arthritis

Logistic regression pointed out significant statistical difference for two serological biomarkers: TNFR1 and 14-3-3η Polypeptide. Model performance relative to the capability to discriminate the outcome of UA patients after 6 months has been evaluated by ROC. Area under curve (AUC) was 0.8, classified the model as moderate accurate, following Swets's method (Swets JA. 1979) (Figure 3.3). This AUC was comparable to the one obtained applying ACR/EULAR 2010 criteria.

A



B

Area	Asyntotic Significant	Asyntotic 95% C.I.	
		lower	upper
0.816	0.000	0.727	0.906

Figure 3.3 *Predictive capability of the soluble biomarkers panel in the outcome of UA.* **A.** *ROC curve;* **B.** *Parameters to characterize AUC*

Multifactor Dimensionality Reduction (MDR) application to detect interactions associated with the outcome of Undifferentiated Arthritis (UA)

SNP × SNP interaction identified variant rs7574865 G>T near STAT4 gene, as the most associated with evolution to RA. Following the recessive genetic model, subjects carrying at least one copy of the allele variant T, showed a trend towards association with a 2.10-fold decreased risk of RA (OR=2.098 95%CI (0.777-5.666) p-value=0.144). The best SNP-SNP interaction resulted between HLA-DQB2 rs10807113 A>C and PADI4 rs2240340 C>T, with the latter showing an epistatic effect over HLA-DQB2 variant. In fact, C/C homozygotes for rs2240340 alone associated with a decreased risk of RA developing (OR = 0.381; 95% CI (0.117-1.241), p value=0.109). A/A homozygotes for rs10807113 variant acted as protection against RA development only if combined with C/T homozygotes for rs2240340. These combinations vs all others showed significant interaction value (OR = 0.233; 95% CI (0.084-0.649), p= 0.0053). Although the difference between interaction and PADI4 rs2240340 variant effect was significant (p value=0.042), the combination showed redundancy: -36.1% effect respect to the addition of the individual variant outcomes. No evident interactions were detectable among multiple SNPs.

Introducing some anamnestic data, best interaction turned out between TRAF1 rs3761847 A>G and gender (Figure 3.4). Previous the genotype effect has been described considering the cohort of patients divided in males and females, MDR study introduced the combined interaction effect of genotype and gender together. As reported in the Table 3.7, male A/G heterozygotes among females and A/A or G/G homozygotes among males vs opposite combinations turned out in a 4.36-fold increased risk of RA (OR=4.364 95%CI (1.470-12.951) p-value=0.0079. In fact, as already observed in the single variant association study on gender comparison, TRAF1 A/G genotype was associated

with higher RA risk among males (OR=3.11 95%CI (95%CI 0.720-13.443, p-value=0.129), while was associated with a significant protective effect among females (OR=0.181 95%CI (0.052-0.629), p-value=0.0072).

If no gender stratification was considered, A/G genotype apparently was not influencing risk of evolving to RA phenotype (OR=1.775 95%CI (0.750-4.202; p value=0.193)).

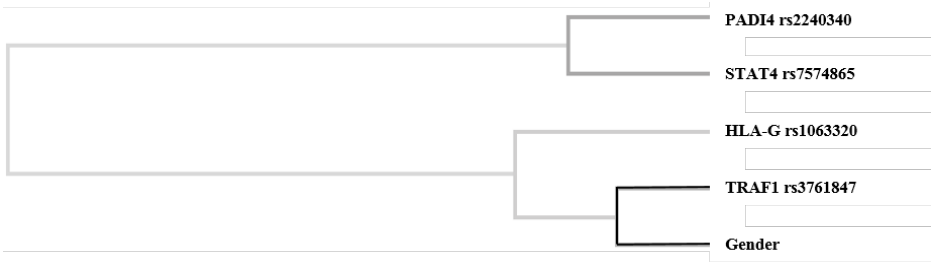


Figure 3.4 *MDR software output. Dendrogram of interaction between genetics parameters and gender associated to RA evolution. Darker and closer lines are referred to the strongest association: TRAF1 rs3761847 and gender.*

Including all serological data into account, top SNP became PADI4 rs2240340 under a recessive model: subjects carrying at least one copy of allele variant T showed remarkable 3.5-fold higher risk to develop RA (OR=3.492 95%CI (0.934-13.054) p-value=0.063), respect C/C homozygotes.

A second interaction came out significant between HLA-DQB2 rs10807113 A>C and high level of Serum Amyloid (over 2 sd), showing statistically significant combination: OR=8.896 (95%CI (2.065-38.316) p-value=0.0028). More precisely, HLA-DQB2 A/A genotype associates with an increased risk of RA development only in presence of normal level of Serum Amyloid, while with high level of Serum Amyloid did not have any significant effect (Figure 3.5).

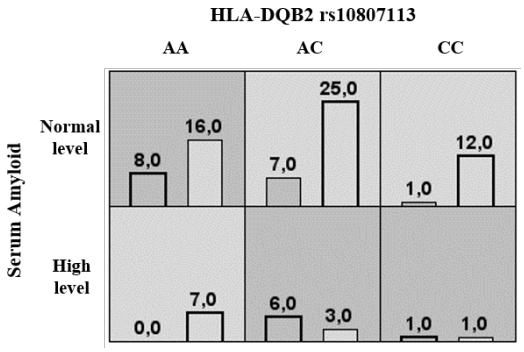


Figure 3.5 *MDR software output. Histogram of interaction between rs10807113 variant and Serum Amyloid associated to RA evolution. Each cell shows counts on left UA whom have developed RA and on right UA stabilized.*

3.4.2 Disease's Progression Evaluation of Early Rheumatoid Arthritis (ERA) patients

ERA subject involved in the study have been stratified evaluating the responsiveness to administered therapy, in terms of Disease Activity Score 28 variation (ΔDAS28). Independently to the specific therapy, responders-to-treatment patients were 88%, whereas non-responders were 12%.
Characteristics of the two group were showed in the Table 3.9.

	ERA Responders	ERA Non-Responders	p
Age	63 (51-73)	59 (51-72)	NS
Gender, %	76	74	NS
BMI Kg/m²	25.26 (22.50-28.91)	26.01 (24.02-28.96)	NS
Smokers %	44	73	0.003
Length Disease (weeks)	13 (8-24)	11 (8-24)	NS
RF + (%)	51	65	NS
ACPA + (%)	50	63	NS
TJC/28	8 (3-12)	4 (1-12)	NS
TJC/44	11 (5-16)	7 (4-16)	NS
SJC/28	6 (3-10)	41 (0-8)	0.020
SJC/44	8 (4-13)	6 (2-12)	NS
GH	50 (40-80)	50 (28-70)	NS
HAQ	1.12 (0.63-1.75)	1.13 (0.75-1.88)	NS
CDAI	27.0 (18.0-36.6)	19.0 (10-38.0)	NS
SDAI	15.3 (11.2-21.7)	20.1 (11.3-38.8)	NS
VAS	50 (45-80)	50 (35-70)	NS
DAS28	5.39 (4.43-6.27)	4.64 (3.28-5.91)	0.011
ESR (mm/h)	39 (21-56)	21 (15-46)	0.031
APC (mg/dl)	1.45 (0.69-4.37)	0.80 (0.29-3.06)	NS

Table 3.9 *Characteristics of ERA patients Responder and ERA patients Non-Responders to therapy. steady Data were expressed as median (25°-75° percentile). $p<0.05$ was considered significant.*
***BMI**: Body Mass Index; **RF**: Rheumatoid Factor; **ACPA**: Anti-Citrullinated Protein Antibody; **TJC 28/44**: Tender 28/44-Joints Count; **SJC 28/44**: Swollen 28/44-Joints Count; **GH:** General Health score; **HAQ**: Healthy Assessment Questionnaire; **CDAI**: Clinical Disease Activity Index; **SDAI**: Simplified Disease Activity Index; **VAS**: Visual Analogue Scale; **DAS28**: Disease Activity Score on 28 Joints; **ESR**: Erythrocyte Sedimentation Rate; **APC**: Activated Protein C.*

Significant differences between the two groups concerned the percentage of smokers' patients, numbers of swollen joints involved, the disease activity score index and the rate of erythrocyte sedimentation.
Out of those parameters, smoking and SJC resulted significant higher in patients Non-Responders to the therapy, whereas DAS28 and ESR showed higher values in Responders group.

Genetic Analyses

Single variant association study

Genetic association between 11 candidate gene-variants and disease progression have been carried out stratifying patients according to the variation of DAS28 after 6 month (follow-up T6).

Overall, 17.15% subject were non-responders, 26.09% were low responders and 56.77% were high responders. In this context, analyses have been performed following two models.

- ***Non-responder patients versus all others***, to characterize the subjects' genotypes panel regarding the disease progression (Table 3.8).
- ***Non-responders and low responder patients versus high responders***, to determinate the genetic profile of best responders' subjects (Table 3.9).

OR, 95% C.I. and the p value for the codominant genetic model for each variant were reported in Table 3.10, referred to "***Non-responder patients versus all others"*** pattern.

Candidate Gene Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G 14 bp (rs66554220)				
	DI/DD	1.272	0.672-2.410	0.469
	II/DD	0.976	0.430-2.134	0.957
HLA-G rs1063320				
	GC/GG	1.300	0.704-2.401	0.409
	CC/GG	1.599	0.667-3.639	0.282
HLA-G rs1233334				
	GC/GG	0.916	0.439-1.913	0.827
	CC/GG	n/a	n/a	n/a
HLA-DRB1 rs6910071				
	AG/AA	0.904	0.453-1.801	0.786
	GG/AA	2.969	0.734-10.555	0.109
HLA-DRB1 rs660895				
	AG/AA	1.077	0.576-2.015	0.828
	GG/AA	2.659	0.531-11.032	0.208
HLA-DQA2 rs9275595				
	TC/TT	1.072	0.594-1.934	0.828
	CC/TT	2.123	0.524-7.112	0.261
HLA-DQB2 rs10807113				
	AC/AA	1.250	0.686-2.276	0.476
	CC/AA	1.331	0.535-3.047	0.530
PADI4 rs2240340				
	CT/CC	**0.539**	**0.299-0.974**	**0.040**
	TT/CC	**0.342**	**0.115-0.843**	**0.035**
TRAF1 rs3761847				
	AG/AA	0.937	0.527-1.668	0.837
	GG/AA	0.462	0.145-1.185	0.150
PTPN22 rs2476601				
	GA/GG	1.370	0.643-2.919	0.422
	AA/GG	n/a	n/a	n/a
STAT4 rs7574865				
	GT/GG	0.794	0.437-1.443	0.459
	TT/GG	1.009	0.287-2.818	0.989

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

Table 3.10 *Single variant association study in the totality of patients: "non-responders versus all others" model.*

Genetic association between candidate genes variants and RA progression did not reveal any significant data in codominant model. However, PADI4 rs2240340 noticed the strong trend toward RA disease progression: homozygotes T/T patients were associated to lower probability to be non-responder, than C/C subjects. In others words, T/T patients showed 2.9-fold higher increased DAS28 variation and responsiveness (OR=2.92, 95% CI= 1.186-8.728; p value= 0.035), respect C/C wild type genotype.

Subsequently, analyses have been performed regarding the model "***Non-responders and low responder patients versus high responders"***, considered as *Best Responders* profile. OR, 95% C.I. and the p value for the codominant genetic model for each variant, were reported in Table 3.11.

Candidate Gene Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G 14 bp (rs66554220)				
	DI/DD	0.725	0.452-1.163	0.183
	II/DD	0.763	0.427-1.335	0.359
HLA-G rs1063320				
	GC/GG	0.848	0.542-1.327	0.480
	CC/GG	1.298	0.697-2.453	0.424
HLA-G rs1233334				
	GC/GG	1.111	0.649-1.902	0.715
	CC/GG	n/a	n/a	n/a
HLA-DRB1 rs6910071				
	AG/AA	1.125	0.683-1.853	0.657
	GG/AA	2.587	0.888-9.039	0.108
HLA-DRB1 rs660895				
	AG/AA	0.840	0.346-1.352	0.482
	GG/AA	2.633	0.831-10.744	0.138
HLA-DQA2 rs9275595				
	TC/TT	0.752	0.480-1.176	0.214
	CC/TT	0.927	0.292-2.752	0.903
HLA-DQB2 rs10807113				
	AC/AA	1.091	0.702-1.697	0.711
	CC/AA	0.800	0.401-1.524	0.542
PADI4 rs2240340				
	CT/CC	0.964	0.600-1.550	0.889
	TT/CC	**0.361**	**0.167-0-706**	**0.006**
TRAF1 rs3761847				
	AG/AA	0.780	0.498-1.222	0.282
	GG/AA	0.811	0.431-1.480	0.515
PTPN22 rs2476601				
	GA/GG	0.941	0.509-1.738	0.856
	AA/GG	0.263	0.016-2.280	0.293
STAT4 rs7574865				
	GT/GG	1.014	0.655-1.572	0.954
	TT/GG	1.005	0.435-2.226	0.991

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

Table 3.11 *Single variant association study in the totality of patients: "Best Responders" model. OR>1 was considered related to higher disease progression. p<0.05 was considered significant and only is this case is reported.*

The "*Best Responder*" model did not find significant data concerning genetic association and RA progression. However, as described for the previous model, PADI4 rs2240340 variant revealed remarkable results, to be underlined.

Acting under a recessive genetic model, homozygotes T/T subjects for the variant showed statistical significant 2.7-fold higher probability to increase DAS28 variation (OR=2.704, 95% CI= 1.499-4.879; p value= 0.001), respect subjects carrying at least on copy of the wild type allele C.

In terms of clinical translation, patients with genotype T/T were associated to best response and low disease progression and severity.

Serological Analyses

ERA patients have been stratifying considering the response to treatment after 6 months (follow-up T6). Responders cohort was statistically significant different from non-responders group for two soluble biomarkers: TNFR1 and Resistin.

As showed in Table 3.12, TNFR1 concentration was lower in patients non-responders to treatment (p=0.0121), whereas Resistin level was higher (p=0.0100).

	ERA Responders	ERA Non-Responders	p
EGF pg/ml	147.3 (84.2-232.7)	180.3 (99.57-218.5)	NS
Leptina pg/ml	11486 (4977-22371)	12348 (4331-21523)	NS
TNFRI pg/ml	4595 (3460-6252)	4519 (3518-5464)	0.0121
VEGF pg/ml	97.88 (64.75-157.4)	83.98 (57.58-119.5)	NS
VCAM-1 ng/ml	864.9 (642.1-1084.9)	762.7 (584.1-1066)	NS
IL-6 pg/ml	12.35 (6.57-24.96)	9.92 (4.6329.28)	NS
MMP-1 pg/ml	8337 (4535-13626)	5772 (2610-11234)	NS
Resistina pg/ml	13104 (9554-18610)	17869 (13343-24013)	0.0100
MMP-3 pg/ml	22691 (13869-40633)	22036 (12865-40924)	NS
Angiopoietina-2 pg/ml	2164 (1658-2868)	2557 (1709-2972)	NS
IL-15 pg/ml	15.60 (9.15-26.68)	15.60 (5.40-30.36)	NS
YKL-40 pg/ml	43824 (24453-73154)	37590 (21542-99463)	NS
MIP-3 beta pg/ml	81.97 (55.03-131.3)	127.6 (67.76-160.1)	NS
SAA ng/ml	4296 (1643-8209)	2822 (1012-7463)	NS
14-3-3Eta Polyp. pg/ml	48 (0-929)	0 (0-523)	NS
S100 A8/A9 ng/ml	8822 (5390-15316)	7972 (4990-16557)	NS

Table 3.12 *Soluble Biomarkers levels in ERA patients Responders and Non-responders to therapy. Results were expressed as median (25°-75° percentile). p<0.05 was considered significant.*

Predictive capability of the soluble biomarkers panel in the disease progression of patients

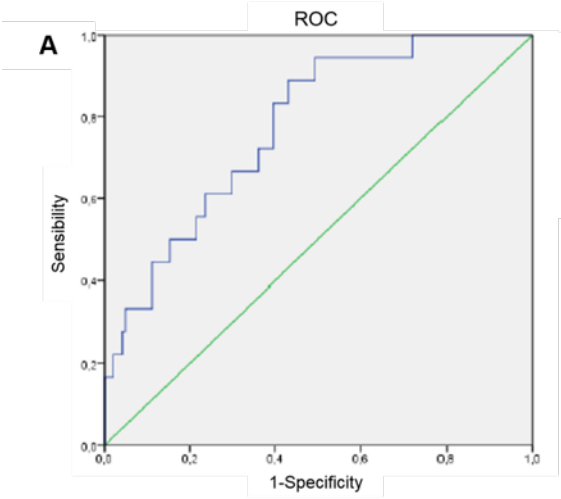
Analyses on predictive capability have been performed considering either the totality of subject (UA+ERA) and ERA patients only. Logistic regression pointed out for both cohorts the significant

role of the Resistin: UA+ERA: p-value= 0.053 (data not showed); ERA: p-value= 0.025 (Figure 3.6 A).

Model performance relative to the capability to determinate the disease progression and the responsiveness to treatment in terms of DAS28 reduction, has been evaluated by ROC.

Area under curve (AUC) was 0.776, classified the model as moderate accurate, following Swets's method (Swets JA. 1979) (Figure 3.6 B).

A



B

Area	Asyntotic Significant	Asyntotic 95% C.I.	
		lower	upper
0.776	0.000	0.674	0.878

Figure 3.6 *Predictive capability of the soluble biomarkers panel in the outcome of UA.*
A. *ROC curve;* **B.** *Parameters to characterize AUC*

Multifactor Dimensionality Reduction (MDR) application to detect interactions associated with disease progression of patients

1. ***Non-responder patients versus all others***

MDR analysis confirmed SNP rs2240340 C>T near PADI4 gene as the variant most related to RA progression. Patients carrying at least one copy of the allele variant T, showed a strong trend toward association with a 2-fold higher increased DAS28 variation and responsiveness (OR=2.032, 95% CI (1.062-3.887); p value= 0.032), respect wild type homozygotes C/C.

The best SNP-SNP interaction resulted between PADI4 rs2240340 C>T and HLA-G rs1063320 G>C (Figure 3.7 A): patients with at least one copy of T allele for rs2240340 showed association to higher ΔDAS28 only if they were homozygous G/G or heterozygous for rs1063320, whereas C/C subjects for rs2240340 manifested responsiveness only in combination with C/C homozygosity for rs1063320 (Figure 3.7 B) This pattern vs other combination noticed statistically significant 3.24-fold increased DAS28 variation (OR=3.24 95%CI (1.716-6.123) p-value=3E-04). The difference between the effect of PADI4 rs2240340 variant and the interaction was not significant (p-value=0.054) (Figure 3.7 C). Nonetheless, the interaction showed synergy, exceeding 17% of the result that would has obtained if the interaction had been additive.

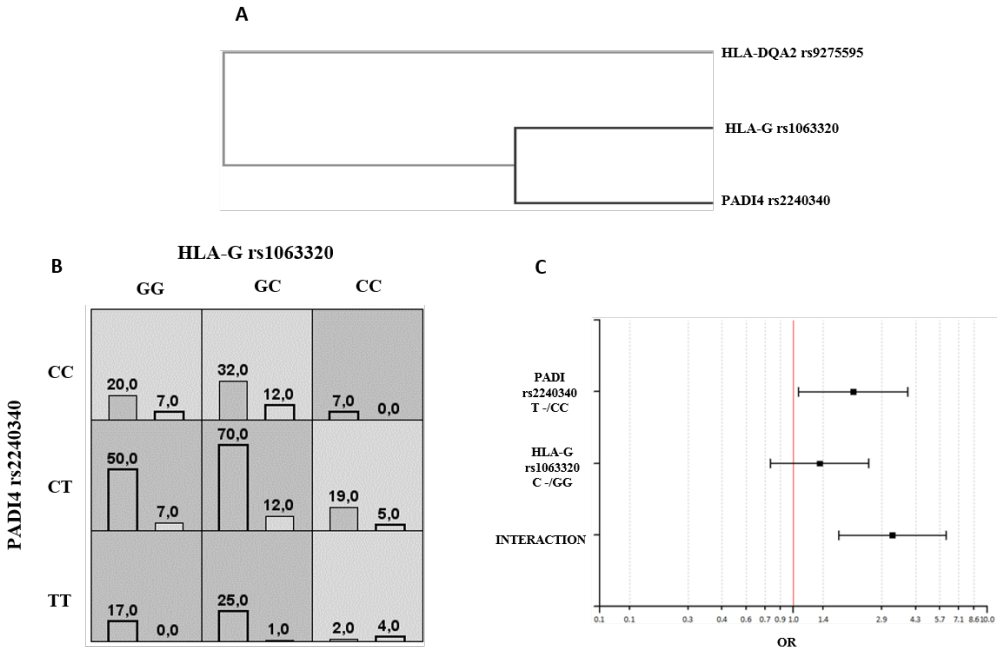


Figure 3.7 *Gene-gene interaction.* ***A****. Dendrogram of MDR software output. Darker and closer lines were referred to the stronger association: PADI rs2240340 and HLA-G rs1063320.* ***B****. Histogram of MDR software output. Each cell shows on left counts of ERA with DAS28 reduction and on right ERA no responders.* ***C.*** *Forest plot with association results of single variants and interaction.*

Taking into account serological parameters, MDR analysis found Interleukin 6 as the single factor most associated to clinical response. In details, subjects with higher levels of IL-6 (over 2 sd) showed 2-fold higher probability to do not progress the RA disease (OR=0.486 95%CI (0.248-0.955) p-value=0.036), leading to good prognosis.

No evident interactions were detectable among multiple genetic variant and serological parameters.

2. Non-responders and low responder patients versus high responders

Subsequently, MDR analyses was carried out considering subjects with higher DAS28 reduction, called *Best Responders*, compared with all others patients, with mild or moderate DAS28 decrease.

The gene variant most associated to identify *Best Responders* was confirmed PADI4 rs2240340 C>T, following recessive genetic model. Subjects T/T allele showed 2.7-fold higher probability of increased DAS28 reduction and responsiveness to treatment (OR=2.704, 95% CI (1.499-4.879); p value= 0.001).

The best SNP-SNP interaction individuated through MDR, resulted between HLA-DQA2 rs9275595 T>C and HLA-DRB1 rs660895 A>G. The compresence for both variants of wild type homozygotes or heterozygotes or homozygotes for the variants vs all other combinations were associated to statistically significant decrease responsiveness and lower ΔDAS28 (OR=0.633 95%CI (0.206-

0.639) p-value=5E-04). The interaction revealed synergic effect, showing statistically significant differences between the effect of single variants and the combination, with p values 0.002 and 1E-04, respectively for rs9275595 and rs660895.

Taking into account serological parameters, MDR analysis found a strong and significant interaction between two serological parameters: Rheumatoid Factor (RF) and Resistin (Figure 3.8). The probability to progressive DAS28 reduction resulted associated to higher levels of Resistin in presence of physiological concentration of RF or to regular levels of Resistin in subjects with increased levels of RF (OR=2.883 95%CI (1.734-4.794) p-value=5E-05).

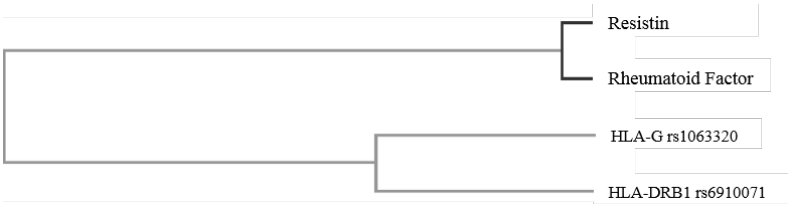


Figure 3.8 *MDR software output. Dendrogram of interaction between genetics and serological parameters associated to RA progression: Best Responders model. Darkest and closest lines referred to the strongest association.*

This crosslinked interaction between Resistin and RF was remarkable, considering the evidence that MDR output found interplay with genotype HLA-DRB1 rs6910071 A>G. This variant is in linkage-disequilibrium with the HLA-DRB1 shared epitope, involved in the etiopathology of RA.

MDR results showed that in subject with higher levels of Resistin, Best Responders were essentially determinate by normal concentration of RF and HLA-DRB1 genotype did not play a role; whereas in cases with physiological levels of Resistin, the best DAS28 reduction was influenced by interaction between RF levels and HLA-DRB1 genotype. In fact, Best Responders resulted associated to higher levels of RF in compresence of HLA-DRB1 wild type allele A or, on the other side, regular concentration of RF was associated to HLA-DRB1 heterozygotes A/G (OR=2.262 95%CI (1.247-4.101) p-value=0.007) (Figure 3.9).

The interaction between RF and HLA-DRB1 rs6910071 came out hypostatic respect to Resistin, appearing only in cases with regular level in serum of this parameter. It was particularly remarkable the evidence of cases with high levels of Resistin in serum, corresponding to 25.75% of subjects, or regular concentration of FR, corresponding to 59.5% of cases: 11.0% of patients showed these parameters together and their combination was associated with significant increased probability of DAS28 reduction (OR=25.714 95%CI (6.70-98.66) p-value=3E-06) and responsiveness. For the others cases, with regular concentration of Resistin (74.3%), Best Responders have been detected by the interaction between RF and HLA-DRB1 rs6910071 A>G.

Overall, the algorithm corresponding to darker squares in the Figure 3.13 below was able to identify Best Responders with the ratio 4:1 (OR=3.803 95%CI (2.250-6.430) p-value=1E-06) (Figure 3.10).

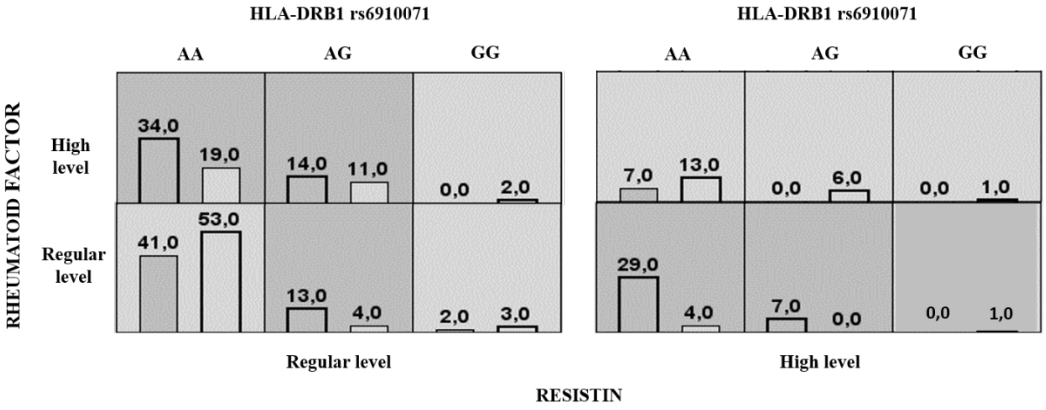


Figure 3.9 *Gene-gene interaction. Histogram of MDR software output. Each cell shows on left the count of ERA with higher DAS28 reduction and on right the count of ERA no-responders or with low DAS28 reduction.*

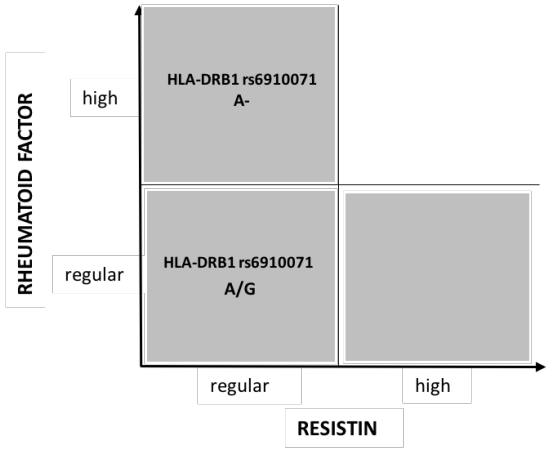


Figure 3.10 *Predictive algorithm to detect "Best Responders" patients in dark grey squares.*

3.4.3 HLA-DRB1 Shared Epitope Association

The sequencing of HLA-DRB1 Shared Epitope (SE) has been performed in enrolled patients, subdividing the groups of UA and ERA subjects at the first visit. Patients carrying at least one Shared Epitope allele were considered SE positive (SE+), whereas subjects without any copy were SE negative (SE-) (Holoshitz J. 2010). According to what has reported by Pratesi F at al (Pratesi F et al., 2013, Tables), SE+ were associated to higher rate of RA predisposing risk, whereas SE- included sequences with different grade and severity of RA susceptibility. DRB1*1303 was borderline to neutrality/predisposing effect, DRB1*0403, *0407, *0411, *03, *15, *0901, *1401, *1404, *1101, *1104, *12, *16 alleles were all related to neutrality, DRB1*07 was borderline to neutrality/protective effect and [71]DEERA[74] sequence showed protection in developing RA.

The distribution and the frequency of SE+ and SE- in the two cohorts of patients enrolled in the study, have been showed in Table 3.13.

Locus	SE alleles	UA patients T0		ERA patients T0	
		N	***%***	***N***	***%***
HLA-DRB1 SE+		40	30.30	145	48.17
QRRAA	**0101, *0102, *0404, *0405, *1402*	25	18.94	100	33.22
QKRAA	**0401*	15	11.36	45	14.95
HLA-DRB1 SE-		92	69.70	156	51.83
DKRAA	**1303*	12	9.09	19	6.31
QRRAE	**0403, *0407, *0411*	22	16.67	39	12.96
QKRGR	**03*	3	2.27	6	1.99
QARAA	**15*	6	4.55	11	3.65
RRRAE	**0901, *1401, *1404*	21	15.91	48	15.95
DRRAA	**1101, *1104, *12, *16*	22	16.67	29	9.63
DRRGQ	**07*	0	0	0	0
DERAA	**0103, *0402, *1301, *1302, *1323*	6	4.55	4	1.33

Table 3.13 *Distribution of the HLA-DRB1 Shared Epitope Alleles in patients enrolled, divided in UA and ERA al the first visit (T0). SE+ is referred to positive SE allele presence, whereas SE- is referred to absence of SE allele.*

Concerning UA cohort T0, 30.30% of patients reported at least one copy of SE+ alleles, whereas 69.70% did not. Data reflected undifferentiated group heterogeneity, which could be related to absence of typical clinical features and could include several symptoms phenotypes, not necessarily associated to a further RA diagnosis.

With regard to ERA group, Shared Epitope frequencies followed roughly the same pattern in both SE positive and SE negative groups, respectively 48.17% and 51.83%. Overall, the sequence with higher frequency was ^{70}QRRAA^{74}, while ^{70}DRRGQ^{74} sequence was never been noticed.

Subsequently, genetic association between SE+ and clinical evaluation has been performed.

Evolution of Undifferentiated Arthritis (UA) to Rheumatoid Arthritis (RA)

Firstly, it has been considered UA cohort. The evaluation of SE alleles presence has been carried out stratifying patients with the end point of RA diagnosis or stabilization/remission in the follow-up 6 months (Table 3.14).

A

	UA T0 → UA T6	UA T0 → RA T6
SE+	30	10
SE-	73	19

B

OR	95% CI	p-value
0.781	0.325-1.875	0.592

Table 3.14 *HLA-DRB1 Shared Epitope Alleles in Undifferentiated Arthritis patients.*
A. *Stratification of stable UA or RA-diagnosed in the follow-up T6 months.* ***B.*** *Association study*

Association analysis did not reveal statistically significant result. However, OR data showed patients carrying SE+ alleles with low probability to remit or stabilize the disease after 6 months.
In others word, SE+ subjects manifested 1.3-fold higher probability to develop RA (OR=1.281 95%CI (0.533-3.075)), respect SE- patients.

Disease's Progression of Early Rheumatoid Arthritis (ERA) patients

Furthermore, has been considered the ERA subset. The association analyses with SE+ presence have been performed stratifying patients according to the variation of DAS28 after 6 month. The evaluation has been reported following the two models, as previous accomplished: "*Non-responder patients versus all others"* and *"Non-responders and low responder patients versus high responders"*. OR, 95% C.I. and the p value concerning the "*Non-responder patients versus all others"* pattern, were described in Table 3.15.

A

	ERA T6 non-responders	ERA T6 low or high responders
SE+	18	127
SE-	30	126

B

OR	95% CI	p-value
1.680	0.891-3.167	0.109

Table 3.15 *HLA-DRB1 Shared Epitope Alleles in Early Rheumatoid Arthritis patients.*
A. *Stratification according to DAS28 reduction in the follow-up T6 months.* ***B.*** *Association study*

OR analysis did not report statistically significant result. ERA patients carrying SE+ alleles showed slightly higher probability to DAS28 reduction, link to low or high response-to-treatment (OR=1.68 95%CI 0.533-3.075), respect SE- subjects. OR, 95% C.I. and the p value concerning the "*Non-responders and low responder patients versus high responders"* model, were reported in Table 3.16.

A

	ERA T6 non or low responders	ERA T6 high responders
SE+	57	88
SE-	70	86

B

OR	95% CI	p-value
1.257	0.794-1.988	0.334

Table 3.16 *HLA-DRB1 Shared Epitope Alleles in Early Rheumatoid Arthritis patients.*
A. *Stratification according to DAS28 reduction in the follow-up T6 months.* ***B.*** *Association study*

Association analysis did not reveal statistically significant result. OR data noted patients carrying SE+ alleles with a mild higher probability to DAS28 reduction after 6 months, leading to best responder's subjects. However, this data was a purely observation and OR was almost close to irrelevant value, as p value confirmed.

3.5 Discussion and Conclusion

Individuating early symptoms of rheumatoid arthritis (ERA) or, even previous, a pattern of features arthritis-like (UA) without fulfill completely the classification criteria, is useful and remarkable to reach benefits of large significance, especially to determine occurrence risk profile and to evaluate promptly the adequate treatment. Benefits of therapies in the window of opportunity (within 3/6 months from symptoms onset) include higher response rate, decreased disease activity score, prevention of bone erosions and less disability, increased rate of remission, even in drug-free remission and larger improvement in globally healthy quality scores (Regueiro C et al., 2017; Finch A et al., 2006). Several advances have contributed to achieve the goal of diagnosis in early stages of rheumatoid arthritis (RA) disease; however, there are still rooms for improvement, especially related to individuate a particular profile associated-risk and hallmarks of severe condition development; and no study has yet done these specific approaches.

In this view, our study enrolled a total of 501 UA/ERA Italian patients and analyses have been performed considering firstly the evolution of UA patients to established RA vs stabilization or remission; secondly the disease progression of ERA subjects in terms of disease activity score (DAS28) improvement. Both approaches were evaluated after 6 months from the first visit (follow-up T6) and included genetics, serology and the combined model.

Concerning the cohort of UA subjects and the evolution to diagnosed RA disorder after 6 months, no significant result was found thus far regarding single candidate gene variants. One of the reason for the association absence may be due to the small sample size. Only STAT4 rs7574865 noticed a trend toward association: taking the G/G genotype as the reference, T/T homozygotes showed more than 5-fold probability of symptoms stabilization (OR= 5.729 (95%CI 1.100-46.947) p-value= 0.068). However, this result should be considered more properly as a simple suggestion, without reaching the statically significant value and without confirming our previous evidence. In fact, in the case-control observational study, described in the *CHAPTER 2 "CHARACTERIZATION OF THE OCCURRENCE RISK PROFILE IN RHEUMATOID ARTHRITIS PATIENTS: FROM HEALTH TO DISEASE"*, it has been reported STAT4 rs7574865 as associated to significant higher RA risk, following the dominant genetic model (OR=1.5 (95%CI 1.189-2.119) p-value=0.0018). These not clear results could be justified by the completely different casuistries analyzed: in fact, UA patients already manifest a rheumatic symptomatology and some *red flags* referred to RA disease; whereas controls were matched subjects in healthy conditions, and precisely because of this they did not show inflammation or other related symptoms. According to our suggestion, Ghosh and colleagues reported that the prediction models depend on the selected cohort of patients with undifferentiated arthritis (UA) (Ghosh et al., 2006). This evidence is sustained even by the absence of the predominant association of HLA-DQA2 rs9275595 variant and RA risk, observed in our *CHAPTER 2*; confirming the intrinsic selection in UA subgroup, not comparable to healthy matched subjects.

The patients' stratification according to the sex, revealed the significant effect of TRAF1 rs3761847 variant in a sex-specific manner (p-value of the sex comparison= 0.0037). More precisely, male heterozygotes showed 3.11-fold higher RA risk (OR=3.11 (95%CI 0.720-13.443) p-value=0.129), whereas female heterozygotes reported 5.27-fold decreased RA susceptibility (OR=0.181 (95%CI 0.052-0.629) p-value=0.0072). To our knowledge, no prior report has linked RA development risk and TNF receptor-associated factor-1 according to the sex, but studies claimed that various gender-specific mechanisms, such as the effect of sex hormones and differences in lifestyle and physiology could influence the pathways involvement of numerous diseases, severity and presence of comorbidities (Emamifar A et al., 2017; Summart U et al., 2017; Khalifa O et al., 2016). Moreover, sexual dimorphism may operate in certain rheumatic and inflammatory diseases which occur more frequently in women than men, as RA (Masi AT et al., 2015).

Serological result revealed TNFR1, as the main associated parameter: its serum concentration was significant higher in UA patients whom have developed RA (p value= 0.012). This finding strengthened our result on TRAF1 gene involvement and it was consistent with other studies which have analyzed the mechanism of the Fn14-TRAF-TNFR axis, reporting interaction between certain expression profile of TNF receptors and TRAF, which participated in various inflammatory and immunological processes (Wang X et al., 2017).

The analysis of combination between genetic and serology, provided a significant interaction between HLA-DQB2 rs10807113 and Serum Amyloid, with the latter showing an epistatic effect over HLA-DQB2 variant. In fact, A/A homozygotes for rs10807113 associated with higher risk of developing RA only in presence of normal concentration of Serum Amyloid (OR=8.896 (95%CI 2.065-38.316) p-value= 0.0028), while with high level of Serum Amyloid, it did not have any significant effect. The finding suggested the preponderant role of this serum parameter on the SNP; indeed, in the study of Targonska and colleagues, it has been observed the strong association between high Serum Amyloid level and RA patients with severe inflammation and increased disease activity score (Targonska-Stepniak B, Majdan M, 2014). Moreover, it has been even reported that Serum Amyloid may play an important and pathogenic role in the proinflammatory cascade in RA course (Connolly M et al., 2012).

Hence, concerning the development from UA to RA established disease after 6 months, our study revealed that serology exerted a preponderance effect respect genetic variants. Monitoring the concentration of some serological parameter at close times, could provide an effort in assessing promptly the very early stage of the RA disease.

With regards to the second approach performed in our study, analysis have been carried out evaluating the disease progression of ERA subjects in terms of DAS28 improvement, comparing the variation of DAS28 (ΔDAS28) after 6 months from the first visit (T0). "*Non-responders versus all others*" model revealed as the main result on single candidate gene variant PADI4 rs2240340,

showing T/T subjects with 2.9-fold increased probability to higher ΔDAS28 and responsiveness (OR=2.92 (95%CI 1.186-8.728) p-value= 0.035), respect C/C wild type genotype. The result was confirmed even in the "*Best responders versus all others"* model, reporting T/T genotype associated to best response and low disease progression (p-value= 0.006). PADI4 gene encodes one of PADI enzymes that catalyze the post-translational modification reaction generating citrulline residues from arginine (Suzuki A et al., 2003) and it has been reported that PADI4 had impact on disease progression and joint damage independently from HLA-DRB1 SE and other serum parameters, such as ACPA status (Suzuki T et al., 2013; Bang et al., 2010). Moreover, several studies reported additional deeper findings considering the relationship between PADI4 gene and RA disease severity in the context of association of PADI4 haplotypes, even with serum factors (Cha S et al., 2007; Harris ML et al., 2008; Hoppe B et al., 2009).

SNP × SNP combination in "*Non-responders versus all others*" model found significant interaction between PADI4 rs2240340 and HLA-G rs1063320 variants: patients carried at least one T allele variant for rs2240340 in presence of G/G or G/C for rs1063320 and C/C for rs2240340 with C/C for rs1063320 showing 3.24-fold increased ΔDAS28 (OR=3.24 (95%CI 1.716-6.123) p-value= 3E-04) respect others combinations. However, this result did not reveal significant p value of the difference between the interaction and PADI4 alone (p value= 0.054), suggesting the effect was ascribable mainly to this genetic variant.

Concerning analysis of interaction in "*Best responders versus all others"* model, it was interesting the combination of two serological parameters Rheumatoid Factor (RF) and Resistin and the gene variant HLA-DRB1 rs6910071. In subjects with high levels of Resistin, Best Responders were essentially determinate by normal concentration of RF and HLA-DRB1 genotype did not play a role (OR=25.714 (95%CI 6.70-98.66) p-value= 3E-06); whereas in cases with regular level of Resistin, Best Responders have been detected by the interaction between RF and rs6910071. The algorithm is able to identify Best Responders with the ratio 4:1 (OR=3.803 (95%CI 2.250-6.430) p value= 1E-06) and it is the most significant finding of this study.

Resistin has been studied in RA as serum adipocytokine associated to inflammatory markers (Forsblad d'Elia H et al., 2008) and clinical activity (Senolt L et al., 2007) and it is included in Multi-Biomarker Disease Activity (MDBA) score which reflects DAS and it is predictive for radiographic progression and risk of flare after drug reduction (Hirata S. Tanaka Y, 2016). RF has been reported to be the one of the most prominent RA-related autoantibodies (Falkenburg WJJ et al., 2017) and the high level of RF in the blood (RF+) is most often associated to tendency toward more severe RA (Firestein G et al., 2012). HLA-DRB1 rs6910071 variant has been studied for its strong association variant to RA disease (Verma A et al., 2016). Thus, separately, these three parameters have been proved to be associated to RA progression and disease activity, but, to our knowledge, no prior report

has linked them together, likely due to a complex interplay between serological factors, genetics and disease activity score variation.

The strength of our study is precisely the interaction and combination of multiple and different parameters, the use of detailed clinical and laboratory information and a moderately large number of participants. On the other side, limitations include the absence of stratification of DAS according to the subtypes of medication assumed and it may not reflect the physiologic processes at play in subjects with late-onset RA. Pharmacogenetics of established RA disease, especially for treatment most commonly use and with high risk-benefit profile, as Methotrexate, remain to be clearly depicted and it could be further analyzed.

Hence, although our results require validation and should be investigated further in other independent larger groups, the formulation of predicting algorithm for *Best responder* patients including serological and genetic parameters could play an additional role strengthening the early therapeutic choice. It might be the key in the development of particular profile, as envisioned in the era of precision medicine.

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CHAPTER 4

PHARMACOGENETICS AND TREATMENT RESPONSIVENESS IN RHEUMATOID ARTHRITIS PATIENTS

FROM DISEASE TO REMISSION/STABILIZATION

"It is easy to get a thousand prescriptions,
but hard to get a single one remedy"

Ancient Proverb

CHAPTER 4

PHARMACOGENETICS AND TREATMENT RESPONSIVENESS IN RHEUMATOID ARTHRITIS PATIENTS

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Table of Contents

Abstract

Background: Rheumatoid Arthritis (RA) is complex disease, with heterogeneous clinical aspects including mild forms up to erosive and severe phenotypes, associated to a large variability of treatment responsiveness and efficacy. New directed therapies, usually comprising Methotrexate (MTX) as first-line treatment, combined with biological Disease Modifying Anti-Rheumatic Drugs (bsDMARDs), have changed the RA manage with great impact for patients' outcome and the quality life assessment. However, a significant percentage of subjects do not adequately respond to treatment. Identified in advance biomarkers related to treatment efficacy should give to clinicians the necessary instrument to evaluate immediately the best therapeutic choice and to prompt remit or control the disease.

Objective: To investigate the potential role of three HLA-G gene variants and the combination of their haplotype and diplotypes, in order to determinate MTX Response in a cohort of RA Italian patients. Moreover, to explore a gender-medicine approach, focusing on female RA patients.

Materials and methods: 308 patients with diagnosis of RA were enrolled. From each subject clinical data along with female patients obstetric history and whole blood sample were collected. Genomic DNA was extracted and the three variants site in HLA-G gene: -725C>G>T (rs1233334), 14bp indel and +3142 G>C (rs1063320) were assessed by allelic discrimination. The study population was divided into 2 groups: MTX-Responders and MTX Non-Responders, according to the ACR/EULAR Response criteria after 6 months of therapy and association analyses were been performed.

Results: Significant differences were found between MTX-Responders and MTX Non-Responders in the alleles and genotypes for 14bp indel and in three-point haplotypes. A borderline trend was observed for +3142 G>C (rs1063320) variant and no association for -725C>G>T. Stratifying results by sex, MTX effect associated to 14bp indel was mostly amenable to female patients, whereas male trend of association was completely opposite. Investigating on women previous obstetric history, it came out the association between the ascertained pregnancies, HLA-G 14bp D/I genotype and MTX inefficacy. In fact, the association of those three parameters shown 3.36-fold higher risk to do not response to MTX treatment (95% CI 1.199-7.557) exhibiting a significant p-value (0.003). In addition, considering women with miscarriage event, results noticed a strong and significant association with MTX inefficacy and DI/DD genotype (OR=18.33 (95% CI 2.016-166.7) (p=0.009)).

Conclusion: Beyond HLA-G gene immunomodulatory involvement in the etiopathology of RA, results showed association with MTX Response. Data revealed a different response in RA patients according to genotype and gender. This suggests HLA-G variants as genetic markers of predictable MTX efficacy and to direct therapeutic strategies in advance, especially in women patients.

4.1 Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disease with autoimmune pathogenesis, characterized by a proliferative synovitis with progressive and destructive evolution. The disorder has a systemic feature, involving both joints and different organs and apparatus (Gonzalez-Gay et al., 2005). The main objective of clinical practice and RA patients management is the prevention of articular damage and disability, but the therapeutic choice is also directly related to the pharmaceutical and social costs saving. RA is a multifactorial disease, associated with both environmental and genetic factors: it is generally accepted that 50-60% of risk of developing that disorder is attributable to genetics, in particular many risk's alleles have been identified in HLA region (Human Leucocyte Antigen), such as HLA-DRB1 and HLA-G (Ding J et al., 2015). HLA-DRB1 represents the locus mainly associated in RA occurrence. 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). Main amino-acidic sequences SE predisposing to risk are ^{70}QRRAA^{74} and ^{70}QKRAA^{74} (Bang et al, 2010; Konda Mohan et al. 2014), whereas the presence of DEERA sequence is associated to protection (Carrier et al., 2009).

HLA-G belongs to non-classical class I molecules, its genetics variant is low and the expression in normally limited to specific organs or cutaneous appendix (Wisniewski A et al., 2015). Among its functions, HLA-G regulates the immune response in physiological (maternal-fetal interface) and pathological conditions, as well as various autoimmune diseases like RA and Systemic Lupus Erythematosus (SLE) (Baricordi OR et al., 2008). HLA-G gene has relevant polymorphic sites, related to several mechanisms of tolerance (Zambra FM et al., 2016) and they could be potentially involved in the biological feature of the gene (Donadi EA et al., 2011). More precisely, in HLA-G gene it is known, in the exon 8 at the 3'-untranslated region (UTR), a insertion(ins)/deletion(del) variant of 14 base pair sequence (rs371194629, +2960). The presence of 14bp insertion allele (5′-ATTTGTTCATGCCT-3′) is associated to an unstable mRNA and to reducing gene expression (Mariaselvam CM. et al., 2015). In addition, other variants in the same region appear to affect the HLA-G mRNA stability, suggesting a different effect and implication in autoimmune disorders (Yie Sm et al., 2006). HLA-G +3142 G>C (rs1063320) is located in the 3'-UTR of HLA-G gene, near HLA-G 14bp indel, in the seed region of target site for three microRNAs (miRNAs): miR-148a, miR-148b, and miR-152; this variant could be involved influencing their expression (Tan Z et al., 2007). HLA-G -725C>G>T (rs1233334) is associated with reduced expression of HLA-G, because of epigenetics aspect already described (Castelli EC et al., 12014).

Moreover, several studies shown association between HLA-G 14bp variant with autoantibody production in RA and juvenile idiopathic arthritis, and the influence in response to Methotrexate (MTX) in RA patients (Veit TD et al., 2008).

MTX is the *conventional* Disease-Modifying AntiRheumatic Drug (csDMARD) recognized as the anchor treatment for RA, used in monotherapy and combination therapy with other DMARDs (Kremer JM et al., 2016). csDMARD category includes MTX, Sulfasalazine, Leflunomide, Hydroxychloroquine, Cyclosporine, Gold, involved in the pathways of immunomodulation or immunosuppression. In lower levels, MTX is an anti-inflammatory and immunomodulatory agent, whereas higher doses lead to cytotoxicity and antineoplastic effect. The doses generally used for RA treatment are 7.5-25 mg/weekly depending on patient status and the assumption is oral. MTX plays a role as folic acid antagonist, necessary for purine and thymidine synthesis: more precisely the drug is a competitive and reversible inhibitor of several enzymes folate-dependent, as dihydrofolate reductase (DHFR), involved in 5-methyltetrahydrofolate (5-MTHF) synthesis, and Thymidylate synthase (TYMS), implicated in purine neo-synthesis (Chan et al., 2002). The inhibition stops the B cells proliferation and interferes with reparation and synthesis of DNA, influencing the immune response (Cronstein., 2005). Revu et al., (2013) reported the effect of MTX reducing the inflammation in synovial liquid and lowering mRNA and proteins production. Monitored MTX treatment is required, due to several side effects, as mucositis, bone marrow suppression and liver toxicity. Although it is well known the main pharmacological action of MTX to inhibit multiple enzymes involved in de novo biosynthesis of nucleotides, the mechanism of the anti-inflammatory effect in RA therapy remains incompletely understood (Kremer JM et al., 2016; Inoue K et al., 2014). In long-term therapy, approximately 10-30% of the patients fail to attain remission because of MTX inefficacy or due to development of adverse events (Muralidharan N et al., 2015). This might suggest a different ability to counteract inflammation between genotypes.

Nowadays, technological improvement developed a new drugs group, called *biological* Disease-Modifying AntiRheumatic Drug (bsDMARD), able to imitate endogenous molecules produced by the immune system (Marije et al., 2015). Those compounds are directed to specific targets, increasing the therapy efficacy and reducing side effects. The pathways involved are inhibition of several cytokines, as TNFα, IL-1 and IL-6 or against T and B lymphocytes, causing lower inflammation and pain reduction. Moreover, several studies have been reported the concomitant use of MTX increased the effectiveness of biological drugs (Daïen and Morel, 2014; Narváez et al., 2015). Therapeutic decision, firstly, is oriented to MTX assumption; the shift to bsDMARds or other combined strategy has to be evaluate in case of MTX inefficacy, side effect or toxicity events.

Furthermore, the decision of treatment choice is modulated accordingly to woman desire to undertake a pregnancy. Some drugs, including MTX, are teratogenic, could affect the correct development of fetus causing irreversible damages and problems, such as sexual function reduction, ovulation dysfunction, hormonal alteration and possible production of antibodies against spermatozoids. Italian Society of Rheumatology (SIR) suggests beginning a pregnancy when disease activity is low, due to the possibility to suspend treatment minimum 6 months before the conception. Around 70% of RA

patients observed symptoms remission during pregnancy, determined by hormonals maternal change, increasing of cortisol production and progestogens role. The outcome is the reduction of general inflammation and joints phlogosis, caused by the cytokines production shifted from T-helper type 1 (Th1) to T-helper type 2 (Th2) lymphocytes. However, within 3-4 months after childbirth, 90% of women observed a flare of disease and symptoms. During pregnancy, the Th2 humoral activation prevails respect Th1 cellular-mediated response (Ragupathy R et al., 2001). Th1 cells activation produces cytokines, such as IFN-γ, TNF-β, IL-2 e TNF-α and macrophages modulation, all implied in cellular immunity, in intracellular pathogens infections resistance, cytotoxic reactions and delay oversensitivity. On the other side, Th2 cells mediate IL-4, IL-5, IL-6, IL-10 and IL-13 secretion, involved in humoral immunity and linked to antibodies production. The ratio among different several cytokines seems to be relevant for the pregnancy success (Vince GS et., 1996).

In addition, studies reported the immunological effect of hormones during pregnancy and post-partum seem to play a role on epigenetic modulation involved in autoimmune elaboration, mostly in susceptible subjects. Hughes GC et al. (2014) observed the role of estrogens and progesterone in RA risk modulation and development. Estrogens are associated to higher RA risk in predisposed women through IFN pathway, T CD4+ lymphocytes differentiation and autoreactive B cell survival. On the other side, progesterone seems to counteract those effects. During pregnancy, Non-Inherited HLA-Antigens from the Mother (NIMA) appear to be involved in RA development risk and to interact with HLA-DRB1 SE. The mother immune system is strongly connected to the fetus, creating bidirectional flows of cells, antigens and antibodies, and the exposition to NIMA might determine a long life immunomodulatory effect. NIMA containing DEERA sequences exerts a protective effect on RA risk through the pathway of B and T cells tolerance induction and microchimerism. In addition, inherited paternal HLA alleles effect on RA risk is marginal respect NIMA: it has been reported subjects DEERA negative with mother DEERA positive shown lower RA risk compared subjects DEERA negative with father DEERA positive (Feitsma AL et al., 2007). Besides, NIMA containing SE sequences has been associated to higher RA risk (van der Horst-Bruinsma IE et al., 1998).

All these evidences suggested a possible link among several aspects of Rheumatoid Arthritis disease, as MTX treatment, HLA-G role and some characteristic related to female gender.

4.2 Rationale and Aim

Based on many reports and on the previous findings (Rizzo R et al., 2006; Baricordi OR et al., 2007) the main purpose of this study is to confirm the role of HLA-G 14bp ins/del in the response of MTX treatment in a larger number of RA Italian patients.

We hypothesized that also HLA-G rs1233334 and HLA-G rs1063320 could be involved in a pharmacogenetics function.

According to that, the objective is to investigate whether haplotypes and diplotypes of these variants are associated with efficacy of MTX drug in the Italian patients.

Besides, the second aim is to explore deeper the possible link between HLA-G role and specific characteristics of women, such as pregnancy and obstetric history, in order to assess some connections and to put forward the value of the gender medicine model.

4.3 Materials and Methods

Subjects

The cohort of patients included 308 Italian individuals 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). Patients were enrolled in the Rheumatology Unit of Sant'Anna University Hospital of Ferrara, with the collaboration of Prof. *M. Govoni*.

Among them, 74 were males and 234 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). Disease Activity was assessed by Disease Activity Score 28 criteria.

All patients were treated according to standard protocol with MTX. The response to therapy was evaluated according to EULAR response criteria after 6 months of therapy (Prevoo ML et al., 2015).

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 recruited. To each subject enrolled were been collected clinical data, along with obstetric history for female patients and whole blood sample. It has been assigned a code and sensible data were been 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 manufacturers' 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), were followed:

- HLA-G rs1233334 G>C
- HLA-G 14bp indel D>I
- HLA-G rs1063320 C>G

Using Genome Browser (http://genome.ucsc.edu/), has been obtained the nucleotide 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 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' (Wisniewski A et al., 2015) 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 224bp for Ins/Ins (I/I) and 210bp for Del/Del (D/D) and both 224bp and 210bp for Del/Ins (D/I) genotypes. The PCR products were visualized using silver staining.

Linkage disequilibrium analysis and haplotype construction

Linkage disequilibrium (LD) analysis was performed for the three-polymorphic site of HLA-G gene on chromosome 6. Haplotype blocks were constructed using Haploview software v4.2

The haplotype predictions revealed the block formed by HLA-G SNPs with a high LD (Figure 4.1). (https://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview).

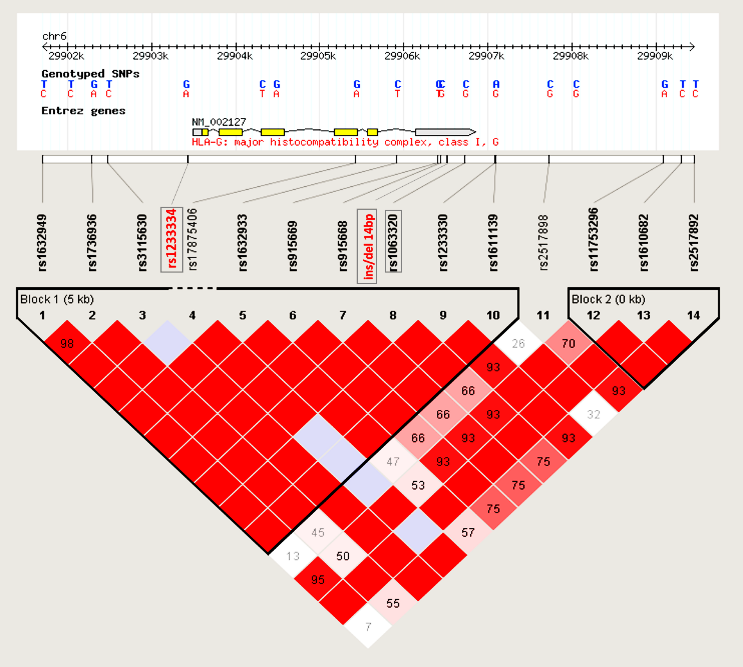

Figure 4.1
Linkage Disequilibrium (LD) of human leucocyte antigen (HLA)-G variants. Color scheme of the LD map is based on the standard D'/LOD (logarithm of odds) option in the Haploview software. Dark squares indicate high r2, and bright squares indicate low r2 values. Values in squares are D' between single markers.

Statistical Analyses

For each SNP, in each subjects' cohort, 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.

The analysis between RA patients Responders and non-Responders to MTX treatment was calculated for each genotype by Odds Ratio (OR) with 95% Confidence Interval (CI). A p value < 0.05 was considered significant. For statistical analysis, odd ratio (ORs), 95% Confidence Intervals (95% CI) and p value was used using MS™Excel and GraphPad packages.

When required, Bonferroni correction has been performed as well as the comparison between two ORs (ex: in sex stratification analyses) with the correspondent p-value of the comparison.

LD was tested for three SNPs (Haploview software version 4.2) and given the LD between alleles from analyzed variant, but unknown genetic phase, the software PHASE version 2.1 (Stephens M et al., 2003) was used to estimate the most likely haplotypes pair for each sample, haplotypes frequencies and to assign the two most probable diplotypes.

Graphs have been carried out using the software Origin Pro version 8.

4.4 Results

A total of 308 patients, 234 females and 74 males, were enrolled in the study. The baseline characteristics of the patients included in the study are summarized in Table 4.1. Young onset RA when onset was ≤60 years and late onset RA when onset was >60 years of age (Mueller RB et al., 2014). MTX efficacy was evaluated according to EULAR response criteria after 6 months of therapy (Prevoo ML et al., 2015).

Characteristics		RA (n=308)
Demographic		
	Female : Male	3.16:1
	Age (years) (mean ± SD)	58.89 ± 13.60
Clinical		
	Young onset RA	54.22%
	Late onset RA	45.77%
MTX-Treatment Response		
	Responders	55.84%
	Non-Responders	44.16%

Table 4.1 *Characterization of RA Italian subjects studied.*

Subsequently, it has been evaluated if the responsiveness to MTX treatment should be linked to the sex, independently to the genotype. The patients' cohort has been sex stratified (Table 4.2).

A

Cohort	Responders to MTX	Non Responders to MTX
Male	49	25
Female	123	111
tot	172	136

B

OR	95% CI	p-value
1.769	1.024-3.052	0.040

Table 4.2 *Sex and MTX responsiveness.* ***A.*** *RA patients' stratification;* ***B.*** *Association study*

In male group, 66.21% were MTX Responders, whereas female responders were 52.5%. OR association study revealed female subjects with 1.77-fold higher probability to be not responders respect to male group, reaching the statistical significant value (p value= 0.040). The result suggested the sex influence on MTX response and genetic variants might be determinants to evaluate MTX efficacy sex-genotype dependent.

The three HLA-G gene variants -725C>G>T (rs1233334), 14bp indel, +3142G>C (rs1063320), were characterized in patients, subdivided in two groups according to the response to MTX treatment. The distribution and the frequency of genotypes and alleles in the two groups RA patients was showed in Table 4.3. The order of the three variants follows the sequence in the human genome.

Variant	RA patients Responder to MTX		RA patients non-Responder to MTX	
	N	%	*N*	%
HLA-G rs1233334				
GG	133	77.3	105	77.2
GC	39	22.7	31	22.8
CC	0	0	0	0
G	305	88.7	241	88.6
C	39	11.3	31	11.4
HLA-G 14 bp (rs66554220)				
DD	68	39.5	40	29.4
DI	67	39.0	70	51.5
II	37	21.5	26	19.2
D	203	59.0	150	55.1
I	141	41.0	122	44.9
HLA-G rs1063320				
CC	44	25.6	24	17.6
CG	79	45.9	73	53.7
GG	49	28.5	39	28.7
C	167	48.5	121	44.5
G	177	51.5	151	55.5

Table 4.3 *Distribution of the HLA-G variants. rs1233334, 14bp and rs1063320 genotypes and allelic frequencies in RA Italian patients, responder and non-Responder to MTX treatment*

HLA-G rs1233334 variant shown genotype and alleles frequencies following the same pattern in both groups. G or C carriers noticed the equal frequencies, respectively 88.7% vs 88.6% and 11.3% vs 11.4% comparing MTX responders and MTX non-Responders.

Regarding the HLA-G 14bp variant, the distribution was different between the aforementioned cohorts, especially for DD homozygous and DI heterozygotes. They were respectively 39.5% vs 29.4% and 39.0% vs 51.5% in responders vs non-responders subsets.

In MTX responder group, Chi-square test χ^2 and Hardy-Weinberg equilibrium reported significant results, respectively χ^2= 6.53 and p value= 0.038, suggesting an unbalance distribution.

Lastly, the HLA-G rs1063320 variant noticed diverse frequencies concerning CC homozygotes, reporting 25.6% vs 17.6% between groups. Hardy-Weinberg results were in equilibrium either with p-value respectively 0.599 and 0.572.

4.4.1 Evaluation of Methotrexate Response

Haplotypes-Diplotypes Analyses

Analyses were performed comparing HLA-G variants between Responders and Non-Responders patients and besides examining the individual variants of the HLA-G gene, the combined haplotype/genotype and diplotype/genotype have been analyzed, in order to individuate the merged influence on the effect of MTX Response.

A total of eight haplotype structures were observed and designated Hap A-H (Table 4.4), the variants have been reported in the same order in the genome.

Haplotype	SNP ID			Number (%) of haplotypes in RA patients	
	HLA-G rs1233334	HLA-G 14 bp	HLA-G rs1063320	Responder to MTX	non-Responder to MTX
Hap A	G	D	C	**126 (36.6)**	**88 (32.4)**
Hap B	C	D	C	35 (10.2)	30 (11.0)
Hap C	G	D	G	40 (11.6)	31 (11.4)
Hap D	C	D	G	2 (0.60)	1 (0.40)
Hap E	G	I	C	5 (1.50)	3 (1.10)
Hap F	G	I	G	**134 (39.0)**	**119 (43.8)**
Hap G	C	I	G	1 (0.30)	0 (0.00)
Hap H	C	I	C	1 (0.30)	0 (0.00)
Total patients number				172	136

Table 4.4 *Haplotypes references of HLA-G 14bp, HLA-G rs1063320, HLA-G rs1233334 in RA Italian patients*

The major haplotypes identified were 4: Hap A (GDC), Hap B (CDC), Hap C (GDG), Hap F (GIG). Among them, Hap A (GDC) and Hap F (GIG) were found to be almost exclusively associated with G allele of HLA-G rs1233334 variant, with a frequency respectively of 36.6% vs 88% and 39% vs 43.8% in the MTX responders group and MTX non-responder cohort. The other two more frequent, Hap B (CDC) and Hap C (GDG) were associated with D allele of HLA-G 14bp variant. Both Hap B and Hap C shown a similar distribution between the two groups: 10.2% vs 11.0% and 11.6%vs 11.4% respectively. Considering the others 4 haplotypes, the frequency among patients was very low and they failed to reveal any interesting results. With regard to genotype of HLA-G variants, referred as diplotype, 36 diplotypes composed of 8 haplotypes were identified. However, results showed 12 diplotypes constituted by 6 haplotypes, because the 24 rare diplotypes were excluded (Table 4.5).

Diplotype	Number (%) of diplotypes in RA patients		Diplotype comparison*		
	responder to MTX	non-responder to MTX	*OR*[a]	95% CI[b]	P value
Hap A/A	25 (14.5)	10 (7.35)	**2.143**	**0.986-4.657**	**0.054**
Hap A/B	15 (8.72)	13 (9.56)	0.904	0.413-1.979	0.812
Hap A/C	16 (9.30)	11 (8.09)	1.166	0.520-2.611	0.723
Hap A/E	3 (1.74)	1 (0.74)	2.396	0.246-23.30	0.460
Hap A/F	41 (23.8)	43 (31.6)	0.677	0.391-1.173	0.165
Hap B/C	8 (4.65)	4 (2.94)	1.610	0.474-5.468	0.454
Hap B/F	12 (6.98)	13 (9.56)	0.710	0.312-1.615	0.421
Hap C/C	3 (1.74)	1 (0.74)	2.396	0.246-23.30	0.460
Hap C/D	1 (0.58)	1 (0.74)	0.789	0.049-12.74	0.877
Hap C/F	9 (5.23)	13 (9.56)	0.522	0.216-1.265	0.151
Hap E/F	2 (1.16)	2 (1.47)	0.788	0.110-5.670	0.825
Hap F/F	34 (19.8)	24 (17.65)	1.150	0.634-2.084	0.659
Total patients number	172	136			

[a] Odds Ratio
[b] Confidence Interval 95%
* Each diplotype was compared with others diplotypes

Table 4.5 *The distributions and association of HLA-G diplotypes in RA Italian patients, responder and non-responder to MTX treatment*

The 12 diplotypes were: Hap A/A (GDC/GDC), Hap A/B (GDC/CDC), Hap A/C (GDC/GDG), Hap A/E (GDC/GIC), Hap A/F (GDC/GIG), Hap B/C (CDC/GDG), Hap B/F (CDC/GIG), Hap C/C (GDG/GDG), Hap C/D (GDG/CDG), Hap C/F (GDG/GIG), Hap E/F (GIC/GIG), Hap F/F (GIG/GIG). Overall, more frequents diplotypes in MTX-responders and MTX non-responder groups were next, in decreasing order: A/F (23.8% vs 31.6%), F/F (19.8% vs 17.65%), A/A (14.5% vs 7.35%), A/B (8.72% vs 9.56%), A/C (9.30% vs 8.09%) and lastly, B/F (6.98% vs 9.56%). The frequencies distribution followed the same pattern in both cohorts. Comparing each diplotype with all others diplotypes, the major result regarded Hap A/A (GDC/GDC). It revealed lower frequency in MTX Non-Responder than Responder group (OR=0.467, 95% CI (0.215-1.014), p value=0.054), noticed association trend with treatment efficacy. In other words, taking Hap A/A as a reference, patients showing one of the others diplotypes, presented more than 2-fold higher probability to MTX inefficacy (OR=2.143, 95% (CI 0.986-4.657)).

Single Variant Analyses

The exploration of the haplotypes and diplotypes of HLA-G variants, individuated the combined effects on MTX response and noticed the main contributor among the three HLA-G variants analyzed. However, the single SNP association value could explain deeper the HLA-G gene pharmacogenetics role. Odd Ratios analyses have been performed comparing HLA-G variants genotypes between Responders and Non-Responders patients, considering the codominant genetic model (Table 4.6).

Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G rs1233334				
	GC/GG	1.007	0.598-1.722	0.982
	CC/GG	-	-	-
HLA-G 14 bp (rs66554220)				
	DI/DD	**1.776**	**1.062-2.971**	**0.028**
	II/DD	1.195	0.630-2.256	0.597
HLA-G rs1063320				
	CG/CC	1.694	0.939-3.057	0.080
	GG/CC	1.459	0.793-2.799	0.243

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

Table 4.6 *Occurrence results of MTX treatment (responder and non-responder) in the totality of RA Italian patients*

HLA-G 14bp indel revealed that DI genotype, taking DD as a reference, was significantly more frequent in MTX non-responder group compared to MTX responder cohort (51.5% vs 38.9% OR= 1.776, 95% CI (1.062-2.791), p value=0.028); it followed the dominant genetic model (I-DD OR= 1.569, 95% CI (0.972-2.534)). HLA-G rs1063320 showed a trend of higher frequency of CG genotype in non-responder cluster, linked to MTX inefficacy (53.7% vs 45.9% OR= 1.694, 95% (CI 0.939-3.057)), but it did not reach the statistical significance (p value=0.080). The variant followed

the dominant genetic model (G-CC OR= 1.604, 95% CI (0.918-2.804)). Lastly, HLA-G rs1233334 variant failed to reveal any association with afore-mentioned MTX Response categories and MAF of C determined the inability to define the genetic model.

Furthermore, maintaining the subdivision based on MTX Response, subjects have been stratified according to the sex (Table 4.7).

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 rs1233334								
	GC/GG	1.303	0.455-3.737	0.635	0.975	0.519-1.832	0.943	-
	CC/GG	-	-	-	-	-	-	-
HLA-G 14 bp (rs66554220)								
	DI/DD	0.534	0.178-1.595	0.264	**2.534**	**1.369-4.598**	**0.002**	**0.014**
	II/DD	0.620	0.148-2.303	0.505	1.467	0.722-3.069	0.295	-
HLA-G rs1063320								
	CG/CC	0.683	0.205-2.277	0.564	**2.214**	**1.116-4.394**	**0.023**	0.096
	GG/CC	0.625	0.159-2.321	0.502	1.939	0.980-4.141	0.071	-

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

Table 4.7 *Occurrence results of MTX treatment (responder and non-responder) sexr-related in RA Italian patients.*

Results underlined a complete opposite association between sex cohorts. Male patients did not reveal any difference of distribution and occurrence into the two groups, reporting not significant p-values; on the other side, female patients association analyses showed remarkable results. Following the trend in the totality of subjects, HLA-G 14bp indel revealed higher frequency of DI genotype in MTX non-responders group (OR=2.534, 95% CI (1.396-4.598), p value=0.002), the dominant genetic model confirmed the result (I-/DD OR=2.141, 95% CI (1.231-3.725), p value=0.007).

A significant association has been also noted for HLA-G rs1063320: taking CC genotype as the reference, in female patients the CG genotype was higher in non-responders than responders subjects (OR=2.214, 95% CI (1.116-4.394), p value=0.023). The genetic dominant model was followed (G-/CC OR= 2.112, 95% CI 1.102-4.048, p value=0.024). As expected, no occurrence was found in HLA-1233334 variant in female patients.

To evaluate if the sex difference was statistically significant, the sex-compared p value has been calculated. As reported in Table 4.7, for HLA-G 14 bp variant, the difference between sex was statistically significant: taking DD genotype as the reference, DI heterozygous patients showed p value=0.014. Considering the dominant genetic model, the compared p-value was 0.021. On the other side, the difference between sex for HLA-G rs1063320 was not statistically significant, with p value=0.096 and p value=0.077 respectively for heterozygous CG and carrier the variant C.

To summarize, the higher MTX inefficacy in female patients has been evaluated significant different respect to male subject only for HLA 14bp variant, for D/I heterozygotes.

4.4.2 Evaluation of Methotrexate Toxicity

Each treatment has intrinsically side effects and the variable risk/benefit ratio should be linked with a particular subjects' characteristic, independently from genotypes.
Thus, the presence or not of toxicity events after MTX therapy (6 months evaluation) has been evaluated according to the patients' sex, following the results obtained for MTX response (Table 4.8).

A

Cohort	MTX Toxicity	MTX Not Toxicity
Male	11	63
Female	51	183
tot	62	246

B

OR	95% CI	p-value
1.596	0.783-3.252	0.199

Table 4.8 *Sex and MTX responsiveness.* **A.** RA patients stratification; **B.** Association study

In male group, around 15% have shown toxic effect, whereas female 21.8%.
Association study revealed female subjects with a trend of 1.60-fold higher probability to present toxic events or side effects respect to male patients, the p value was not significant.

Thereafter, Methotrexate therapy has been analyzed in terms of toxicity accordingly to the three HLA-G variants genotypes. Association analysis have been performed, firstly, in the total cohort of patients (Table 4.9), secondly stratifying according to the sex (Table 4.10).

Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G rs1233334				
	GC/GG	0.596	0.285-1.245	0.170
	CC/GG	-	-	-
HLA-G 14 bp (rs66554220)				
	DI/DD	0.992	0.536-1.839	0.982
	II/DD	0.697	0.276-1.580	0.426
HLA-G rs1063320				
	CG/CC	1.448	0.700-2.996	0.323
	GG/CC	0.883	0.386-2.057	0.783

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

Table 4.9 *Occurrence results of MTX toxicity in the totality of RA Italian patients.*

Results did not show any particular influences or associations with the three HLA-G variants analyzed. All ORs data were close to 1 value, meaning the absence of strong interaction between adverse events and genotypes. P values were not significant.

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 rs1233334								
	GC/GG	0.938	0.223-3.938	0.936	0.534	0.224-1.273	0.158	-
	CC/GG	-	-	-	-	-	-	-
HLA-G 14 bp (rs66554220)								
	DI/DD	0.600	0.143-2.511	0.494	1.111	0.558-2.214	0.777	-
	II/DD	0.600	0.077-3.536	0.613	0.729	0.258-1.836	0.539	-
HLA-G rs1063320								
	CG/CC	1.607	0.288-8.965	0.601	1.398	0.624-3.129	0.423	-
	GG/CC	1.125	0.195-7.600	0.908	0.835	0.326-2.155	0.722	-

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

Table 4.10 *Occurrence results of MTX toxicity sex-related in RA Italian patients.*

Considering the sex stratification, male and female results did not reveal significant opposite association data. HLA-G 14bp variant individuated similar values for each genotype: taking DD genotype as the reference, only DI heterozygotes showed OR higher respect all others (OR=1.111, 95% CI 0.558-2.214), but not statistically significant (p value=0.777). Regarding HLA-G rs1063320 variant, the comparison between se and genotype did not identify any significant association. Taking CC genotype as the reference, male and female CG heterozygous followed the same pattern, whereas GG homozygous behavior seemed a little different between groups, but this observation was not supported by significant p value. HLA-G rs1233334 variant failed to reveal any association with MTX Toxicity and MAF of C determined the inability to define the genetic model.

4.4.3 Obstetric History Analyses in female Rheumatoid Arthritis cohort

Previous results reported significant association between HLA-G 14bp variant and MTX response only in female RA patients, showing higher MTX inefficacy in heterozygotes women (DI/DD OR=2.534, 95% CI 1.396-4.598, p value=0.023)). It became evident the possibility of the interaction with specific sex parameters. More precisely, when available, the previous obstetric history of female RA patients has been analyzed, stratifying the group in women who had or had not ascertained pregnancies (Table 4.11).

	Pregnancies	
Cohort	**Yes**	**No**
Females	137	12

Table 4.11 *Pregnancies in RA Italian female case-series.*

Subsequently, occurrence analysis has been performed considering the effect of pregnancies on MTX treatment efficacy dependent on HLA-G 14bp genotype of female RA patients (Table 4.12).

Variant	Codominant Genetic Model	YES PREGNANCIES Test of association			NO PREGNANCIES Test of association			compared P value
		OR [a]	*95% CI* [b]	*P value*	*OR* [a]	*95% CI* [b]	*P value*	
HLA-G 14 bp (rs66554220)								
	DI/DD	**3.366**	**1.199-7.557**	**0.003**	3.000	0.188-47.96	0.448	0.937
	II/DD	1.554	0.600-4.154	0.379	n/a	n/a	n/a	-

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

Table 4.12 *Occurrence results of MTX treatment responsiveness according to obstetric history of female RA patients.*

Taking DD genotype as a reference, HLA-G 14bp indel results revealed that heterozygous women DI was associated to MTX inefficacy. Deeper, RA female who had pregnancies showed 3.36-fold higher probability to not response (95% CI 1.199-7.557) exhibiting a significant p value (0.003). The variant followed the dominant genetic model (I-DD OR= 2.641, 95% CI 1.252-5.573) and the p value was significant (0.011). The group of "No Pregnancies" women noticed the same trend of inefficacy for the heterozygous genotype, but it did not reach the statistical significance (OR= 3.000, 95% CI (0.188-47.96) p value=0.448).

Therefore, the female RA patients who had previous pregnancies have been stratified considering the presence or absence of spontaneous miscarriage events during their obstetric history.
Association analysis has been carried out showing the outcome of abortion on MTX treatment efficacy depending on HLA-G 14bp genotype (Table 4.13).

Variant	Codominant Genetic Model	YES ABORTIONS Test of association			NO ABORTIONS Test of association			compared P value
		OR [a]	*95% CI* [b]	*P value*	*OR* [a]	*95% CI* [b]	*P value*	
HLA-G 14bp (rs66554220)								
	DI/DD	**18.33**	**2.016-166.7**	**0.009**	1.800	0.693-4.674	0.229	0.058
	II/DD	**18.75**	**4.481-209.6**	**0.002**	0.700	0.197-2.329	0.058	**0.017**

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

Table 4.13 *Occurrence results of MTX treatment responsiveness according to abortion events of female RA patient.*

Considering the codominant model, both DI heterozygous and II homozygous patients shown significant association between MTX inefficacy and presence of previous spontaneous abortion events, respect their absence. Women with previous abortion event noticed more than 18-fold higher risk to MTX inefficacy, respect the other group. The dominant genetic model I-/DD reported significant OR= 18.46 (95% CI 2.145-158.9) and the p value was significant (0.008). The compared p value between the same genotype into the two groups were statistically significant for I/I homozygous (p value= 0.017), and for the dominant genetic model I-/DD (p value= 0.028), suggesting the complete opposite direction between the afore-mentioned cohorts.
Figure 4.2 below displayed graphically and summarized the obtained results.

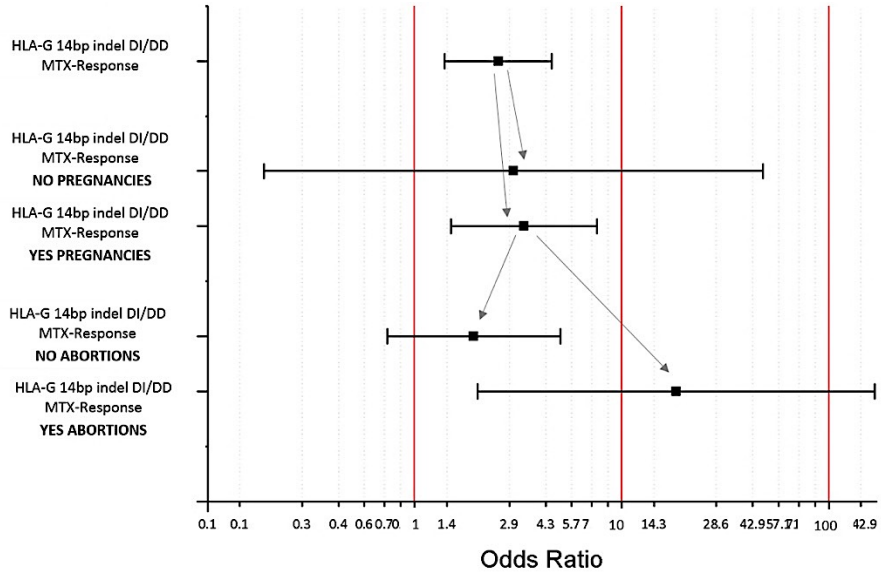

Figure 4.2 *Summary of RA female stratification and OR results.*

To crosscheck results and to underline the additional value of pregnancy and spontaneous miscarriage events, the obstetric history effect on MTX responsiveness has been evaluated independently from HLA-G 14bp genotypes (Table 4.14).

A

Cohort	Responders to MTX	Non-Responders to MTX
Yes Pregnancies	67	64
No Pregnancies	5	6
tot	71	70

B

OR	95% CI	p-value
0.796	0.231-2.738	0.730

Table 4.14 *Pregnancies and MTX responsiveness.* ***A.*** *RA patients stratification;* ***B.*** *Association study*

As depicted, the subjects size among groups of MTX responders and MTX non-responders according to previous pregnancies was superimposable, with around 50/50 percentage of distribution in all cohorts. The result suggested the irrelevance of pregnancies events on MTX efficacy in female RA patients. On the other side, occurrence analysis of pregnancy and HLA-G 14bp genotypes it has been performed without considering MTX efficacy (Table 4.15).

Variant	Codominant Genetic Model	Test of association		
		OR [a]	*95% CI* [b]	*P value*
HLA-G 14 bp (rs66554220)				
	DI/DD	0.889	0.237-3.337	0.871
	II/DD	2.311	0.522-21.79	0.386

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

Table 4.15 *Occurrence of pregnancy effect (yes pregnancies/no pregnancies) according to HLA-G 14bp genotypes.*

Results shown absence of interaction between HLA-G 14bp genotype and obstetric history regarding previous ascertained pregnancies. ORs were close to 1 value or the 95% CI were larger, indicating the complete insignificant effect between the variant and pregnancy presence.

4.5 Discussion and Conclusion

The present study originates from the knowledge of the crucial role of methotrexate (MTX) in the treatment of rheumatoid arthritis (RA). Despite the development of biologic-era (bsDMARds), MTX (csDMARD) persists as the key drug for both first-line choice therapy in newly diagnosed RA and as a combination therapy with others csDMARds or bsDMARDs (Becciolini A et al., 2016). Next to MTX long-term effectiveness and its widespread use in clinical practice, its side effects should be taken into account: find predictors for MTX efficacy and candidate of risk to develop adverse events is remarkable and should direct treatment choices in advance. The anti-rheumatic effect of MTX is ascribed to several pathways as inhibition of purine and pyrimidine synthesis and metabolism, inhibition of T cell proliferation and alterations of inflammatory mechanisms (Cronstein BN, 2005). Moreover, it has been demonstrated the MTX effect on production of soluble HLA-G (sHLA-G) isoforms (Rizzo R et al., 2006). In RA, HLA-G gene is involved in the immune response through its immunomodulatory and tolerogenic functions (Brenol CV et al., 2012).

In this view, the genetic profile of HLA-G gene variant in the 5'-UTR -725C>G>T (rs1233334) and two variants in 3'-UTR 14bp indel, +3142G>C (rs1063320) were analyzed in a cohort of Italian RA patients. The -725C>G>T variant in the promoter of the HLA-G gene is involved in epigenetic mechanism, where substitution of C to G in this position creates a CpG island at nucleotides -726 and -725: HLA-G transcription is inhibited by DNA methylation (Wisniewski A et a., 2010; Moreau P et al., 2003). In the 3'-UTR, 14bp indel affect mRNA stability (Rousseau Pet al., 2003) and the +3142G>C variant influences the affinity of HLA-G mRNA targeted by micro RNA, more precisely the +3142G allele downregulating the expression of HLA-G (Veit TD et al., 2009). In addition, considering the strong linkage disequilibrium (LD) of these variants, their combination would predict individual subjects' efficacy to MTX treatment.

The Italian RA cohort assessed in this study was divided into two subgroups according to MTX response, this stratification revealed around half of patients were MTX Non-Responders, corroborating the evidence of MTX efficacy lack (Muralidharan N et al., 2015). In the totality of RA patients assessed in this study, strong LD between HLA-G variants was observed (Figure 4.1). Three-point (−725C>G>T, 14bp indel, +3142G>C) haplotype frequency estimation revealed a prevalence of four different haplotypes, out of eight Hap A-H. Hap A (GDC) and Hap F (GIG) were associated with G allele of HLA-G −725C>G>T variant, whereas the other two more frequent haplotypes Hap B (CDC) and Hap C (GDG) showed both the presence of D allele of HLA-G 14bp variant. At first sight, the −725C>G>T variant seems to determine the character of the haplotype for the first two, although for the other two more frequent haplotypes, HLA-G 14bp variant is involved.

Regarding genotype as diplotype, six diplotypes were more frequent, in decreasing order: A/F, F/F, A/A, A/B, A/C and B/F. Among them, the comparison between each diplotype vs all others, showed Hap A/A (GDC/GDC) associated with almost significant result of MXT efficacy (OR=0.467, 95%

CI 0.215-1.014, p value=0.054). On the other side, Hap A/F (GDC/GIG) and Hap C/F (GDG/GIG) revealed higher distribution in MTX Non-Responder than Responder subjects, indicating a trend of MTX-inefficacy: respectively, OR=0.677, 95% CI (0.391-1.173), p value=0.165; OR=0.522, 95% CI (0.216-1.265), p value=0.151. This might indicate that the second position of diplotype, occupied by HLA-G 14bp variant, is involved and should interfere with the MTX efficacy. In fact, the presence of DD homozygosis (Hap A/A) suggests to be "efficacy-related", whereas DI heterozygosis (Hap A/F and Hap C/F) is associated to higher inefficacy trend of MTX treatment. The first position of haplotypes and diplotypes suggests that HLA-G rs1233334 variant does not influence MTX response, −725G is present in all three combination, probably because of low MAF C. The haplotype and diplotype studies suggest that the specified constellation of HLA-G variants seems to influence the MTX response; hence, the association analysis for each single SNP has been performed.

HLA-G 14bp results showed statistically significant difference either in genotypes and alleles distribution between Responders and Non-Responders groups: DI heterozygosis is significant associated with MTX inefficacy (OR=1.776, 95% CI 1.062-2.791, p value=0.028). Our data are consistent with other previous reports in RA pharmacogenetics (Rizzo R et al., 2006; Baricordi OR et al., 2007). Rizzo et al. reported that patients responsive to MTX were more likely to have 14bp DD genotype compared to non-responders and attributed it to the ability of this genotype to produce more sHLA-G in the presence of IL-10. This evidence confirms results previously exposed in this dissertation concerned diplotypes.

Additional analyses were performed considering the frequency of RA disease in women population, where the female-to-male ratio is 3/4:1 (Alamanos Y et al., 2005). Our results reported that HLA-G pharmacogenetics effect, described in the totality of patients, is mostly related to females, whereas male data showed opposite trend. In fact, HLA-G 14bp variant in women revealed D/I heterozygous significant associated with MTX inefficacy (DI/DD OR=2.534, 95% CI (1.369-4.598), p value=0.002). The dominant genetic model I-/DD reported statistically significant association either (OR=2.141, 95% CI (1.231-3.725), p value=0.007)). Regarding HLA-G +3142G>C variant, our data showed association in female patients only. Even though in all RA patients results did not show significant association, stratifying by sex indicate that this variant could be involved in the MTX Response. Both, C/G heterozygosis and the dominant genetic model G-/CC were significant associated with MTX inefficacy in female RA subjects (CG/CC OR=2.214, 95% CI (1.116-4.394), p value=0.023; G-/CC OR=2.112, 95% CI (1.102-4.048), p value=0.024).

However, the sex-compared p value was significant only for HLA-G 14bp variant (0.014).

These findings are interesting evidences on sex-dependent effect to MTX treatment. The lack of effective response in women should be connect to some specific characteristics of females, such as hormones changes in physiological life phases and especially during pregnancy. Several studies reported that HLA-G is selective expressed at the fetus-maternal interface (Kovats S et al., 1990) and

play a role in the immunological interaction between mother and child inhibiting mother's cytotoxic T-cell response (Parham P., 1996), moreover HLA-G is implicated in maternal immune system suppression preventing NK cells attack against fetus.

In this view, analysis have been performed considering obstetric history of female RA patients and in particular investigating on previous pregnancies and miscarriage events. Results showed 3.36-fold higher significant probability of MTX inefficacy in women with previous pregnancies and presenting D/I genotype, respect to D/D (95% CI 1.199-7.557 (p-value=0.003)). This evidence suggested a particular role of pregnancy in treatment response, linked to the ability to counteract the inflammation and immune system reaction. Furthermore, the RA women case-series has been stratified focusing on previous spontaneous miscarriage events. Results showed a complete different association direction: female RA with abortion case noticed important and remarkable higher risk to not respond to MTX treatment respect to women without previous abortions. Taking D/D as the reference, women carried the allelic variant I (D/I or I/I genotype) showed more than 18-fold up significant higher risk to MTX inefficacy, without allelic dose dependency (codominant model: DI/DD p value= 0.009; II/DD p value= 0.002)). The results should be explained trough the consideration that pregnancy is the more common source of microchimerism, in which fetal cells are able to flow into maternal bloodstream. Fetal cells, having semi allogenic nature, might be recognized as external factors, unleashing the maternal immune response. Effectively, this suggestion has been supported by the evidence that in RA women it has been found the presence of microchimerism (Østensen M et al., 2011). HLA-G role should be modulated by the fetal cells existence, especially if they are localized in synovial membrane; the effect of this alteration could influence the RA development and the treatment responsiveness. The replication in independent and larger case-series could confirm the hypothesis and could explain the major incidence of RA in female population. Moreover, the evidence of the relation among HLA-G 14bp genotype, MTX response and previous abortion events suggested that miscarriage could be referred to a particular subgroup of RA female patients, with a specific etiology and a different MTX pharmacogenetics. Further analysis should be performed comparing RA cohort with a matched group of healthy subjects, in order to evaluate eventual occurrence risk dependent only on miscarriage.

In conclusion, results of the present study on genetic analyses confirmed the importance of precision medicine in RA patients, underlying the capability to predict in advance the methotrexate efficacy. HLA-G variants and haplotypes findings put forward a potential role of immunomodulation and autoimmune context. HLA-G variants and in particularly HLA-G 14bp indel should be considered as genetic biomarkers of response-to-treatment in a specific group of patients, suggesting the new frontier of sex-pharmacogenetics.

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CHAPTER 5

THE GENDER-MEDICINE VIEW

AUTOIMMUNITY, PREGNANCY, MICROCHIMERISM

"Be like the stem cell,
differentiate yourself from others"

Ancient Proverb

CHAPTER 5

THE GENDER-MEDICINE VIEW:

AUTOIMMUNITY, PREGNANCY, MICROCHIMERISM

Table of Contents

Abstract

Background: Women are more susceptible to develop several autoimmune diseases, as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE). This evidence suggests the particular interaction between genetic profile and risk factors specific for female gender, as pregnancy and previous miscarriage episodes. Ascertained pregnancies may be considered as vehicles through acquiring circulating fetal cell in mother's bloodstream, this microchimeric condition could determine allogenic input or their own could be an allogenic target, even many years after pregnancies. Nowadays, the role of fetal microchimeric cells (fMC) in the etiology and pathogenesis of RA and SLE is not fully understood.

Objective: To investigate the male fMC presence in the peripheral blood of female patients affected by RA or SLE disorders, correlating it with causative role in etiopathology. Secondly, the purpose was to assess the association between previous women obstetric history and the positivity to blood fMC detection.

Materials and methods: Y-Chr detection has been carried out on each subject enrolled in the study. Cases included 238 women affected by RA and 146 women affected by SLE, along with blood sample. In a subgroup of both cohorts, previous obstetric history details have been collected. The corresponding matched control group comprised 104 healthy female subjects. A total of 175 women whom have experienced spontaneous or voluntary miscarriage have been considered as a comparable group to evaluate the effect of abortion independently of autoimmune disease. Occurrence analysis and association with previous pregnancies and miscarriage events have been performed.

Results: The presence of male fMC in SLE patients bloodstream was 3.483-fold significant higher than healthy female group (95% CI 1.833-6.618, p value=0.00016). Stratifying by previous pregnancies, twin gestation shown a trend of 5-fold higher probability to detect Y-Chr positivity, without reaching the significant value. Considering previous miscarriage events, induced episodes shown a positive trend of male fMC presence (OR=6.333 95% CI 0.848-47.311, p value=0.072) respect spontaneous abortions. Concerning RA cohort, the affected female subjects did not reveal significant association compared with healthy controls and even stratifying according to pregnancies and miscarriages.

Conclusion: Significant positive finding of male fMC in bloodstream of SLE female subjects is dependent on autoimmune disorder, showing trend of association with elective pregnancy loss. The widespread symptoms of SLE could account for the higher presence of fMC than which has detected in RA patients' bloodstream. Investigation on localized RA affected synovium might be crucial to understating fMC role.

5.1. Introduction

Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are long-term diseases, resulted from immune system mistakenly attacking the own body. Autoimmune pathologies often share common clinical and pathological features such as chronic inflammation, innate immune response activation, and development of specific autoantibodies (Shah NR et al., 2017). In particular, RA and SLE conditions are characterized by inflammatory status affecting joints, but RA usually, firstly occurs in specific localization, whereas lupus symptoms can vary widely from subject to subject and more than joints, it can affect many other part of the body, such as skin manifestations, renal alteration and gastrointestinal involvement. In fact, lupus has been called "the great imitator", because it could seem like RA, but not just (Kurien BT, Scofield RH, 2006). Furthermore, both diseases are much more common in women, who are 3-4 times more likely than men to be affected by RA and they also are up to 9 times more likely to get lupus.

RA is distinguished especially for chronic synovium inflammation characterized by interactions of Fibroblast-Like Sinoviocytes (FLS) cells with cells of the innate (macrophages, dendritic cells, mast cells and NK) and adaptive immune system (B and T lymphocytes). These mechanisms lead to the destruction of cartilage and bone and they are associated to the skeletal remodeling, which includes four pathological phases; all of them show inflamed synovial tissue attached to the bone surface to form a covering called pannus (Liu D et al., 2017). Etiopathology factors are still not fully understood, but it has been postulated that a high-risk genetic background, in combination with epigenetic treats and environmental exposure, leads to a cascade of events inducing synovitis and consequent severity and destructive arthritis (Angelotti F et al., 2017).

SLE is an autoimmune and systemic chronic pathology, characterized by wide clinical polymorphism and different autoantibodies, some responsible for cytotoxic damage, others implicated in the formation of immune complexes. SLE is a long-term disease with variable severity progression: the preclinical phase is marked by the presence of autoantibodies found also in other systemic autoimmune pathological condition, then it assumes a more specific and manifestly SLE immune connotation (Bertsias GK et al., 2010). During its course, SLE shows the alternation of flares and remission periods, culminating in damage related to disease and therapeutic choices, such as: alopecia, erythematous lesions, cognitive disorders, avascular necrosis, tendon rupture and osteoporosis. Earlier damages are mainly due to the SLE disease, whereas delayed alterations, such as infections, atherosclerosis, malignant neoplasia are usually attributable to immunosuppressive therapy complications. The disease etiology is still unknown, but it includes genetic and environmental factors, which combined they could determinate the irreversible breakdown of extrinsic immune tolerance resulting in an autoimmune response directed against endogenous nuclear antigens.

Moreover, knowing that rheumatic autoimmune diseases affect women several times more frequently than men, it should be important to focus especially in some specific characteristic and condition of women patients, as first of all, pregnancy (Krause ML et al., 2016). Many women struggle to find adequate information to guide them on pregnancy planning and careful monitoring in relation to their chronic disease, to ensure the best possible management for their own condition and fetal outcome (Iijima S, 2017). Pregnancy represents a physiological context requiring immune tolerance toward the fetus, whereas RA are SLE are characterized by pathogenic mechanisms interfering in regular immune system response. The coexistence of the two conditions could be linked to severe outcomes, such as spontaneous abortion, intrauterine death, preeclampsia, intrauterine growth restriction (IUGR), preterm birth and disease symptoms reactivation. Pregnancies in RA or SLE affected women are higher risk events; however, has been observed an increasing number of patients whom begin and complete a successful pregnancy, due to a constant monitoring and a contemporary clinicians support (Lateef A et al., 2013; Clowse ME et al., 2007).

From immunologic point of view, pregnancy is an allograft status: fetus carries 50% of paternal antigens, fetus-maternal interface presence as a connection between fetal and maternal antigens, hormonal setting characterized by higher estrogens and progesterone levels. Mother's immune system has high plasticity, assuring tolerance development and protection against infections, due to antibodies production B cells-mediated and through the T helper costimulatory activity. During pregnancy, Th2 cells are predominant, they are responsible of T cytotoxic cells suppression and they inhibit immune response against fetus cells. It has been observed SLE patients in the third trimester of pregnancy showing lower levels of estrogens, progesterone and cytokines Th2 derived than healthy subjects (Doria A et al., 2004). SLE pregnant women manifest a particular condition: on one side, during pregnancy, T-reg cells guarantee fetus-maternal tolerance development, on the other side it has been observed lower concentration of this lymphocyte population in SLE disease. Therefore, in pregnancies begun during inactivity phase, T reg cells are the prevalent population assuring a normal tolerance; but if the mechanism is altered due to the presence of SLE acute phase, it could be link to complications, such as preeclampsia, preterm birth, miscarriage (Aluvihare VR et al., 2004). Some authors sustained women affected by SLE produced a dysfunctional tolerance, affecting gestational adaptability and it has been suggested to undertake a pregnancy after SLE remission periods (Tower C et al., 2011). Pregnant SLE women shown increased levels of IL-17, IL-6, IL-10, TNF, suggesting immune system hyperactivity, which could lead to complications (Lee YK et al., 2009).

Moreover, placenta plays a role in estrogens and progesterone production, able to influence immune response. Higher estrogens levels induce the cellular shift to Th2 lymphocytes, exacerbating SLE disease, mediated specifically by this population (Torricelli M et al., 2011).

The evidence is completely opposite respect pregnant RA women, whom observe a symptomatology

betterment: the gain is ascribable just a Th2 shift, cause RA inflammatory pathway is characterized by Th1 cells subgroup (Nelson JL et al., 1997; Østensen M, 1999).

Placenta ensures the relation between mother and fetus, it allows the nutritive transition and the correct fetus development and it acts as a barrier to separate the two immune systems. Different cells and chemokines appear in the fetus-maternal interface, with several functions: to guarantee the selection of leucocytes transiting placenta, to permit the trophoblast invasion, to allow the placental angiogenesis and to ensure the recruitment and activation of immune cells promoting a positive pregnancy environment (Du MR et al., 2014). Concerning HLA complex, HLA-E and HLA-G subtypes are the main involved. More precisely, HLA-G recovered only in placenta, could be crucial for tolerance maintain toward fetal antigens. Immune tolerance is obtained due to HLA molecules functions as ligands for NK cells and macrophages receptors (Blaschitz A et al., 2001). Placental alterations are mediated through IgG, IgA, IGM, C3 actions and they could interfere with the normal fetus development (Hanly JG et al., 1988). In the meanwhile, fetus promotes tolerance through fetal cells migration and cell-free fetal DNA (cffDNA) in maternal bloodstream, creating a state called microchimerism persistent even after many years (Munoz-Suano A et al., 2011; Bianchi DW et al., 1996).

Microchimerism (MC) is the condition in which is possible to identify cells or DNA belonging to genetically different subject. Physiologically, during pregnancy exists a bidirectional trafficking of cells and DNA in the fetus-maternal interface that can results in naturally acquired microchimerism in both mother (fMC) and fetus (mMC) (Chan WFN et al., 2012). This interaction normally starts around the 7^{th} week of gestation, it increases through the pregnancy reaching the higher peak during the childbirth (Ariga H et al., 2001). Typically, the flow is asymmetric, because higher number of fetal cells have been shifted to the mother (Lo YM, 2000). Moreover, other fMC sources are represented by iatrogenic procedures, such as transplantations or transfusions, eventually from a twin or brother/sister or unconfirmed pregnancy, interrupted before ending. This suggestion should justify fMC phenomenon in women whom do not declare children.

Studies detected in mothers' hematopoietic fetal cells, nuclear erythrocytes, leucocytes (Bianchi DW et al., 1990; Bianchi DW et al., 1996; Maloney S et al., 1999; Mueller UW et al., 1990), $CD3^+$ and $CD4^+$ cells (Kremer Hovinga IC et al., 2006) and it is known that fetal cells persist in mother body many years after the childbirth (Bianchi DW et al., 1996). Although immediately after the birth, maternal immune system induces the fetus cells apoptosis, this process is not sufficient to remove them completely (Kolialexi A et al., 2004). The methodology mostly used to identify a presumed fMC consists in male DNA or male entire cells isolation from the maternal blood or tissue. The Y-Chromosome presence leads to the observation that fMC is common, resulted positive in 70-80% of tested cases (Lo YM et al. 1997). Moreover, several studies observed that pregnancies complicated by preeclampsia (Holzgreve W et al., 1998), preterm birth (Leung TN et al., 1999) and intrauterine

growth restriction (IUGR) (Al-Mufti R et al., 2000) shown an increased amount of fetus cells or cffDNA. Nonetheless, applications of this finding are controversial: some study reported it should be use to individuate women with preterm birth risk (Jakobsen TR et al., 2012), or preeclampsia (Clausen FB et al., 2013), others did not find as much (Stein W et al., 2013). Those evidences underlined the debatable situation regarding fMC, on one side it seemed correlating to higher probability of obstetric complications, melanoma and neoplastic formation in breast, uterine cervix and thyroid (Kallenbach LR et al., 2011), on the other side it has been proposed that fetal cells should be involved in maternal wound healing (Nassar D et al., 2012).

Thus, hypothesis on fMC role are many and different. The main ones, with particular regard on the influence on autoimmune diseases, such as RA and SLE, are following:

- ***fMC induces graft-versus-host reaction (GVH)***: fetal chimeric T lymphocytes (graft), recognize maternal cells (host) as extraneous, stimulating specifics antibodies production.
- This is supported by several similarities between autoimmune diseases and a transplantation condition, known as graft-versus-host disease (GVDH), assuming that a GVH reaction should contribute to the autoimmune disease pathogenesis (Chosidow O et al., 1992; Gratwhol AA et al., 1977). GVH reaction is satisfy by three conditions (Jimenez SA et al., 2005): mother accepts chimeric cells, chimeric cells are immunologically competent T-lymphocytes (Kremer Hovinga IC et al., 2006) and fetal cells recognize mother as extraneous. Studies reported fMC presence in SLE women bloodstream (Gannage M et al., 2002; Mosca M et al., 2003; Miyashita Y et al., 2000).
- ***fMC induces host-versus-graft reaction (HVG):*** fetal chimeric cells are the target of maternal immune response, as a result of direct action against fetus antigens or due to a molecular mimicry mechanism. Direct action occurs when fetal chimeric cells are recognized as non-self by mother immune system, cause paternal antigens existence. Renal biopsies of women affected by lupus nephritis shown chimeric cells presence (Kremer Hovinga IC et al., 2006), stimulating immune activation and promoting chronic forms. Molecular mimicry appears due to the chimeric cells surface contemporary expression of antigens similar to mother ones and different, from paternal derivation. Cross-reactive antigens endorse maternal T naïve lymphocyte or antibodies production by autoreactive B lymphocytes against self-molecules, leading to autoimmune effect.
- ***fMC cells repairs damages tissues:*** fetal chimeric cells differentiate from progenitors to distinct phenotypes, included endothelial cells (Mahmood U et al., 2004), neurons (Zeng XX et al., 2010), smooth muscle cells and cardiomyocytes (Bayes-Genis A et al., 2005; Kara RJ et al., 2011). Considering that mesenchymal cells from fetus have been found in the bone marrow of women with at least one son (O'Donoghue K et al., 2004), pregnancy should be considered as a physiological mechanism to acquire fetal cells population able to repair maternal injured tissues (Bianchi DW et al., 1996).

Lam GK et al., (2005) reported that women affected by lupus nephritis with high concentration of fetal chimeric cells are associated to creatinine lower level, whereas women with the absence of fMC owned higher creatinine values. fMC should performs a protective function: in response to tissues damage, mother organism recruits chimeric cells in inflammation situs, in order to assess the correct injury resolution (Stevens AM et al., 2006). Nonetheless, after reparation, fMC could act as a trigger for the HVG reaction, as described before.

Lastly, it has been proposed the fMC cells role as "innocent bystander", establishing an autoimmunity epiphenomenon not influencing clinical manifestations. The supposition is supported by the chimeric cells presence in healthy subject, even if in a very low number respect diseased women. Those observations convey in the controversial function of fMC; further and deeper studies are necessary to decode its role in the autoimmune disease context and, more specifically, in RA and SLE.

5.2. Rationale and Aims

The main aim of the present study is to explore the presence of fMC in blood of women RA patients in the active phase of the disease. More precisely, objectives are:

- to define the role of fMC in the etiopathology, following a case-control model and comparing fMC results which a matched healthy group;
- to investigate the association of fMC with previous obstetric history of female RA patients;
- to individuate the fMC presence in SLE, another autoimmune disease, in order to assess the fMC role linked to systemic or localized disease;
- to determinate the association of fMC with previous obstetric history of female SLE patients;

Lastly, to evaluate the eventual fMC presence in women with spontaneous or voluntary interruption of abortion event, in order to understand fMC role independently from the autoimmune diseases.

5.3 Materials and methods

Subjects

- *Rheumatoid Arthritis patients*

The study included a cohort of 238 female 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). Subjects were been enrolled 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 recruited.

Based on 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).

To each subject, whole blood sample has been collected, but only in a subgroup of 143 patients, clinical data along with previous obstetric history have been obtained. Deeper analysis described further has been carried out in this subgroup.

- *Systemic Lupus Erythematosus patients*

The study included a cohort of 146 female Italian patients with diagnosis of SLE, satisfying the American College of Rheumatology and Systemic Lupus International Corroborating Clinics (SLICC) for SLE criteria (Ines L et al., 2015). Subjects have been enrolled 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 recruited. To each subject, whole blood sample has been collected, but only in a subgroup of 28 patients, clinical data along with previous obstetric history have been obtained. Deeper analysis described further has been carried out in this subgroup.

- *Spontaneous Abortions/Voluntary Abortions cases*

A total of 175 women with episode of spontaneous miscarriage before 12 weeks of gestation or who had voluntary miscarriage event have been recruited. Subjects have been enrolled in the Gynecology and Obstetrics Unit of Sant'Anna University Hospital of Ferrara, with the collaboration of Dr. *R. Capucci.* 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 recruited. Overall, 81 women experienced a spontaneous abortion event, whereas 94 women had a voluntary interruption of pregnancy (VIP). Each case into each cohort comprised two biological sample: whole blood from the woman and the corresponding abortion tissue from the developing fetus, obtained after uterine cavity curettage. Both spontaneous and elective samples were collected by using disposable Safetouch system collection bottles and they have been suspended in normal saline solution and placed in the refrigerator until procession.

The investigation comprised even a matched group of 104 *female healthy controls*, in order to perform a retrospective observational case-control study.

A code has been assigned to each subject enrolled in each cohort described and sensible data were been separated from biological samples. Biological samples were storage at -20°C until processed for genomic DNA extraction.

Genomic DNA isolation

-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 lower amount of blood sample, gDNA was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer'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.

-Abortion tissues

Tissue samples derived from abortion during the first trimester of pregnancy, from both spontaneous and elective material, were sectioned accordingly to the histological specimens. Firstly, maternal decidua has been divided from fetus chorionic villae using stereomicroscope (Leica Microsystem Srl, ALL Microscopy and Histology, Milan, Italy); then, chorionic villae material was manually selected with disinfected forceps. After homogenization, genomic DNA was extracted using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) according to the manufactures's instructions, as earlier describes. Due to technical problems, insufficient amount or poor quality of abortive material, analyses have been performed on 54 and 80 chorionic villae tissue, respectively from spontaneous and elective abortive specimens.

Male fMC Analysis

The analysis of male fetal microchimerism (fMC) was based on the detection of Y-Chr sequences and it was carried out using the Custom Assay PrimeTime Standard qPCR (IDT, Tema Ricerca, Italy) and the specific PCR Master Mix (IDT, Tema Ricerca, Italy). The Custom Assay was specific for TSPY-1 gene, present in 35 copies localized in Y-Chromosome. The assay was composed by two primers 5'-TTCCCCTTTGTTCCCCAAA-3' and 5'-CATCCAGAGCGTCCCTGG-3' and the Prime Time Assay Std probe: /56-FAM/CGAAGCCGA/ZEN/GCTGCCCATCA/3IABkFQ/ requiring HPLC purification. Analyses to investigate the presence of fMC have been performed using 10ul of gDNA at 5ug, corresponding to 50ng in total. PCR condition for all reaction were as follow:

95°C for 3 min, then (95°C for 15 seconds and 60°C for 1 min) x 55 cycles and the final hold was 25°C for 10 minutes, using 7300 Real Time PCR System (Applied Biosystems by Thermo Fisher Scientific). The output was analyzed on the 7300 System Software (Applied Biosystems by Thermo Fisher Scientific). fMC experiment included scalar dilutions of male DNA (range 10ng/ul-10^{-6}ng/ul) in female DNA samples 10ng/ul to carried out calibration curve. Number of cycles (n°ct) needed to detect the rise of the curve and the point of flush has been identified for each sample and according to Y-Chr signal level, the number of male genome equivalents was determined.

β-Globin Analysis

In the same male fMC running plate, in separates wells, β-globin assay was performed for each sample, as internal control to check the effective presence of gDNA. It has been used a Custom Assay PrimeTime Standard qPCR (IDT, Tema Ricerca, Italy) and the specific PCR Master Mix (IDT, Tema Ricerca, Italy). The assay was composed by two primers 5'-GTGCACCTGACTCCTGAGGAGA-3' and 5'- CCTTGATACCAACCTGCCCAG-3' and the Prime Time Assay Standard probe: /56-FAM/AAGGTGAAC/ZEN/GTGGATGAAGTTGGTGG/3IABkFQ/ requiring HPLC purification. Analyses to investigate the presence of β-globin have been performed using 10ul of gDNA at 5ug, corresponding to 50ng in total. PCR condition for all reaction were as follow: 95°C for 3 min, then (95°C for 15 seconds and 60°C for 1 min) x 55 cycles and the final hold was 25°C for 10 minutes, using 7300 Real Time PCR System (Applied Biosystems by Thermo Fisher Scientific). The output was analyzed on the 7300 System Software (Applied Biosystems by Thermo Fisher Scientific). β-Globin experiment included scalar dilutions of male DNA (range 10ng/ul-10^{-6}ng/ul) to carried out calibration curve. Number of cycles (n°ct) needed to detect the rise of the curve and the point of flush has been identified for each sample to evaluate the effective gDNA concentration.

Statistical Analysis

The quantification of fMC has been performed referring to scalar dilutions of male DNA (range 10-10^{-6}) in female DNA samples and considering genome equivalents concerning to Y-Chromosome. β-Globin has been used as internal control to assess the exact concentration of sample gDNA. Logistic regression has been performed in order to measure the relationship between the categorical dependent variable of Y-Chr detection and the gDNA concentration, using MS™Excel and GraphPad packages. Multiparametric and statistical analyses of Odds Ratio (OR) with 95% Confidence Interval (CI) and p-value with Fisher's exact test have been carried out using MS™Excel and GraphPad packages. A p value < 0.05 was considered significant.

5.4. Results

Calibration Setting

For the best interpretation, calibration curve with serial dilution has been reported in the Figure 5.1.

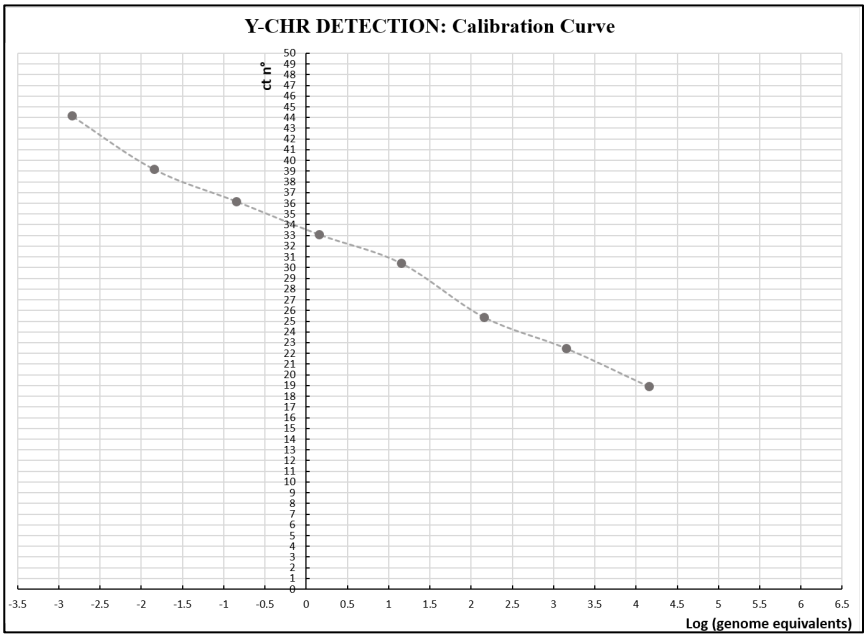

Figure 5.1 *Y-Chr calibration curve referred to logarithm of genome equivalents for serial dilution (10ng/ul-10^{-6}ng/ul) from right to left side.*

As shown, genome equivalents have been considered in logarithm scale and the value 4.15 corresponded to the genomes number in 50ng of total gDNA. Briefly, 1 genome equivalent is equal to 0.0035ng, so considering the gDNA concentration equal to 50ng (it has been used 5ul of gDNA with the concentration 10ng/µl as the first value), in diploid genetic patrimony it corresponded to a total number of 14285.71 genome equivalents. Repeating the same reasoning for all gDNA serial dilution (10ng/µl-10^{-6}ng/µl), the number of genome equivalents comes out for each concentration used. Shifting all those values into logarithm scale, calibration curve has been created showing each point corresponding to the logarithm of genomes equivalents, referring to each serial decreased concentration of gDNA (10ng/µl-10^{-6}ng/µl) (X-axis). Y-axis reported the correspondent n°ct needed to detect the rise of the curve and the point of flush.

On the base of the calibration curve obtained, logistic regression has been created: both, the logarithm of genome equivalents for each dilution and the number of cycles allowed finding the line equation (y=mx+q), the corresponding point-slope (m) and the slope-intercept with X axis (q). Thus, establishing a scalar line based on the decimal scale logarithms (x) and using both, m and q values, it was possible to find correspondents y values and to build the regression line (Figure 5.2).

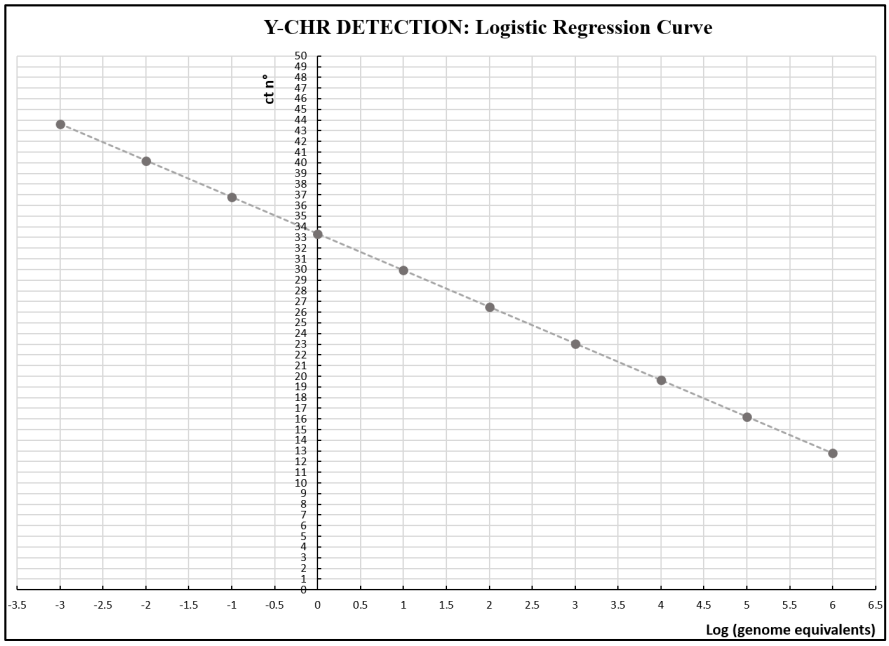

Figure 5.2 *Y-Chr logistic regression curve referred to logarithm of genome equivalents for decimal scalar dilution (10ng/µl-10-6ng/µl) from right to left side.*

Analyses to investigate the presence of male fMC in subjects enrolled in this study have been carried out. The number of cycles needed to detect the rise of the curve and the point of flush (n°ct) has been identified for each sample and accordingly the number of genome equivalents present. Considering the calibration and logistic regression curves, the threshold necessary to define positivity was set on 32 cycles, corresponding, roughly, to find 1 male cell in 10^3 females cells.

5.4.1 Male fMC detection in Rheumatoid Arthritis female Italian patients

A total of 238 RA female patients were enrolled in the study. The baseline characteristics of the patients included in the study are summarized in Table 5.1. In the subgroup of 143 RA subjects obstetric history details have been collected.

Characteristics		RA (n=238)
Demographic		
	Age (years) (mean ± SD)	58.34 ± 12.76
Clinical		
	Young onset RA	41.56%
	Late onset RA	58.44%
Obstetric Characteristics		RA (n=143)
Previous Ascertained Pregnancies		
	Yes	93.00%
	No	7.00%
Previous Miscarriage Events		
	Yes	33.09%
	No	66.91%

Table 5.1 *Characterization of RA Italian female subjects studied.*

Analyses to investigate the presence of male fMC in RA patients and healthy matched individuals, have been carried out, in order to evaluate a potential role in etiopathology (Table 5.2).

A

Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Controls	15	89	104
RA	46	192	238

B

OR	95% CI	p-value
1.422	0.754-2.682	0.281

Table 5.2 *Male fMC detection in female RA patients and female controls.*
A. *Subjects stratification;* ***B.*** *Association study.*

Female RA subjects shown 1.42-fold higher probability to manifest male fMC than healthy women (95% CI 0.754-2.682). The p-value was not significant, but this should be an evidence of a positive trend of Y-Chr presence in women with RA, suggesting the possible interaction between microchimerism detection and autoimmune disease.

Moreover, RA patients have been stratified according to previous ascertained pregnancies (Table 5.3) and association analysis have been carried out.

A

RA Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Ascertained Pregnancies	25	108	133
No Ascertained Pregnancies	0	10	10

B

OR	95% CI	p-value
n/a	n/a	n/a

Table 5.3 *Male fMC detection in RA patients stratified by previous pregnancies.*
A. *Subjects stratification;* ***B.*** *Association study*

The ratio between previous pregnancies or their absence was 13.3.
The association analysis was not possible to carried out: in fact, as noticed in Table 5.3, no one RA women whom had not experienced previous ascertained pregnancies shown positive Y-Chr signal, all were grouped in the negative fMC cohort. This result is remarkable in order to find eventual possible link between pregnancies and microchimerism presence in RA patients blood.

Furthermore, RA women subjects have been subdivided according to previous miscarriage events (Table 5.4 A) and association analysis has been carried out (Table 5.4 B).

A

RA Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Abortions	9	36	45
No Abortions	14	77	91

B

OR	95% CI	p-value
1.357	0.545-3.472	0.511

Table 5.4 *Male fMC detection in RA patients stratified by previous abortions.*
A. *RA stratification;* ***B.*** *Association study*

In the totality of patients analyzed, the ratio between absence of previous miscarriages or their presence was around 2. Considering only the group showing positive Y-Chr signals, the ratio

between whom have had no previous abortion episode compared to their presence, was 1.5 times more. OR analysis did not reveal any significant association. It has been reported that female patients with previous miscarriages shown higher probability to manifest positive Y-Chr signal than whom had not experienced abortion episodes (OR= 1.375, 95% CI 0.545-3.472), without reaching the statistical significance (p value= 0.545).

5.4.2 Male fMC detection in Systemic Lupus Erythematosus female Italian patients

A cohort of 146 SLE female patients have been recruited for the study. The baseline characteristics of the patients included in the study are summarized in Table 5.5. Main data concerned to age and SLE disease onset, whereas in the subgroup of 28 SLE subjects' obstetric history details have been collected.

Characteristics	SLE (n=146)
Demographic	
Age (years) (mean ± SD)	64.46 ± 11.67
Clinical	
SLE diagnosis age (mean)	40.38 ± 12.83
Obstetric Characteristics	SLE (n=28)
Previous Ascertained Pregnancies	
Yes	82.14%
No	17.86%
Previous Miscarriage Events	
Yes	46.43%
No	53.57%

Table 5.5 *Main Characteristics of SLE Italian female subjects studied.*

Deeper data about the subgroup of 28 SLE patients were as followed.

Regarding to previous obstetric history: the menarche average age was 12.5 years; among 28 patients, 24 women were already under menopause with mean age of 45.5 years, of which 18 subjects reached spontaneously menopause state, whereas remained 6 after surgical operation.

16 patients referred to do not use o had not use either progestogens contraceptives, the remaining 12 women mentioned to have used at least once in life; they have not always been able to determine with certainty the assumption duration.

Additional features concerned comorbidities, showing 6 patients affected by others rheumatic diseases, in particular: 1 by fibromyalgia, 2 by Sjögren Syndrome and fibromyalgia, lastly, 3 by osteoporosis and Sjögren Syndrome.

About therapies, 3 subjects reported to do not currently assume any type of treatment for SLE symptoms control, 7 women informed to use corticosteroids (Medrol® or Deltacortene®), 3 affirmed to be under hydroxychloroquine (Plaquenil®) and the remaining patients to take both drugs typologies.

The association analysis among female SLE patients and female controls considering the positivity of Y-Chr signals and the presence of male fMC has been performed, in order to evaluate a potential role in the disease etiopathology (Table 5.6).

A

Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Controls	15	89	104
SLE	54	92	146

B

OR	95% CI	p-value
3.483	1.833-6.618	2E-04

Table 5.6 *Male fMC detection in female SLE patients and female controls.*
A. *Subjects stratification;* ***B.*** *Association study*

Female SLE patients shown around 3.5-fold higher statistical significant probability to present positive Y-Chr signal than healthy female individuals (95% CI 1.833-6.618). The p-value was significant (0.00016), showing the strong association of SLE subjects to manifest male fMC in systemic bloodstream.

Subsequently, SLE patients have been stratified according to previous ascertained pregnancies (Table 5.7 A) and association analysis has been carried out (Table 5.7 B), in order to determinate any connection with male fMC or eventually a causative fMC role.

A

SLE Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Ascertained Pregnancies	5	20	25
No Ascertained Pregnancies	1	2	3

B

OR	95% CI	p-value
2.000	0.150-26.735	0.613

Table 5.7 *Male fMC detection in SLE patients stratified by previous pregnancies.*
A. *Subjects stratification;* ***B.*** *Association study.*

The ratio between previous pregnancies or their absence was 8.3. Considering the group showing positive Y-Chr signals, the ratio between whom have had previous ascertained pregnancies compared to no one, was five times more. OR analysis did not reveal any significant association. It has been reported that female patients with ascertained pregnancies noticed higher probability to manifest negative Y-Chr signal than whom had not experienced gestations (OR= 2.000, 95% CI 0.150-26.735), without reaching the statistical significance (p value= 0.613).

It could be referred to higher number of pregnancies compared to just 3 cases without none: confidence interval was wide, due to low samples size.

Keeping close attention to reproductive history and considering only completed gestations, 22 women have had at least one child; among them, 10 subjects have undertaken and concluded a second pregnancy. Out of 10, 4 women reported a twin pregnancy: 3 cases with dizygotic twins (male-male; female-female; male-female) and 1 case with monozygotic twins (female-female). Third child events were attributable exclusively to twin gestations.

This observation lead to the intention to stratify SLE patients according to twin pregnancies and to perform analysis considering male fMC presence (Table 5.8).

A

SLE Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Twin Pregnancy	2	2	4
No Twin Pregnancy	4	20	24

B

OR	95% CI	p-value
5.000	0.535-46.720	0.159

Table 5.8 *Male fMC detection in SLE patients stratified by previous twin gestations.* ***A.*** *Subjects stratification;* ***B.*** *Association study*

SLE patients reporting twin gestation shown 5-fold higher probability to manifest positive Y-Chr signal compared to women without twins (95% CI 0.535-46.720). P-value was not significant (0.159) and the confidence interval was wide, due to low samples size.

Furthermore, SLE patients have been stratified considering previous miscarriage events (Table 5.9 A) and association analysis has been performed (Table 5.9 B).

A

SLE Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Abortions	3	9	12
No Abortions	3	13	16

B

OR	95% CI	p-value
1.444	0.236-8.844	0.704

Table 5.9 *Male fMC detection in SLE patients according to previous abortions.* ***A.*** *SLE patients stratified;* ***B.*** *Association study*

The ratio between no abortion event or their presence was around 1 (1.33). Considering the group showing positive Y-Chr detection, the number of patients whom have had previous miscarriage events compared to no one, was exactly the same. Association analyses did not reveal significant result: in fact, SLE women who had previous abortion events showed around 1.5-fold higher probability to manifest Y-Chr positivity than whom had not experienced miscarriage (OR= 1.444, 95% CI 0.236-8.844), without reaching the statistical significance (p value= 0.704).

Globally, 12 cases with abortion have been observed, among them 7 were spontaneous, whereas 6 electives; 1 patient reported to have experienced both the events.

Concerning spontaneous events, mean patients age was 26 years, or on average 12 years before SLE diagnosis. Among 7 cases, 1 women reported the episode of ectopic gestation ended in spontaneous miscarriage, whereas the others 6 subjects did not document precise cause of such occurrences.

Regarding elective episodes, they were performed by patients at an average age of 36 years, or on mean 3 after before SLE diagnosis. Deeper, among 6 events, 4 women have asserted to voluntarily interrupted pregnancy due to SLE disease and worries about fetus repercussions.

According to these observations, SLE patients have been stratified considering if it should be difference in terms of male fMC in the bloodstream of SLE women comparing spontaneous abortions episodes (Table 5.10) or elective abortions events (Table 5.11).

A

SLE Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Spontaneous Abortion	0	7	6
No Spontaneous Abortion	6	15	22

B

OR	95% CI	p-value
n/a	n/a	n/a

Table 5.10 *Male fMC detection in SLE patients according to previous spontaneous miscarriages.* ***A.*** *SLE patients stratified;* ***B.*** *Association study*

A

SLE Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Yes Voluntary Abortion	3	3	6
No Voluntary Abortion	3	19	22

B

OR	95% CI	p-value
6.333	0.848-47.311	**0.072**

Table 5.11 *Male fMC detection in SLE patients according to previous voluntary miscarriages.* ***A.*** *SLE patients stratified;* ***B.*** *Association study*

Results referred to spontaneous miscarriage episode failed to reveal any association: as shown in Table 5.10, no one patient with spontaneous abortion presented positive Y-Chr, defining the impossibility to evaluate OR. Concerning data reported in Table 5.11, SLE women who had previous elective abortion events, showed a nearly significant trend of more than 6-fold higher probability to manifest Y-Chr positivity than whom had not experienced voluntary miscarriage (OR= 6.333, 95% CI 0.848-47.311). The p-value was borderline without reaching the statistical significance (p value= 0.072). Considering the global results, the compared p value between spontaneous and induced abortions events was not been calculated.

5.4.3 Male fMC detection in women with spontaneous or voluntary abortions

It has been evaluated the association of spontaneous or elective miscarriage events (VIP) with the positive Y-Chr detection and the presence of male fMC in women whom have experienced these conditions, but not affected by RA or SLE diseases (Table 5.12). The analysis has been performed in order to assess the contribution of this previous obstetric complication independently from the presence of rheumatic autoimmune pathology.

A

Women Control Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Spontaneous Abortion	1	80	81
Voluntary Abortion	4	90	94

B

OR	95% CI	p-value
3.556	0.389-32.475	0.264

Table 5.12 *Male fMC detection in women control cohort, according to previous miscarriages.* ***A.*** *Women control cohort stratified;* ***B.*** *Association study*

Overall, only 1 woman with spontaneous event shown Y-Chr presence, whereas in the elective group, 4 subjects manifested the positivity. Association analysis did not reveal any significant result. Nonetheless, data reported that cohort with voluntary miscarriage 3.5-fold higher probability to find male fMC respect the group of spontaneous episodes. 95% CI were wide and p value was not significant (0.264).

Results suggested that abortion event did not influence alone the positive detection of fMC in bloodstream of women without RA or SLE.

Furthermore, taking advantage of the abortive tissues availability and in particular the chorionic villae specimens, after gDNA extraction and Y-Chr assay, it has been possible evaluate the sex of the conception product, both for spontaneous and elective cases (Table 5.13).

Abortion Cohort	Positive Y-Chr signal	Negative Y-Chr signal	tot
Spontaneous Abortion	32	22	54
Voluntary Abortion	39	41	80
tot	71→Males	63→Females	

Table 5.13 *Male detection in abortions cases, according to spontaneous or elective miscarriages.*

In this investigation, the threshold to evaluate the Y-Chr presence was fixed at the same n°ct needed to detect the Y-Chr positivity in each cell. Cause technical problems, insufficient amount or poor quality of abortive material, analyses have been performed using 5 ng of gDNA for each sample. Thus, in diploid genetic patrimony, 5ng of gDNA corresponds to find a total number of 1428.571 genome equivalents. Considering the calibration curve, the number of cycles needed to detect the rise of the curve and the point of flush corresponds to 23 ct. As shown in Table 5.13, positive Y-Chr signal has been found in 71 miscarriage tissues, whereas negative cases were 63. Accordingly, 71 samples were male conception products and 63 were females.

Consequently, it was possible to evaluate if the sex of the conception product was significant related to fMC detection in mother's blood, accordingly to whom have experienced spontaneous or elective episode (Table 5.14).

Cohort	SPONTANEOUS MISCARRIAGE vs VOLUNTARY MISCARRIAGE Test of association			Sex compared P value
	OR [a]	95% CI [b]	P value	
Male Abortions	2.583	0.256-26.120	0.429	-
Female Abortions	n/a	n/a	n/a	

[a] Odds Ratio

[b] Confidence Interval 95%

Table 5.14 *fMC detection in women with spontaneous or elective event, considering the conception sex.*

Male conceptions from voluntary abortion event shown more than 2-fold higher probability to manifest fMC in mother's bloodstream, compared spontaneous miscarriages (OR= 2.583, 95% CI 0.256-26.120); the result did not reach the statistical significance (p value= 0.429). Female conceptions failed to reveal any association result, due to the absence of positive Y-Chr detection in voluntary abortion cases. In spontaneous miscarriage group, only 1 case has given a borderline n°ct, due to possible presence of some Y-Chr positive cells derived from a previous male pregnancy.

Considering the global results, the sex-compared p value was not possible to be calculated.

5.5 Discussion and Conclusions

Microchimerism (MC) is a particular status characterized by the presence in an individual of a small amount of genetically distinct cells originating from a "donor". It is known that MC can develop after pregnancy, blood transfusions and solid organ transplantation. However, the immunological significance of this phenomenon remains to be clarified. From the finding of several studies, which reported autoimmune affected patients with significant positivity for Y-Chromosome microchimerism than in health controls (Mosca Met al., 2003), this study would to explore about MC and rheumatic autoimmune diseases: Rheumatoid Arthritis (RA) for specific joints involvement and Systemic Lupus Erythematosus (SLE) for systemic and widespread symptoms.

Although fetal immune system function has been well studied, not much is known about the functionality of cells that originate in the fetus but are long-term residents within the maternal environment. Some RA epidemiological, immunogenic and MC studies illustrated the potential for beneficial as well as adverse consequences of maternal and fetal origin MC (Nelson JL; 2012).

The investigation about previous obstetric history of two separately female case-series, affected respectively by RA and SLE, has made possible the identification of specific role of pregnancy and the miscarriage, linking them to the male fetal microchimerism detection in mother's bloodstream.

Concerning RA cohort, results indicated association to positive Y-Chr detection respect to healthy women (OR=1.422 95% CI 0.754-2.682, p value=0.281). The data is perfectly in agreement with what is reported by Kekow M et al (2013), which have found the presence of fetal microchimeric cells in whole blood of female RA patients in 18% of cases, significant higher respect to 3.7% revealed in control female subjects. Stratifying our RA case-series according to the presence of previous abortions events, it came out women with miscarriage episodes shown around 1.4-fold higher probability to be fMC positive. It has been claimed that natural acquisition of cellular MC occurs during pregnancy and women could maintain the presence of fetal cells after pregnancy ending (Gammil HS, Harrington WE, 2017). The investigation of the particular role of miscarriage in autoimmune disorders is ongoing, but studies reported acquisition of microchimeric cells during pregnancy loss (Peterson SE et al., 2013).

Regarding SLE cohort, the collection of clinical and obstetric characteristics and the multiparametric analysis, allowed to confirm some evidences already known concerning epidemiology and reproductive history of women affected. The mean age of the patients at the time of disease diagnosis was 39.6 years, appearing superimposable with data reported by references, which found 65% of women showing first symptoms between 16 and 55 years. Moreover, this study confirmed data regarded preterm birth: among 28 patients analyzed, 3 reported this condition (10.7%), whereas European data on this eventuality documented a percentage of 5% in healthy population. Concerning fertility rate, lupus women and general female subjects manifest approximately the same value,

corresponding 1.37 children for woman (source: Istat, 2004) and in fact, in our case-series the children number for patient is 1.42. Subsequently, this study shown the strong presence of fMC in bloodstream of SLE patients respect to healthy individuals: in fact, SLE affected subject shown higher statistical significant probability to present positive Y-Chr signal than healthy female individuals (OR=3.483 95% CI 1.833-6.618, p value=0.00016). Several studies confirmed our finding: they reported that the amount of male microchimeric cells of fetal origin, detected in the peripheral blood of women diagnosed with SLE, was significantly higher when compared with those of healthy women (Abbud Filho M et al., 2002) and postulating fMC involvement in SLE pathogenesis (da Silva Florim GM et al., 2016).
The attention, then, has been focused specifically on twins: in general population, the frequency of twin births appears 1.80; in this study, on the contrary, altogether on 36 total childbirths, it has been found 4 twin births, always in the second pregnancy. The frequency of this event is remarkable and appears to be greater than expected, but due to the reduced sample size, it cannot be asserted that it is statistically significant (p value=0.082). Concerning, the number of dizygotic twins (3 out of 4) in our case-series, it is in accordance with is normally found in twin births, referring the incidence of monozygotic twins is about one-third respect dizygotic twins (Charles E Boklage, 2009).

Moreover, only 2 out of 4 twin births, seem correlate to the presence of male cells in mother's bloodstream; whereas, the other 2 twin births do not reveal positivity to male cells in mother's blood. The results do not allow to conclude a specific association between twins and microchimerism detection (OR= 5.000, 95% CI 0.535-46.720; p value= 0.159), but suggested the possible "overload" of fMC which could play a role as a disease rescue or enhancer, as already observed (van Dijk BA, 1996). Considering miscarriage event, it has been noticed in 10% of begun pregnancies in SLE affected women. In the study, on a totality of 22 women with previous ascertained pregnancies, 7 cases reported spontaneous abortions, showing a frequency of 32%. To fully understand the scope of this observation, SLE patients recruited in the study have the age range gestations 20-29 years; in this age-window Istat reported the spontaneous abortions frequency equal to 12.7% (Istat, 2013). Although the remarkably high percentage of the cases is attributable to its elusiveness, it confirms the evidences already been stated in the literature on pregnancies in SLE (de Jesus GR et al., 2015).

Concerning the 7 spontaneous abortions cases, it is to underline that the only one reported in the patient with both, spontaneous and elective events, gives positive detection of male cells in mother bloodstream; whereas in the others 6 cases, no chimeric detection have been reported above the threshold that defines fMC positivity. With regard to voluntary interruption of pregnancy, out of 22 cases of ascertained gestations, 6 women refer to being subjected, corresponding to 21.42%. Mean age of patients at the time of VIP, was 36 years and in this age range Istat refers a VIP rate equal to 9.12/1000 women (Istat, 2013). More precisely, in Italy has been reported 7.06 VIP cases every 1000

women, for subjects between 15 and 49 years old. Among 6 elective cases, 5 have been fulfilled after disease onset and again, among them, 3 events correspond to the 3 cases out of 4 associated to higher fMC positivity. Others remarkable results concerned the 3 elective abortions linked to a strong fetal microchimerism detection: each of them was performed on average four years after the pathology onset. At the time of blood sampling investigated for fMC, the mean time spent by the disease onset was equal to 21 years and the mean time spent by voluntary interruption was equal to 17 years. Male cells were found in maternal blood, in fact, from a minimum of 11 to 28 years after the VIP. This finding, therefore, is perfectly in agreement with what is claimed in the literature, repeatedly observing a male genetic material persisting in the maternal body for years after pregnancy (Kristina M et al., 2009). Considering what it has been described so far, it should be emphasized that, according to the cases presented by this study, abortion exhibits relationship with microchimerism only when it is voluntary. Association result shows elective events with more than 6-fold higher probability to detect fMC, compared to spontaneous miscarriage (OR= 6.333, 95% CI 0.848-47.311), the p-value does not reach the statistical significance, but it is strongly borderline (0.072). Iatrogenic procedure as uterine scraping may, in fact, be the cause of the dissemination of male cells, not completely eliminated by the maternal immune system. However, limitations of the effective microchimerism-voluntary abortion link have been given to the evidence that only in 3 of the 5 cases have shown the real presence of male genetic material, it might be due to the small sample size or the used technique which do not allow detection of female chimeric cells.

Moreover, 1 case has revealed the presence of positive fetal microchimerism even if the women did not report previous abortions events; this result could be justified by the transition and failure elimination of child's cells derived from a previous male pregnancy. In fact, this one case described 2 sons before the blood sampling. The finding is corroborated by the literature studies which refer positive fMC even in women who state they have not had any abortions, either voluntarily or spontaneously interrupted, and who are not affected by SLE or other autoimmune diseases (Gammill HS, Harrington WE, 2017).

Results lead to reflections on microchimerism function. In fact, it should be noted that 3 of the 4 patients with the highest levels of male genetic material in bloodstream have experienced not only voluntary abortion, but also they are the 3 cases who currently do not take any particular therapy for SLE: 2 are not under treatment, 1 only under corticosteroid. This reflects the quite stable and satisfactory health condition distinguishing those patients, as emerged from the analysis of their clinical records, which shows a nearly constant stationary state of well-being and over the years. It should be noted, moreover, that the other positive microchimerism women in the sample usually show a history of disease not particularly aggressive and stable over time.

Results concerning the male fMC detection in women with spontaneous or voluntary abortions, not affected by RA or SLE, did not shown association to fMC detection independently from autoimmune

disease. The evaluation of developing fetus sex did not reveal association to spontaneous or elective events, suggesting that male microchimerism presence is linked exactly to autoimmune disease. This finding is controversial with what has previous reported: among women without sons, male DNA has been found in peripheral blood in almost a quarter of those who had spontaneous abortions and more than half with induced abortions (Yan Z et al., 2005). The statement could be referred to the different age sample or considering that the composition of MC acquired by women who have had induced or spontaneous abortions has not been fully studied (Nelson JL. 2012).

Overall, such findings would seem to support the "repair" hypothesis of microchimerism: the chimeric cells would be able to provide a tissue injury and flushing resolutions associated with SLE, ensuring symptoms reduction and low doses or no therapeutic treatment. Subsequently, it could even identify potential and unexpected therapeutic tools in the voluntary interruption of pregnancy and in subsequent circulating chimeric cells. Obviously, the voluntarily gestation renounce in absence of any other condition or complication is not reliable from practical and ethical point of view. However, it might be to take into account the opportunity to include the assumption of chimeric cells as an implementer therapeutic tool for SLE stabilization or remission. The less feasible results reported in RA case-series are probably due to the less deeper information about obstetric history and the fact that inflammation is mainly localized in the specific site of synovium. Peripheral blood sample could be a good specimen in case of blood diseases or more systemic and widespread pathologies, such as SLE. Besides fMC in bloodstream of RA women patients, it should be interesting and remarkable performing further analysis specifically in joints synovial tissue, in order to related fMC to patient's inflammatory status and to understand much more better its own role.

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CHAPTER 6

THE CORE DIRECTION:

FROM SYSTEMIC AFFECTION TO LOCALIZED INFLAMMATION

"The value of a college education
is not the learning of many facts,
but the training of the mind to think"

Albert Einstein

CHAPTER 6

THE CORE DIRECTION:
FROM SYSTEMIC AFFECTION TO LOCALIZED INFLAMMATION

Table of Contents

Abstract

Background: Synovial tissue (ST) is the main area affected in Rheumatoid Arthritis (RA) disease, characterized by inflammation, infiltration of several cell populations and progressive damage leading to chronic condition and mobility reduction. Fetal microchimeric cells (fMC), acquired especially through pregnancy, have been found in the ST of female patients, suggesting their potential role in RA disorder. In the ST were localized immune system cells, which contribute to the RA progression and increasing inflammation.

Objective: The main purpose was to explore if the male fMC presence in ST of RA subjects could contribute to the etiopathology of the disease, and if fMC might be considered as causal or casual factor. Second aim was to investigate about markers applied in immune-histological analyses of ST and the correlation of the synovial infiltration with different therapeutic treatment choices.

Materials and methods: Synovial biopsies were obtained from 6 RA diagnosed patients, along with peripheral whole blood sample, clinical data and therapeutic treatments previously assumed. From female subjects have been collected obstetric history details. Y-Chromosome detection has been carried out in blood and each tissue specimen. ST section were analyzed through Hematoxylin/Eosin (H/E) and then by immunohistochemistry (IHC) using monoclonal antibodies against CD4 T-lymphocytes, CD20 B-lymphocytes and CD68 macrophages.

Results: Y-Chr failed to reveal positive detection in female synovium and even in blood. H/E analysis noticed the two RA cases with stable therapies showed low total immune cells presence, whereas the other four cases manifested clearly infiltration. IHC results on these four cases revealed CD4+ T-lymphocytes widespread in all ST patients, although the patterns of CD20+ B-lymphocytes and CD68+ macrophages were different among cases. The two patients without treatment presented high CD20+ infiltration and not considerable CD68+ detection, whereas cases whom have changed several therapies, showed the opposite aforementioned features.

Conclusion: The absence of male fMC in women subjected to arthrocentesis may suggest that microchimeric cells can exert a rescue role, acting as stem cells repairing tissues injuries; thus, it has possible to detect fMC cells only in slight or mild RA cases. Histological analyses of RA patients ST suggest the remarkable value to identify promptly the effective therapy, in order to avoid any invasive procedures. Future studies may support fMC hypothesis and may clarify the relation between immune cells subgroup infiltration and treatments.

6.1. Introduction

Rheumatoid Arthritis (RA) is a multifaceted disease, characterized by autoimmune origin and joints chronic relapsing-remitting inflammation. The pathology affects approximately 1 percent of the adult population, but mostly female subjects (Lawrence RC et al., 1998). This evidence suggests the presence of interaction between genetic profile and risk factors specific for female sex, especially related to obstetric history. During the first trimester of pregnancy exists a bidirectional trafficking of cells and DNA in the feto-maternal interface that can results in naturally acquired microchimerism in both mother (fMC) and fetus (Chan WF et al., 2012). Women could maintain the presence of fMC after pregnancy ending for many years and previous studies have suggested that fMC may confer either risk to or protection from RA, depending upon several factors including the HLA specificity of the fMC (Rak JM et al., 2009). The presence of microchimeric cells could be revealed in mothers blood through nested-PCR technique or using TaqMan Probes in Real-Time PCR. Although not absolute detection-rate, the technique generally used consists in the investigation of chromosome-Y sequences.

fMC has been revealed high frequent in the peripheral whole blood of female patients affected by autoimmune diseases, such as RA, Scleroderma, Systemic Lupus Erythematosus (SLE), Sjögren Syndrome and autoimmune thyroiditis (Kekow M et al., 2013). The presence of fetal microchimeric cells in whole blood of female RA patients has been found in 18% of cases, significant higher respect to 3.7% revealed in control females subjects (Kekow M et al., 2013). Serological parameters, such as anti-CCP, ANA, RF did not significant differ in patients fMC+ and fMC- and disease onset was 19-20 years after the first pregnancy in both cohorts.

Actually, the role of microchimeric cells in the etiology and pathogenesis of RA is not fully understood, studies hypothesized that cells could determine allogenic input or their own could be an allogenic target. Male fetal microchimeric cells have been found at low density ($1.2x10^{-6}$) in the synovial tissue in 38.5% of RA female patients (Hromadnikova I et al., 2008). Recent evidences have documented the presence of males cells in rheumatic nodules in 21% of RA female patients and using HLA-specific quantitative PCR has been found microchimerism in rheumatic nodules in 60% of subjects (Chan WF et al., 2012). Among them, chimerism has been ascertained to be fetal in 75% of cases and in the remained 25% it was presumably maternal. In some cases, RA patients not carried HLA-DRB1 Shared Epitope (SE) showed in bloodstream microchimeric leucocytes expressing HLA-DRB1-SE (Rak JM et al., 2009; Yan Z et al., 2011). This evidence suggested the hypothesis that fetal microchimerism exerted a role in the etiopathology of RA: through fMC women not carried HLA-DRB1-SE could acquire risk epitope and the susceptibility to develop RA disease.

Considering RA as a rheumatic disease characterized by prominent synovial tissue (ST) inflammation and by infiltration of many different cells population participating in complex pathophysiological pathways, it could be remarkable shifting from peripheral whole blood analyses

to synovium investigation. Male fMC cells could play a role specifically in the localized tissue and they might influence the progression of RA disease, acting through one of the following hypothesized mechanisms: the induction of graft-versus-host reaction (GVH), stimulating antibodies production (Chosidow O et al., 1992); the induction of host-versus-graft reaction (HVG), promoting the increase of maternal immune response against cells recognized as non-self; the repairing of damages tissues, in which fetal cells could act as rescue for injured joints (Stevens AM et al., 2006). Hence, the RA female patients could exacerbate or reduce the pathology condition according to fMC cells presence in their synovium.

Besides this, studies reported RA disease outcome may variate due to the effect of different treatment, in terms of invasive pannus development and proteinase production which leads to the articular damage, loss of cartilage and bone erosion. Thus, it has become increasingly important to differentiate RA therapies accordingly to the patients' inflammatory status, clinical features, detection of rheumatoid factors and radiographic evaluation over a period of time (Walsh AM et al., 2017).

Inflammatory symptoms are alleviating, firstly, using non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, ketoprofen, and naproxen sodium, typically prescribed to reduce prostaglandin production (Crofford L, 2013) or corticosteroids; then RA patients are treated with conventional disease modifying antirheumatic drug (*cDMARDs)* or biological disease modifying antirheumatic drug (*bDMARDs)* according to subject's state and therapy efficacy. Despite the effective of these treatments relieving the widespread symptoms of RA, they may potentially modulate the ST condition.

Hence, it is clear that analysis of biopsy specimens and especially ST, could help to make complete overview of phlogosis and cells infiltration in joints, assessing differences in the adhesion molecules expression, cytokines and metalloproteinases presence. Rheumatoid synovium undergoes to many typical hallmarks pathological characteristics, such as vascular congestion, fibroblast proliferation, synovial lining layer hyperplasia, mononuclear cell infiltration and fibrin deposition. A wide range of immune cells has been detected in RA articulation, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells and macrophages. Various soluble mediators produced by these immune cells have been shown to correlate with disease progression and/or severity and patients early or long-standing RA (Tak PP et al., 1997). The importance of the immune system in disease pathogenesis is illustrated by the success of biologic therapies that target key inflammatory cytokines (inhibitors of TNFα and anti IL-6 therapy), immune molecules and immune cells (B cell depletion). Endothelial cell activation, acute phase reaction and articular damage are caused by the activation of several cells population contributing to the production of proinflammatory cytokines and chemokines (Roberts CA et al., 2015).

T-lymphocytes are one of the main population involved in promoting RA pathology, they undergo to clonal expansion after recognition antigens presented by antigen-presenting cells. In particular, $CD4^+$ T cells exerts a relevant role in the autoimmune response: they are key to the initiation and progression of synovitis and they are frequently found in rheumatoid synovium infiltration. It has been reported that interfering with their activation by blocking costimulatory signal in an effective therapy for RA (treatment with *bDMARDs: Abatacept*) (Fonseka CY et al., 2017; Savola P et al., 2017).

The contribution to the inflammatory process in synovia, even correlated with bone erosions, is provide by T lymphocyte-macrophage interactions, which are complex and bidirectional (Fonsecs JE et al., 2002). On the one hand, interferon-γ (IFNγ) and interleukin-2 (IL-2), produced by CD4+ T lymphocytes of the Th1 subtype, support macrophage activation. On the other hand, IL-4, IL-5, and IL-13, from CD4+ T lymphocytes of the Th2 subset, down-modulate proinflammatory cytokine production by macrophages (van der Graaff WL et al., 1999). Thus, it has been hypothesized the possible regulatory relationship among CD4+ lymphocytes and macrophage is RA synovium. Moreover, a further complexity exists, since both Th1 and Th2 products provide help for B lymphocytes and support antibody production, which may feedback further onto macrophage function.

In fact, a subset of CD4+ T cells, called follicular helper T (Tfh), is specialized to provide 'help' to B cells, creating a T-cell/B-cell interaction (Crotty S, 2014). Tfh cells localize with B cells in follicles and germinal centers in secondary lymphoid tissues and promote the differentiation of activated B cells into memory cells and Ab-secreting (Abs) plasma cells. This ability underlines long-lived memory against pathogen infection, but not all Abs produced by B cells are good. In fact, in the context of autoimmunity, as RA, autoreactive B cells are pathogenic, they produce Abs against antigens recognize as non-self, resulting in severe tissue damage (Tangye SG, 2017).

On the surface of pre-B cells to mature B-lymphocytes are expressed CD20+ antigens. They are not present on stem cells and are lost before differentiation of B cells into plasma cells (Edwards JCW, 2004). Studies reported that autoreactive B cells play an important role in RA process, they are involved in joint damage, through direct action and driven by the T cell to produce IgG autoantibodies (Kotzin BL, 2005).

In blood and in RA synovium, the B-cell depletion, and especially a selective transient depletion of the CD20+ B-cell subpopulation, is correlate to good clinical response, controlling sign and symptoms, improving physical function and preventing the radiological damage (treatment with *bDMARDs: Rituximab*) (Vieccelli D et al., 2016).

Hence, the histological examination of rheumatologic synovial biopsy specimens could be useful to provide insight into pathogenic mechanisms underlying inflammatory arthritis and to evaluate the effects of therapeutic interventions.

6.2. Rationale and Aims

The main aim of the present study is to explore the presence of male fMC in synovial tissue of RA patients in the active phase of the disease. More precisely, male fMC presence will be investigate:

- to define its function in the etiopathology (case-control model considering male and female patients);
- to define its association to the previous obstetric history of female patients;
- to determinate its role in response-to-treatment.

Secondly, the purpose is to explore synovium histologic features, in order to evaluate immune cells infiltrations and to correlate the condition with therapeutic treatment choices.

Finally, to evaluate the expression of $CD4^+$ (T-lymphocytes), $CD20^+$ (B-lymphocytes), $CD68^+$ (macrophages) in RA patients' synovium and to associated the pattern to the therapeutic treatment.

Investigation Phase

The study was initially performed with a small number of cases and it was mainly focused to optimize the preservation and storage conditions of samples and setting up the molecular analyses. Although preliminary results obtained have not the power to reach statistical significance, they could indicate a visible trend and set up a future wider project.

For each patient included in the study anamnesis includes the following:

Clinical data:

- Patients initials and date of birth
- Gender □ F □ M
- Disease Length
- Biopsy localization
- Therapy

Obstetric History (before disease onset):

n° pregnancy: ……..

abortions	yes □ n° …..	no □
sons	yes □ n° …..	no □
daughters	yes □ n° …..	no □
Menopause	yes □	no □

6.3 Materials and methods

Subjects

Subjects were identified at the Gaetano Pini, CTO Hospital, Milan, Italy (Director: Prof. *R. Viganò*), with the collaboration of Dr. *Andrea Fossali*.

Female RA patients were recruited as cases, whereas male RA patients or subject not affected by RA should be used as controls. Subjects enrolled in the project provided biopsy sample of synovial tissue (stored in fixative and lysis buffer) and whole blood sample (EDTA Vacuette® vials). Human tissues were collected from Italian Rheumatoid Arthritis patients whose informed consent was obtained in writing in accordance with the Helsinki Declaration and the institutional review board.

Human tissue specimens of patients were obtained during arthrocentesis of tibial-tarsal, knee or coxofemoral joints. Samples were immediately placed in lysis buffer (pH 7.4) for the subsequent

genomic DNA extraction, in formalin for wax-embedding and corresponding whole blood samples were collected and storage at -20°C. All samples were transported from Milan to Ferrara and then processed.

Genomic DNA isolation

-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 lower amount of blood sample, gDNA was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer'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.

-Biopsy tissues

Tissue samples were sectioned accordingly to the histological specimens, considering for each patient total tissue, synovial tissue and cartilage tissue. The first subdivision has been made using microscope (Nikon Eclipse TS100); then, synovial material, the site of inflammation, was manually selected with disinfected forceps. After homogenization, genomic DNA was extracted using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) according to the manufactures's instructions, as earlier describes. From all the cases identified has been possible obtained the three different specimens.

MICROCHIMERISM DETECTION

Male fMC Analysis

The analysis of male fetal microchimerism (fMC) was based on the detection of Chr-Y sequences and it was carried out using the Custom Assay PrimeTime Standard qPCR (IDT, Tema Ricerca, Italy) and the specific PCR Master Mix (IDT, Tema Ricerca, Italy). The Custom Assay was specific for TSPY-1 gene, present in 35 copies localized in Y-Chromosome. The assay was composed by two primers 5'-TTCCCCTTTGTTCCCCAAA-3' and 5'-CATCCAGAGCGTCCCTGG-3' and the

PrimeTime Assay Std probe: /56-FAM/CGAAGCCGA/ZEN/GCTGCCCATCA/3IABkFQ/ requiring HPLC purification. Analyses to investigate the presence of fMC have been performed using 10ul of gDNA at 5ug, corresponding to 50ng in total. PCR condition for all reaction were as follow: 95°C for 3 min, then (95°C for 15 seconds and 60°C for 1 min) x 55 cycles and the final hold was 25°C for 10 minutes, using 7300 Real Time PCR System (Applied Biosystems by Thermo Fisher Scientific). The output was analyzed on the 7300 System Software (Applied Biosystems by Thermo Fisher Scientific). fMC experiment included scalar dilutions of male DNA (range 10ng/µl-10^{-6}ng/µl) in female DNA samples 10ng/ul to carried out calibration curve. Number of cycles (n°ct) needed to detect the rise of the curve and the point of flush has been identified for each sample and according to Y-Chr signal level, the number of male genome equivalents was determined.

β-Globin Analysis

In the same male fMC running plate, but in separates wells, β-globin assay was performed for each sample, as internal control to check the effective presence of gDNA. It has been used a Custom Assay PrimeTime Standard qPCR (IDT, Tema Ricerca, Italy) and the specific PCR Master Mix (IDT, Tema Ricerca, Italy). The assay was composed by two primers 5'-GTGCACCTGACTCCTGAGGAGA-3' and 5'- CCTTGATACCAACCTGCCCAG-3' and the Prime Time Assay Standard probe: /56-FAM/AAGGTGAAC/ZEN/GTGGATGAAGTTGGTGG/3IABkFQ/ requiring HPLC purification. Analyses to investigate the presence of β-globin have been performed using 10ul of gDNA at 5ug, corresponding to 50ng in total. PCR condition for all reaction were as follow: 95°C for 3 min, then (95°C for 15 seconds and 60°C for 1 min) x 55 cycles and the final hold was 25°C for 10 minutes, using 7300 Real Time PCR System (Applied Biosystems by Thermo Fisher Scientific). The output was analyzed on the 7300 System Software (Applied Biosystems by Thermo Fisher Scientific). β-Globin experiment included scalar dilutions of male DNA (range 10ng/ul-10^{-6}ng/ul) to carried out calibration curve. Number of cycles (n°ct) needed to detect the rise of the curve and the point of flush has been identified for each sample to evaluate the effective gDNA concentration.

Statistical Analysis

The quantification of fMC has been performed referring to scalar dilutions of male DNA (range 10^{-3}-10^{-9}) in female DNA samples and considering genome equivalents concerning to Y-Chromosome. β-Globin has been used as internal control to assess the exact concentration of sample gDNA. Logistic regression, belonged to generalized linear models, has been performed in order to measure the relationship between the categorical dependent variable of Y-Chr detection and the gDNA concentration, using MS™Excel and GraphPad packages. Statistical analyses of Odds Ratio (OR) with 95% Confidence Interval (CI) and p-value with Fisher's exact test have been carried out using MS™Excel and GraphPad packages. A p value < 0.05 was considered significant.

HISTOLOGICAL INVESTIGATION

Hematoxylin/Eosin staining

Rheumatoid Arthritis specimens obtained from arthrocentesis were fixed in 10% buffered formalin and embedded in paraffin. For histological and morphological observation, the sections (5 μm thickness) of the formalin fixed synovial tissue from paraffine blocks were deparaffinized in xylene and rehydrated in alcohol gradients and then stained with hematoxylin and eosin (H/E) (Sigma). Tissue with identifiable synovium was obtained from all 6 subjects and the morphological characteristics of these samples were studied.

Preparation of samples and immunohistochemical staining

Unstained sections of the formalin fixed synovial tissue from paraffin blocks were deparaffinized through xylene immersions for three changes of five minutes each, followed by graded alcohol immersions to water. Immunostaining was done using an automated immunostainer (Ventana BenchMark ULTRA, Tucson, Arizona, USA), according to the manufacturer's instruction and program. IHC has been performed with the collaboration Prof. Lanza, Anatomy Pathology Unit of the Sant'Anna University Hospital, Ferrara.

Antibodies used, clone, and dilutions were as follows: Rabbit Anti-Human CD4 Monoclonal Antibody (Clone SP35) (Spring Bioscience) 1:50; Anti-CD20 primary antibody (Ventana, Tucson, Arizona, USA), 1:20, and Monoclonal Mouse Antibody to Human Macrophage CD68 (Clone PG-M1), (DBS Pleasanton, CA) 1:200. All assays used citrate buffer and low wattage microwaving for antigen retrieval. The enzymatic procedure used the three-step peroxidase-catalyzed reaction and DAB (3,3'-Diaminobenzidina) as a substrate: more precisely, the molecule of interest was targeted by antibody conjugated with a peroxidase enzyme and in the presence of hydrogen peroxide, DAB is readily catalyzed to its oxidized form forming a brown precipitate.

Microscopic analysis of synovial tissue and cell counting

After enzymatic procedure, immunohistochemically stained synovial tissue sections were examined using a light microscope, in a random order and by an observer unware about clinical data. The expression of CD4+ (T cells), CD20+ (B cells) and CD68+ (macrophages) in the sublining was assessed. The manual counts of the obtained images have been performed, which were then processed in Fiji software (imagej.net/Fiji). (Abramoff M. 2004). Although longer and requiring more attention, manual cell counting was demonstrated to be reproducible and effectively superimposable compared to automated cell-counting results. Automation could be considered more practical and an additional technique, in terms of data acquisition (Diem K et al., 2015).

Images were cropped, scaled to μm and separate by color channel, in order to removed artifacts; an area tool was used to select the biopsy region and to calculate the area. Manual counting has been

carried out using the Fiji cell counter tool, which labelled the cells with colored dots. The results were saved to a spreadsheet and screen shots were used to record the session. Count cross checking has been performed with the automated cell counting done with Immunohistochemistry (IHC) Image Analysis Toolbox (imagej.nih.gov/ij/plugins/ihc-toolbox/index.html).

Statistical Analysis

Cells populations number were given and comparisons between patients under different treatment and cell subtypes have been performed using a two-sided unpaired t-Student test. Correlation analyses between age and cell subpopulations were done. All statistical calculations have been performed using Graphpad software. As we undertook multiple comparisons, a Bonferroni adjustment was done and results were considered significant for a probability (p) value of 0.016 (=0.05/3).

6.4. Results

Overall, six case have been identified in the study, two males and four females. Mains clinical subjects' information were showed in the Table 6.1, whereas obstetric history of women RA patients were reported in the Table 6.2 below.

	Cases					
Codes	**RA001**	**RA002**	**RA003**	**RA004**	**RA005**	**RA006**
Age (years)	56	30	72	79	74	76
Gender	F	M	F	F	M	F
BMI	21	26	22	26	23	26
Smoking	N	Y	N	N	Y	N
Treatment: Salazopyrin (1) Methotrexate (2) Hydroxychloroquine (3) Chlorochin (4) Leflunomide (5) Gold (6)	1	2	-	3 2 4	-	3 5 6

Table 6.1 *Subjects basal characteristics.*

	Cases					
Codes	**RA001**	**RA002**	**RA003**	**RA004**	**RA005**	**RA006**
Age (years)	56	30	72	79	74	76
Gender	F	M	F	F	M	F
Menarche (age)	13	-	14	12	-	13
Menopause (age)	50	-	53	50	-	52
Pregnancies (n°) 1° child (M/F) 2° child (M/F)	1 F	-	2 M M	2 M M	-	1 F
Miscarriages (n°) 1°(Spontaneous/Volunteer)	1 V	-	-	-	-	1 S

Table 6.2 *Obstetric History of Female RA patients.*

Concerning previous therapeutic treatment, two patients (RA003 and RA005) did not assume any drug, whereas RA001 and RA002 were under stable therapy, respectively with Salazopyrin and Methotrexate. The remaining two patients RA004 and RA006 noticed continuing shifting therapies. Overall, it has to underline none subject was under therapeutic treatment of blocking $CD4^+$, $CD20^+$, $CD68^+$ pathways, such as Abatacept or Rituximab.

Considering obstetric history of the four female cases, two reported 1 daughter, whereas the other two described 2 sons each. Abortions details regards one case with 1 previous elective event and one case who have experienced 1 spontaneous episode.

6.4.1 MICROCHIMERISM DETECTION

Male fMC analysis

Firstly, calibration curve with serial dilution has been carried out, it has been reported in the Fig. 6.1.

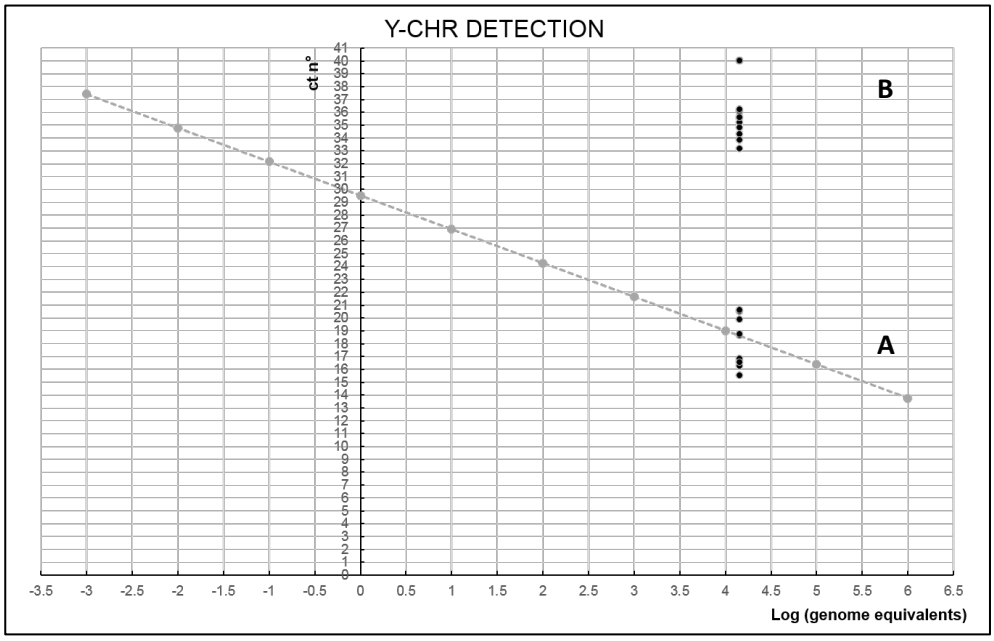

Figure 6.1 *Y-Chr detection referred to logarithm of genome equivalents.*
***A**. Spots group with reliable signal; **B.** Spots group with no detectable signal*

As showed, genome equivalents were considered in logarithm scale and the value 4.15 corresponded to the genomes number in 50ng of total gDNA. Briefly, 1 genome equivalent is equal to 0.0035ng, so considering the gDNA concentration=50 ng (it has been used 5ul of gDNA with the concentration 10ng/ul as the first value), in diploid genetic patrimony it corresponded to a total number of 14285.71 genome equivalents. Repeating the same reasoning for all gDNA serial dilution (10ng/ul-10^{-6}ng/ul), the number of genome equivalents comes out for each concentration used. Shifting all those values into logarithm scale, calibration curve has been created showing each point corresponding to the logarithm of genomes equivalents, referring to each serial decreased concentration of gDNA

(10ng/ul-10^{-6}ng/ul) (X-axis). Y-axis reported the correspondent n°ct needed to detect the rise of the curve and the point of flush. Analyses to investigate the presence of fMC in RA patients were performed using, from each case, multiple genomic DNA (gDNA) samples extracted from different sources: Peripheral Blood, Lysis Buffer of whole tissue sample, Core of whole tissue sample, Synovial tissue and Cartilage tissue. This multiple sampling was in order to detect eventual discrepancies of male fMC across different specimens from the same subject. For male fMC analyses has been used 10ul of gDNA at 5ug, corresponding to 50ng in total. Each spot depicted in Figure 6.1 was a sample and all of them have been localized in the x-value corresponded to the concentration of gDNA used in the analyses. Y-axis reported the number of cycles needed to detect the rise of the curve and the point of flush and earlier the signal has been detected (corresponded to low n°ct), higher was the concentration of Y-Chromosome

According with the calibration curve, the group of samples spots in the range of 15-22 cycles (A group) corresponded to a high presence of Y-Chr and they have been recognized as male subjects RA002 and RA005. The spots group B has been detected in the range of ct n° 33-40 and considering calibration straight line it corresponded to very low concentration of genome equivalents, roughly in the range of 1 male cell in 100000 female cells, comparable to no signal. The results did not reveal male fMC in female RA subjects.

For the best graphical interpretation, the number of genome equivalents detected in each specimen has been reported in the Figure 6.2. The two male patients, RA002 and RA005, were used as full-scale reference. The analysis of the four female cases, RA001, RA003, RA004 and RA006, turned out in no detectable level of Y-Chr sequences, regardless of their obstetric history.

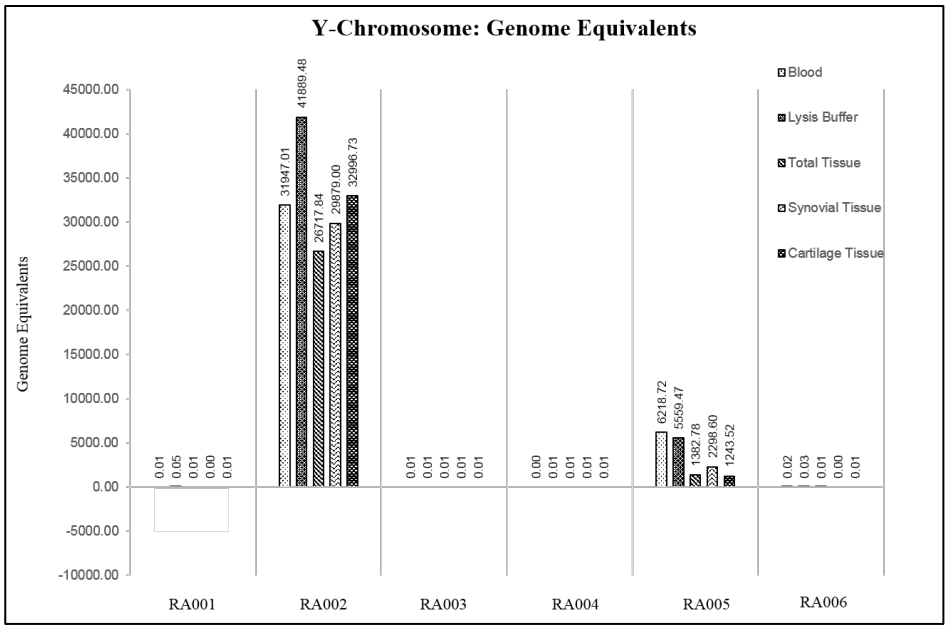

Figure 6.2 *Genomic equivalents for RA subjects, regarding each specimen.*

For female cases, due to complete negative results, stratifying upon sample specimens was inconclusive, as well as performing all the association analysis (case-control model, fMC association with previous obstetric history data, fMC association with therapeutic treatment).
The analysis of Y-Chr level in specimens of the two male cases showed comparable results in peripheral blood and in tissue samples.

6.4.2 HISTOLOGICAL INVESTIGATION

Hematoxylin/Eosin staining results

Along with DNA extraction and evaluation of the presence of male fMC, histological representation of synovial tissue is crucial to assess inflammatory status. Hence, it is interesting to evaluate lymphocyte infiltration in the tissues of different patients and, eventually, to relate their status with treatment efficacy. As depicted by H/E staining (Figure 6.3), the synovial tissues of each patient (reported as Figure 6.3 A, B, C, D, E, F) showed differences in the morphological structure, organization, lymphocytes distribution and infiltration. Considering histological grading, figure 6.3 A (RA001) and B (RA002) showed similar condition, with low levels of infiltration; D (RA004) and F (RA006) were analogue each other with mild, but clearly visible presence of lymphocytes. Pics C (RA003) and E (RA005) exhibited the highest concentration of lymphocytes, that were spread throughout the whole sample.

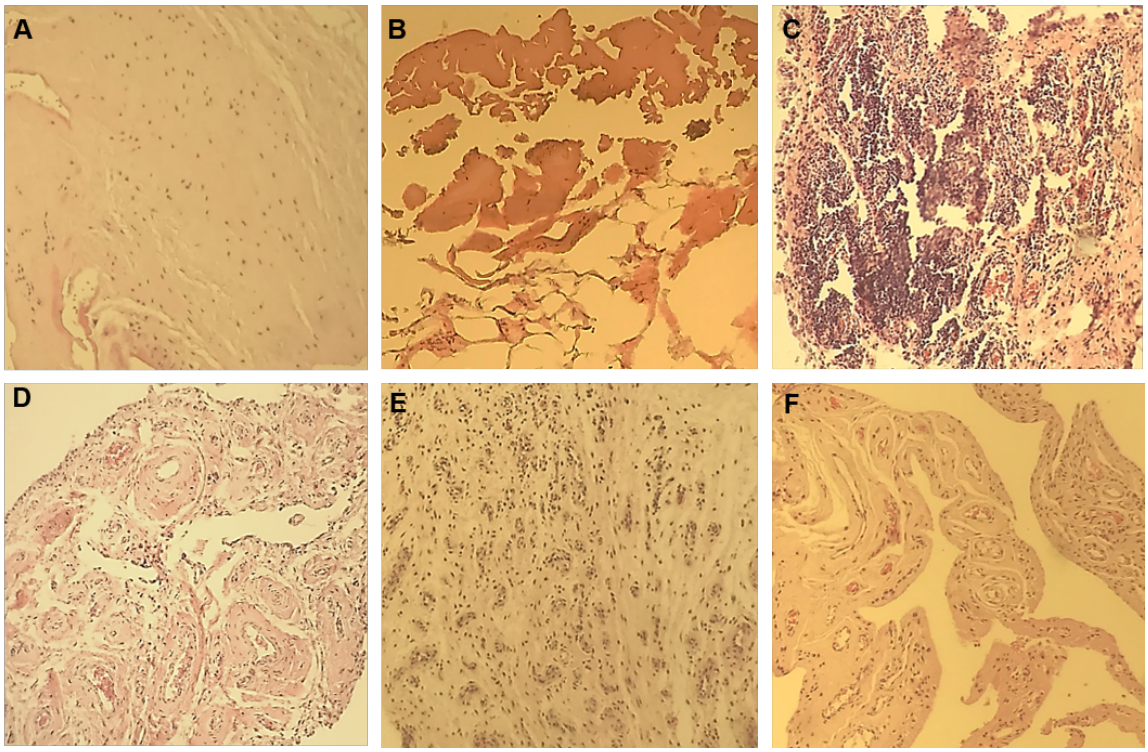

Figure 6.3 *Rheumatoid Synovia, Paraffine section 5µm, haematoxylin-eosin staining, 10x.*
*Histological differences among subjects **A**. RA001; **B**. RA002; **C**. RA003; **D**. RA004; **E**. RA005; **F**. RA006.*

Morphological analysis

Case RA005 was considered for deeper histological investigation of synovial tissue. Figure 6.4 below shows different areas of synovia. Figure 6.4 A displays the areolar type of synovium, the most specialized form, often crimped into folds, which might disappear when stretched or it might contain projections or villi. It was possible to detect a continuous layer of lining cells often two or three deep on the tissue surface and immediately beneath these cell capillaries are shown with a deeper plexus of small arterioles and venules, associated with mast cells. Lymphatic vessels were widespread and numerous and high level of leucocyte infiltration is identified. Figure 6.4 B shows the adipose synovium, and Figure 6.4 C the sub-intimal border zone.

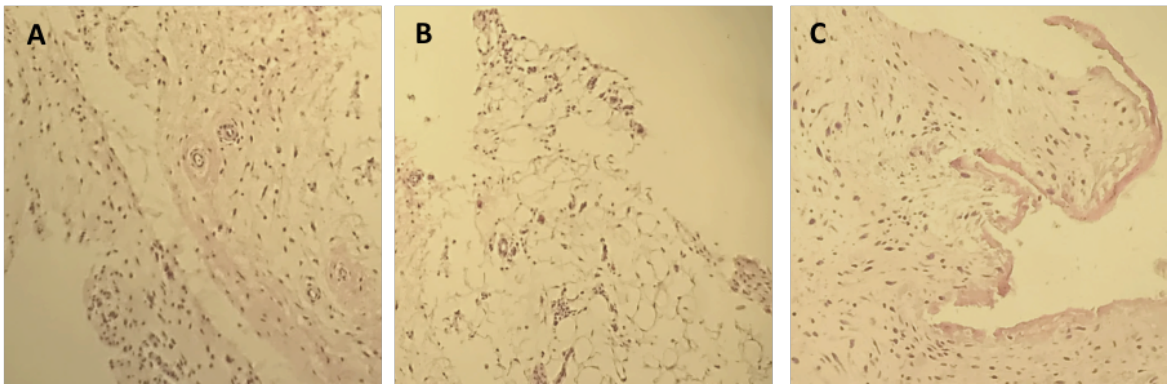

Figure 6.4 RA005. *Rheumatoid Synovia, Paraffine section 5µm, haematoxylin-eosin staining, 10x.*
***A**. Aerolar form; **B**. Adipocytes; **C**. Subintima Border Zone.*

Histological Grading

As previous reported, case RA005 has been taken as a remarkable model to investigate about the RA synovium pattern and histopathological assessment of synovial membrane. The grading of the synovial membrane was carried out on routine haematoxylin and eosin (H&E)-stained slides, according to the three synovial membrane features: synovial lining cell layer (already shown in Figure 6.4), stroma cell density and inflammatory infiltrate. The ranking of alterations being on a scale from none (0), slight (1) and moderate (2) to strong (3) (Figure 6.5). Considering the three synovial membrane features, the grading system came out from the summary of the parameters and interpreted as follows: 0, no synovitis; 2–4, low-grade synovitis; and 5–9, high-grade synovitis (this grading system is based on and resembles an advancement of the grading system first proposed at the 18th European Congress of Pathology (Krenn V et al., 2001).

In our case, we have detected only RA005 histological grading, in order to evaluate lymphocyte infiltration level. As depicted in the Figure 6.5, we have evaluated different condition in the same sample, accordingly to the area of interest. It has been evident the leucocyte infiltration in all the synovium, but the distribution should be dependent on the position of the blood vessels: less concentration far from them (Fig 6.5 A), whereas it rose up closest (Fig 6.5 B). Fig 6.5 C showed the

higher histological grading of leucocyte infiltration, where was possible to see all of the spots centralized in the same area.

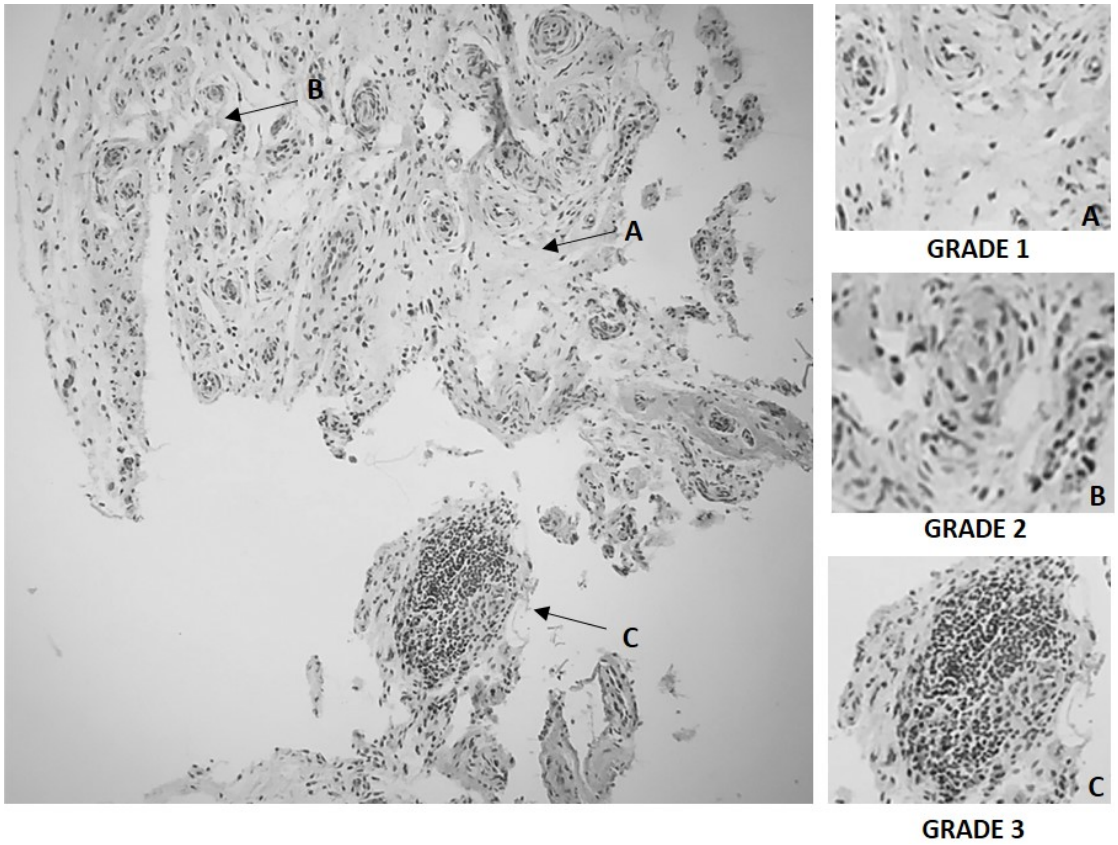

Figure 6.5. RA005. *Rheumatoid Synovia, Paraffine section 5µm, hematoxylin-eosin staining, 10x. Histological Grading: aggregates of Grade 1 (A). Grade 2 (B), Grade 3 (C).*

Immunohistochemical Analysis

The technique has been performed in order to evaluate and discriminate the concentration of lymphocyte subdivided in the different population. The RA joint includes a wide range of immune cell type, such as monocytes, $CD4^+$ T cells both inflammatory and regulatory, $CD20^+$ B cells and macrophages ($CD68^+$). Pictures have been summarized and reported in the Figure 6.6.

As previous described in H/E stained pictures, RA001 and RA002 patients did not show high inflammatory and autoimmune cells infiltration, thus these two cases were not stained with the immune-histochemical markers $CD4^+$ $CD20^+$ and $CD68^+$. Concerning the other four cases, all of them shown high $CD4^+$ T-leucocytes infiltration and aggregation (Figure 6.6 left column).

The pattern of cells distribution was diffused for RA003, perivascular in RA005 case and regarding the other two subjects, CD^+ T cells were localized following the tissue layer and borders; probably due to fragmentary synovium. Considerable variation of synovial tissue between patients were observed for CD20 (Figure 6.6 central column) and CD68 markers (Figure 6.6 right column).

According to results noticed in H/E staining, on one side RA003 and RA005 cases followed a similar direction, showing remarkable concentration of $CD20^+$ B-lymphocytes and very low $CD68^+$ infiltration, comparable to absence.

On the other side, RA004 and RA006 manifested likely the same status, but in contrast respect the previous two: there were suggestions of higher number of macrophages $CD68^+$ than $CD20^+$ B-cells.

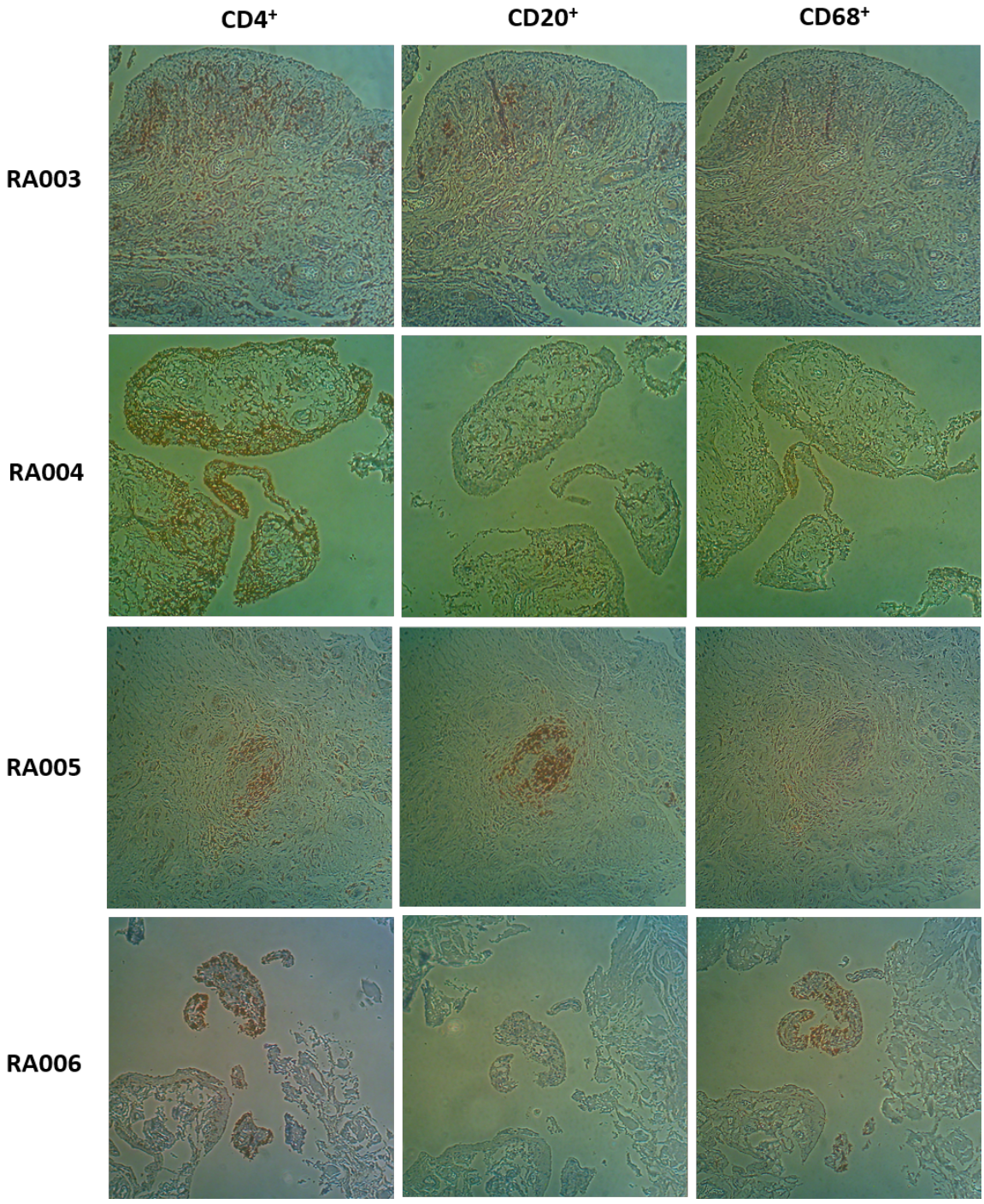

Figure 6.6. *IHC detection of infiltration by CD4+ T cells, CD20+ B cells, CD68+ macrophages in synovial tissue of Rheumatoid Arthritis subjects.*

A summary of cell counts subpopulation, staining positive in the RA synovium, has been provided in Table 6.3. As previous depicted in the Figure 6.6, all cases detected the presence of CD4+ T-lymphocytes, whereas the distribution of the CD20+ and CD68+ cell markers was similar in the cases with similar therapeutic treatment conditions.

Cases	Cells Subpopulations		
	$CD4^+$	**$CD20^+$**	**$CD68^+$**
RA003	33	19	8
RA004	29	5	11
RA005	28	17	6
RA006	14	5	12

Values are expressed in n°cells/mm^2

Table 6.3 *Distribution of cells subpopulation in synovial tissues of RA cases.*

Comparison of the subjects according to whom had not received previous therapies and those whom have shifted several treatments have been reported in the Table 6.4.

Comparison	Cells Subpopulations		
	$CD4^+$	**$CD20^+$**	**$CD68^+$**
Subjects (n=4)			
No therapies (n=2) RA003-RA005	33-28	19-17	8-6
Several therapies (n=2) RA004-RA006	29-14	5-5	11-12
unpaired t-student test	1.138	13.00	4.025
p-value	0.373	**0.006**	0.056

Values are expressed in n°cells/mm^2

Table 6.4 *Comparison of cells subpopulations between RA cases according to treatments.*

On comparing patients regards $CD4^+$ T-lymphocytes infiltration, no significant differences between the two groups were found (p value=0.373). $CD20^+$ B-cells macrophages population noticed higher more infiltration in subjects without therapies, reaching the statistically significant value (p=0.006). There were observations of greater numbers of $CD68^+$ macrophages population in subjects whom have changed several treatments: results shown a remarkable trend almost reaching the statistically significant value (p=0.056), probably due to the small sample size.

There was no correlation between numbers of various cell subpopulations and the age of the subject, gender or with others details on clinical history in either groups (data not shown).

6.5 Discussion and Conclusions

Studies have revealed that long-term persistence of fetal cells in the mother (fetal microchimerism fMC) and maternal cells in her progeny (maternal microchimerism mMC) could be both related to autoimmune diseases development, localizing in the blood circulation and specific affected tissues (Hromadnikova I et al., 2008). It has been reported that higher levels of fMC have been found in peripheral blood and skin from women with various autoimmune disorders, involving mainly systemic sclerosis and systemic lupus erythematosus (Sawaya HH et al., 2004; Gannagé M et al., 2002). Concerning Rheumatoid Arthritis (RA) studies claimed to have individuate fMC presence in rheumatic nodules (Chan WF et al., 2012).

Based on previous finding, the aim was to evaluate fMC manifestation in female subjects affected by RA and either in their synovium. Probably also due to low sample size, our results on women RA blood and synovial specimens did not reveal fMC presence. Nonetheless, this finding could corroborate the repairing hypothesis of fetal cells in case of maternal injures, such as in joint inflammation. In fact, reports suggested that hematopoietic stem cells (CD34β), hematopoietic progenitor cells (CD34β, CD38β), mesenchymal stem cells (MSC) and mesenchymal progenitor cells (MPC) of fetal origin may persist in a maternal stem cell niche. In the eventuality of damages, these cell subtypes home to the damaged organ and differentiate as part of maternal healing response contributing to tissue repair and adopting various phenotypes (Huu SN et al., 2006). MPC also demonstrate high proliferative capacity and they are able to differentiate into several mesenchymal lineages, including bone, cartilage, fat, tendon, and stromal tissue (Pittenger MF et al., 1999). Moreover, MPC also have a great potential to repair damaged bone and cartilage and are likely to contribute to joint regeneration (O' Donoghue K et al., 2003).
Hence, fMC positive detection should be linked to women with mild or moderate status of RA disorder. This subgroup of women might show stable condition or symptoms reduction especially due to fMC action. Besides the hypothesis of fMC cells presence in the RA synovium, it is well known that synovial tissue (ST) is characterized by several immune cells infiltration leading to relevant changes in its architecture, vascular and lymphatic alteration and cytokines and chemokines increased production (Smith MD, 2011).

With regard to H/E staining, results showed a link between infiltration and patients' pharmacological treatment. In fact, the two subjects (RA003 and RA005) with highest total immune cells infiltration did not receive previous therapies; the two cases (RA004 and RA006) whom have changed several treatments showing likely the same mild status. Respectively, they were both under Hydroxychloroquine, then Methotrexate and Leflunomide and finally Chloroquine and Gold, suggesting the necessity to shift or to switch therapies in order to reach the best efficacy. The lastly two patients (RA001 and RA002) exhibited the lower level of leucocytes presence in the synovial

tissue, were under treatment, respectively Salazopyrin and Methotrexate. Considering the poor infiltration, therapies allowed the expected responsiveness and patients did not require the switch to another one. These observations indicate that the good therapy modulate the leucocytes infiltration of synovial tissue. In particularly, the early proper treatment could modify immediately the inflammation status leading to symptoms control and stabilization or remission of the disease. Several studies confirmed the necessity to find out the best effective therapy, able to modulate immune cells infiltration and contributing to subtype cells depletion in synovial layer. (Utomo L et al., 2016).

Concerning morphological analysis and the feature of synovium different area, adipocytes are found mainly in fat pads, as well within villi. There is a complete intimal cell layer and a superficial net of capillaries. The intima might lie directly on adipocytes but is often separated by a band of collagen-rich substratum, while the deeper tissue is fat. Villi usually shows a central arteriole and venule, but could sometimes be avascular. Studies shown that the amount of fat in villi varies and probably decreases with age, with an increase in fibrous tissue (Smith MD. 2011). In RA synovium, intimal cells increase in number. They belong to two cell types: type A and type B sinoviocytes. Type A sinoviocytes have cell surface markers typical for macrophage lineage and presumably derive from blood monocyte. Type B sinoviocytes, instead, have a fibroblast lineage and are locally derived. The increment of synovium intima cells is probably the outcome of several processes, with macrophage influx from the vascular compartment under cytokine and cell adhesion molecule control being the dominant process (Singh JA et al., 2007).

Furthermore, considering several observations of increased production of proinflammatory cytokines of the Th1 subset in RA disease (Janossy G et al., 1981) and of synovial pathologic hallmarks (Brennan FM et al., 1995), CD4+ T cells are considered remarkable in the pathogenesis of RA (Turesson C et al., 2005) and the interplay between $CD4^+$ T cells and monocyte/macrophages could contribute to the synovial phenotypes and patterns. It has been reported that investigating the two populations by IHC with specific antibodies to $CD4^+$ $CD68^+$ molecules would be particularly informative (Kraan MC et al., 1999; Roberts CA et al., 2015). Moreover, a subgroup of $CD4^+$ has found to be involved in the pathway of B-lymphocytes cells, coordinating the production of antibodies and huge number of cytokines. Studies have shown B-cells induced depletion, and in particular the $CD20^+$ subset decreasing, are a visible target to RA therapeutic treatment (Leandro MJ et al., 2006). Based on findings in H/E, which reported the poor cells infiltration in two cases, IHC analysis has been performed in the others four cases, evaluating the presence of three markers, $CD4^+$ T-lymphocytes, $CD20^+$ B-lymphocytes and $CD68^+$ macrophages.

The results of immune-histochemical staining showed different patterns among patients: in fact, two revealed higher amount of $CD20^+$, whereas the other two subjects increased number of $CD68^+$. This finding is supported by the observation that all subjects depicted diffuse and perivascular higher

levels of $CD4^+$ T-lymphocytes, involved in both, B-lymphocytes and macrophages pathways, as previous described. More precisely, considering the relevant role of B-cell depletion in quite stable and satisfactory health condition of RA patients, the two cases presenting lower or almost not detectable amount of $CD20^+$, were corresponding to whom have changed several therapies, in order to reach a symptoms stabilization over time. The CD4/CD68 interplay remained univariate and patients showed these two hallmarks in their synovium. Result confirmation takes place considering H/E staining: the two cases reported total moderate cells infiltration.

On the other side, the two patients not under previous treatment, presented greater CD20+ infiltration and not considerable CD68+ detection, this pattern referred to a more severe condition and phenotype. Among cases, they presented the higher level of synovial tissue widespread infiltration, as depicted in H/E figures. In addition, studies found CD20 antigen expression not exclusive to B cells; there was a small population of circulating T cells and NK cells that expressed low levels of CD20 and they could contribute to more severe phenotypes of patients showing CD20 positivity, exacerbating the RA disorder (Vieccelli D et al., 2016). Shifting observations to statistical results, the comparison between the two groups concerning $CD4^+$ T-lymphocytes was not significant (p value=0.373), confirming the relevant role of this cell subpopulation in RA disorder and its involvement in several pathways, regardless to therapies. The difference on $CD20^+$ B-cells subpopulation between the two groups reached the statistical value (p-value=0.006), whereas the difference on $CD68^+$ macrophages subtypes groups was not significant (p=0.056). The inability to detect differences between $CD68^+$ in the two set may reflect type II error resulting from the small sample size.

These observations indicate that the good therapy modulates the leucocytes infiltration of synovial tissue. In particularly, the early proper treatment could modify immediately the inflammation status leading to symptoms control and stabilization or remission of the disease. Further analyses could be carried out in a larger case-series, it is known there is still much be learned about the immunological microenvironment of synovial articular tissues.

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