CHAPTER 7

ENVIROMENTAL CONTRIBUTION AND EPIGENETICS APPROACH:

COULD WE CHANGE OUR FAITH?

"DNA is recipe, not a blueprint:

a recipe in a kitchen book is in no sense
a project for the cake coming out from the oven"

Richard Dawkins, The Blind Watchmaker,
Norton & Company Inc, 1968

CHAPTER 7

ENVIROMENTAL CONTRIBUTION AND EPIGENETICS APPROACH

COULD WE CHANGE OUR FAITH?

Table of Contents

Abstract

Background: Understanding epigenetic mechanisms in the pathophysiology of autoimmune diseases, as Rheumatoid Arthritis (RA), will help to individuate new pathways of susceptibility risk and to disease severity and to establish more target-directed therapy with reduced systemic side effects. Only few studies explored the global DNA methylation obtained controversial results, and no analyses have been reported on individual specific gene HLA-G and MTHFR methylation.

Objective: To investigate the pattern of methylation status from healthy subjects, passing through early symptoms, to established RA diseased patients. In addition to explore eventually sexual dimorphism and the epigenome variation accordingly to disease activity improvement.

Materials and methods: Genomic DNA was extracted from a total of 160 subjects: 30 RA patients, 6 of them complete of tissue specimens and corresponding blood sample; 50 ERA T0, 50 ERA T6 and 30 healthy matched controls. LINE-1 global methylation and specific HLA-G and MTHFR genes methylation analysis have been performed through Sodium Bisulfite Conversion and Pyrosequencing. Statistical analyses of paired and unpaired student t-tests, Anova and p-values have been carried out using GraphPad Prism 6.0.

Results: Globally methylation of LINE-1 did not reveal differences in the patients' groups comparing to controls, as well as the stratification of ERA T6 accordingly to ΔDAS28 failed to report significant results. Concerning HLA-G methylation, the sex stratification showed significant hypermethylation in female RA patients, respect to RA males (p value= 0.0103); this result was noticed only in the RA case-series. Significant hypomethylation was identified in MTHFR gene, with a progressive rising to the increase of disease establishment: the methylation level was 29.8±5.57% for controls, 19.2±5.42% for ERA T0, 15.8±6.33% for ERA T6 and 7.3±2.11% for RA patients. Hypomethylation of individual gene was associated with increased gene expression and MTHFR is involved specifically in the folic acid cycle, target of methotrexate treatment and essential for pyrimidines and purines synthesis, cell replication and homocysteine metabolism.

Conclusion: Gene expression could be altered and modulated by methylation status, contributing to the pathogenesis of RA. HLA-G different methylation between sex suggests an implication of female parameters and inter-individual differences in the pathophysiology affecting RA development. Our interesting result on progressive MTHFR hypomethylation, underlies that this epigenetic state persists beyond RA stages, inducing a compositional remodeling of DNA methylation pattern.

7.1 Introduction

Genome-wide association studies (GWAS) have identified multiple common genetics risk factors of Rheumatoid Arthritis (RA) occurrence, the strongest of which are in the MHC region of chromosome 6, first of all HLA-DRB1 *0401, *0404 alleles, but also include others non-HLA genes, such as PTPN22, STAT4, PADI4 (Gregersen PK et al., 2009; Viatte S et al., 2013).

Despite the strong genetic basis, the multifaceted nature of RA disorder sustains the favored hypothesis claiming that complex etiology involves multiple genetic and environmentally-induced alteration. These can be expressed through epigenetics modification resulting in immune-dysregulation (Hedrich CM, Tsokos GC. 2011). Epigenetics refers to any process capable of modifying gene expression without altering the sequence of DNA bases and such modification can be traced from the cell to progeny as a result of exposure to specific environmental factors (Dupont et al., 2009). These phenomena are the bases of most cell differentiation processes, they appear to play an important role in fetal programming (Geraghty AA., Et al., 2015) and they can be inherited in a stable way by providing a possible "memory" of molecular exposure. Epigenetics encompasses mechanisms that go beyond the traditional conception of inheritance and is therefore the basis for understanding the relationship between organism and environment (Holliday R., 2006), allowing us to analyze interactions between exposures during the early stages of life and subsequent health outcomes (Yamada L., Chong S., 2017).

Different and several epigenetic mechanisms have been identified: DNA methylation, histone acetylation, phosphorylation, ubiquitination. Epigenetic modifications occur physiologically and are essential for the proper functioning of the body (ex: inactivation of X chromosome), but if they occur improperly, they can be the cause of serious repercussions. Several processes resulting in immune response alterations, such as reduction of DNA methylation, histones hypo or hyper acetylation, overexpression of some miRNAs, all were correlated with the onset and progression of some autoimmune disorders, as RA and SLE (Absher DM et al., 2013)

DNA methylation affects the cytosine base within the cytosine-guanine dinucleotide ("CpG") (Razin A., Cedar H., 1991). From a biochemical point of view, this modification consists of the substitution, by the enzyme DNA methyltransferase (DNMT), of the carbon-linked oxygen atom in position 5 of the cytosine base with a methyl group (with formation of 5-methylcitosan (5-MeCs). Methylation patterns are established at the beginning of development, modulated during specific tissue differentiation and altered in many diseases (Patterson K., et al., 2011). Approximately 80% of the CpG dinucleotides present in the mammalian genome are methylated (Cheung P., Lau P., 2005), while the remaining 20% is non-methylated and predominantly located at high density ("CpG Island") in promoter regions of constituent or inducible genes. Each potential methylation site in the genome is symmetric and it implies the inheritability of this modification; in fact, after duplication of the nucleic acid, DNMT specifics recreate the methylation profile of the parental DNA strand in

the newly synthesized DNA. This epigenetic modification is implicated in many processes, such as transcriptional regulation and modulation of chromatin structure. In addition, it has a stabilizing effect that promotes and guarantees the integrity of the genome, as well as a proper temporal and spatial expression of genes during development (Song F., et al., 2005). DNA methylation can suppress transcription by direct mechanisms (including inhibition of DNA-binding transcription factors) or indirect, due essentially to the effects of proteins that bind to the methylated cytosine base within the CG dinucleotide (Jaenisch R., Bird A., 2003).

In germ cells and embryos at early stages of development, epigenetic reprogramming processes on most of the genome occur through mechanisms of demethylation of the DNA and remodeling of histones; DNA methylation is therefore highly dynamic during development and differentiation in mammals. Restoring methylation levels to undifferentiated germ cells in male embryos takes place before birth, while the corresponding process in female embryos is completed after birth during the growth phase of the oocyte (Liu H. et al., 2016) . Epigenetic programming is a demonstration of how inter-individual variations at the epigenetic profile of newborns can be important to understand the role of epigenetic variations in human pathologies (Saffery R., et al., 2014).

Epigenetics analysis in RA represent a new area of research and recent studies reported widespread DNA hypomethylation in RA synovial fibroblasts, including hypomethylation of LINE1 retrotransposons (Neidhart M et al., 2000) that are repetitive elements normally repressed by DNA methylation. In these cases, loss of the repressive DNA methylation signal results in increased gene expression. A recent genome-wide study on RA synovial fibroblasts revealed a number of differentially (hypo- and hyper-) methylated genomic regions and most of the affected genes appear to be involved in inflammation, matrix remodeling, leukocyte recruitment and immune responses (Nakano K et al., 2013).

LINE-1 (or L1) elements belong to the LINEs (Long Interspersed Elements) class, included in the non-LTR retrotransference family. The human genome contains more than half a million copies (Lander ES, et al., 2001), which contributes to making up about 17% of the genome and they are one of the most abundant repeating elements. The complete element L1 is about 6.000bp and is comprised of an untranslated region in 5' (called 5'UTR, 5'UnTranslated Region), followed by two protein encoding regions important for the retransfer of L1 (Open Reading Frame) (Moran JV., Et al., 1996) and untranslated region 3 '(3'UTR) containing a polyadenylation signal and a rich variable poli-A tail (Babushok DV., Kazazian HH., 2007). It has been reported that L1 elements preferentially integrate into rich genome regions in AT, in accordance with their retrograde mechanism, and these regions generally do not include genes (Lander ES, et al., 2001). Recent studies have shown that the frequency of these elements is much higher than previously estimated and contributes significantly to genetic diversity, leading to no longer considering DNA segments without any influence but important to the genetic diversity, evolution of genomes and to give them a dynamic character

(Abecasis GR., et al., 2010; Iskow RC., et al., 2010). Due to of their frequency within DNA, the methylation state of LINE-1 has been proposed as a marker for estimating the global level of methylation of the DNA (Marques-Rocha JL., Et al., 2016).

Besides the global methylation, it has been mentioned that the majority of sites containing CpG islands are located in promoter region of the genes, and these sites are subject to methylation. The methylation of the CpG islands can interfere with the expression of a specific gene, although genetic repression appears to be largely indirectly through the recruitment of methyl-CpG binding domains (MBD), inducing changes in chromatin whose intensity depends on the density of CpG (Weber et al., 2007).

Studies indicated changes in methylation of methylene tetrahydrofolate reductase (MTHFR) gene promoter may be related to several diseases: a high percentage of methylation of the MTHFR promoter in mothers who gave birth to children with Down syndrome (Coppedé et al., 2016); the hypermethylation of the MTHFR promoter to pre-eclampsia phenomena (Ge et al., 2015). In addition, hypermethylation of the promoter appears associated to male idiopathic infertility (Wu et al., 2010), in recurrent abortions (Rotondo et al., 2012), kidney disease (Ghattas et al. 2014), in colon cancer (Oyama et al., 2004) and hypertension in a study conducted on the Mexican population (Pérez-Razo et al., 2015). Concerning MTHFR and RA disease, several studies have been observed its genetics variants association in the context of methotrexate (MTX) treatment, knowing that MTHFR is an important enzyme in the MTX pathway and is involved in folate metabolism and DNA synthesis (Shao W et al., 2017; Qiu Q et al., 2017); however, from the epigenetics point of view, inconsistent results have been reported. Li H. and colleagues suggested that methylation of molecules involved in the methionine cycle may exert a crucial role on immunocytes developments and activation, influencing the pathological processes and drugs responsiveness in immune disorders, such as RA and SLE (Li H et al., 2017). Thus, further elucidation in this direction may contribute to exploring the impact of MTHFR epigenetics regulation in RA.

With particular regard to the autoimmunity connotation of RA disease, HLA-G has been described as one of the molecules most associated in the regulation of inflammatory process (Catamo e et al., 2014) but so far, no studies have been carried out on its epigenetics affection and involvement. Moreover, it has been reported the link between HLA-G gene and women, especially referred to its role during pregnancy, contributing to maternal tolerance of semi-allogenic fetus (Hashemi M et al., 2017).

Hence, considering a range of environmental exposures could alter the epigenome, which has sufficient plasticity to react to the internal and external environment, and DNA methylation events have critical roles in gene regulation, epigenetic analysis in RA represent a new area of investigation with the potential to answer unsolved questions and likely to provide new insight into the disease.

7.2. Rationale and Aims

The main aim of the present study is to explore the epigenetic variation in different cohorts, evaluating the pattern of methylation from healthy subjects to RA established disease and observing the eventual proceeding in the methylation status, according to the RA progression.

More precisely, specific purposes would determinate:

- the genomic global methylation of LINE-1, to define the pattern of total epigenome in RA and ERA patients and in matched healthy subjects.
- the genomic methylation of the specific candidate genes HLA-G, focusing deeper on the epigenetic variation according to the sex.
- whether DNA methylation of specific candidate gene MTHFR is involved in the disease activity improvement and consequentially in the treatment responsiveness.

7.3 Materials and Methods

Subjects

- *Rheumatoid Arthritis patients (RA)*

RA subjects belonged to cases identified at the Gaetano Pini, CTO Hospital, Milan, Italy (Director: Prof. *R. Viganò*) with the collaboration of Dr. *A. Fossali*. Subjects enrolled in the project provided bioptic 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. In addition, in order to reach a congruous sample size, whole blood sample of RA subject has been obtained from cases 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. All RA cases satisfied the ACR/EULAR classification criteria (Aletaha D et al., 2010).

- *Early Rheumatoid Arthritis patients (ERA)*

ERA subjects were identified in the context of the multicentric Italian *A. Liberati* 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 Ferrara and the written informed consent was taken from all patients recruited. ERA cases satisfied the ACR/EULAR criteria (Aletaha D et al. 2010) and the characterization was evaluated during the first visit first visit (T0) and after 6 months (follow-up T6). Each time considered several clinical parameters associated to disease's state and collection of whole

blood sample. Due to technical problems regarding low sample quality or quantity, epigenetics analyses have been performed in a subgroup of patients.

The investigation comprised even a matched group of 30 *healthy controls*, whom maintained the female-to-male ratio 3:1 as in samples groups.

A code has been assigned to each subject enrolled in each cohort described and sensible data have 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.

-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.

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.

Sodium Bisulfite Conversion (BSP)

After genomic DNA extraction, titration and normalization, samples at 10ng/ul concentration were selected and Bisulfite converted using EZ DNA Methylation Lightning™ Kit (Zymo Research, Irvine, CA, USA) following manufacturer's instruction. Briefly, the ready-to-use Lighting Conversion Reagent was added to the DNA sample and then the reaction continued with DNA denaturation and bisulfite conversion. The processes are combined with added heat to facilitate rapid denaturation. Desulphonation and clean-up of the converted DNA is performed using a low-elution spin column. High yield, converted DNA is ready-to-use using the desired application. Treating DNA with bisulfite chemically modifies non-methylated cytosine into uracil (detected as thymine through PCR), methylated cytosine remains unchanged. Once converted, the methylation profile of the DNA is determined amplifying the region of interest with PCR and then processed for Pyrosequencing®.

Pyrosequencing

After Sodium Bisulfite Conversion, BiSulfite Products (BSP) were analyzed to measure *LINE-1, HLA-G* and *MTHFR* methylation level.

Regarding *LINE-1*, the assay was created according to manufacturer's instruction (IDT, Tema Ricerca), where Reverse primer is biotinylated for later pyrosequencing process. PCR reactions were performed in duplicates with total volume of 50ul containing 5ul of BSP-gDNA, 10XPCR buffer, 50mM MgCl2, 10mM dNTPs, 10pmol of each primer and 1U of Taq polymerase (Invitrogen Co., Carlsbad, CA). PCR cycling profile was following: 27 cycles of 94°C for 15s, 60°C for 30s and 72°C for 30s, followed by 72°C for 2 mins. The amplicon of 247bp was analyzed on 8% polyacrilammide gel using silver staining. The residual PCR product (pPCR) was transferred in two PCR plates with each well containing 37ul of binding buffer, 20ul of RNAase free distilled water, 3ul of sepharose beads containing streptavidin and 20ul of BSP-pPCR. The total volume of 80ul of the mixture was placed on the thermo-mixer. Following this, the pPCR was made single-stranded to act as a template in a pyrosequencing reaction by washing with ethanol 70% and denaturation buffer using a Pyrosequencing Vacuum Prep Tool (Bio-Stage). Two pyrosequencing runs were performed from each PCR reaction on PyroMarkQ96 ID® using PyroMark Gold reagents (Qiagen) using SQA mode. Each well had total volume of 40ul, including Annealing Buffer and Biotinilated sequencing Primer with final concentration of 10uM with suspended beads containing sample DNA.

During pyrosequencing run the dispensation order was: ACTCAGTGTTCAGTCAGTTAGTCTG, (Figure 7.1). The output of the two pyrosequencing run was combined. The average LINE-1 methylation level was calculated as the mean of the proportions of C (%) at the 3 CpG sites analyzed,

which were located at positions +319, +322 and +329 (positions of the corresponding Guanine in the forward DNA strand, in relation to the first nucleotide base of the consensus promoter sequence).

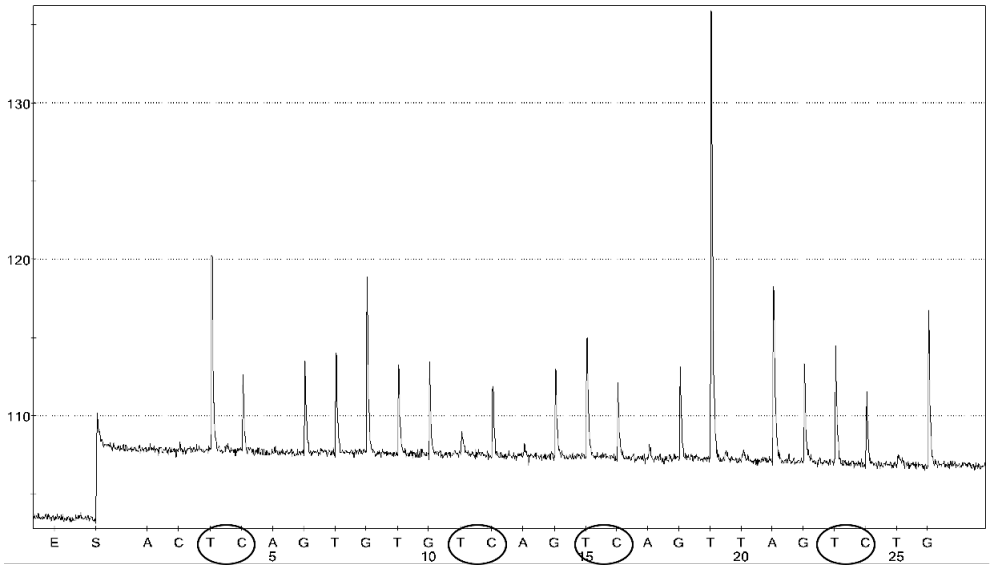


Figure 7.1 *LINE-1 pyrogram obtained from pyrosequencing.*
The nucleotides dispensation order was reported and sites of interest were circled

Regarding *HLA-G* and *MTHFR* regions of interest, the CpG assays were predesigned by Qiagen (PyroMark® CpG Assay, QIAGEN, Hilden DE) and reconstituted according to the manifacturer's protocol. After the same PCR conditions, amplicons of 119bp and 232 bp respectively were analyzed on 8% polyacrilammide gel using silver staining. Then the same procedure up to pyrosequencing results has been followed and methylation levels of HLA-G and MTHFR was calculated considering CpG sites in the sequences. Pyrograms output have been reported below (Figure 7.2 HLA-G; Figure 7.3 MTHFR).

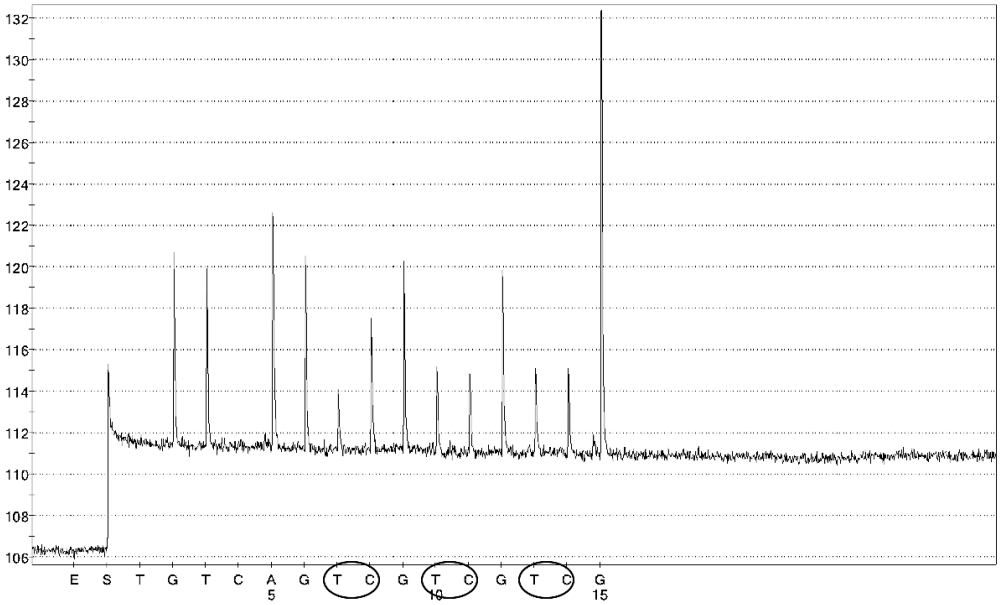


Figure 7.2 *HLA-G pyrogram obtained from pyrosequencing.*
The nucleotides dispensation order was reported and sites of interest were circled.

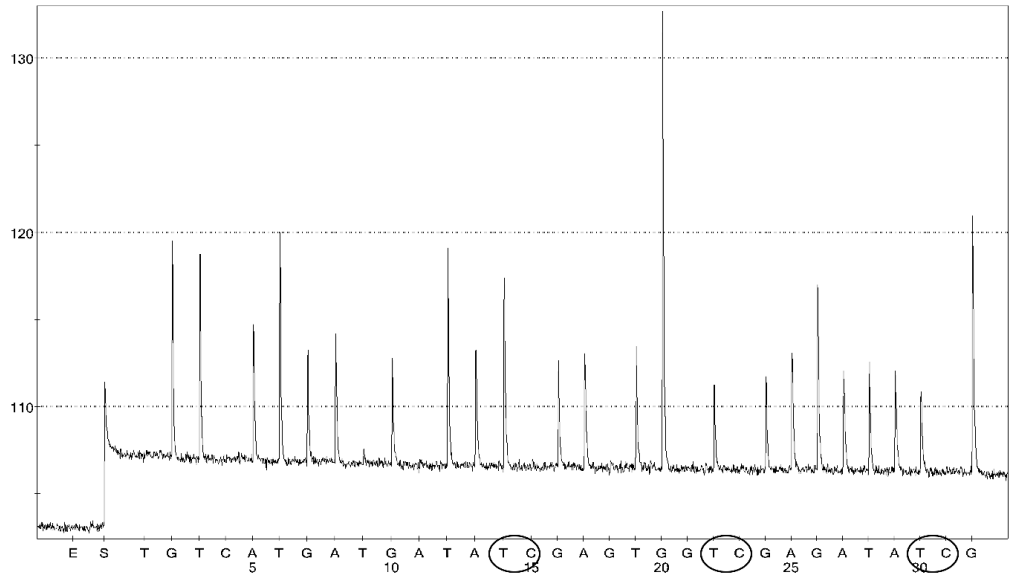


Figure 7.3 *MTHFR pyrogram obtained from pyrosequencing.*
The nucleotides dispensation order was reported and sites of interest were circled.

Statistical Analyses

Each ERA patient included in the study was under pharmacological treatment of steroid (Medrol®). To this, has been added cDMARD (Clorochin/Sulfasalazin/Methotrexate) depending on clinical evaluation. Response to treatment was evaluated accordingly to DAS28 variation in different follow-up points as table down describes (Table 7.1). EULAR Improvement Criteria identifies three groups of patients: Good or High Responders, Moderate Responders, Non-Responders.

Statistical analyses of students' t-test, Bonferroni Correction for multiple comparison, adjusted p value and ANOVA were performed using the GraphPad Prism 6.0. All p-value were 2-sided.

LINE-1, HLA-G and *MTHFR* methylation scores over all CpG islands within a sample were summed and the mean value was obtained. The average was compared between methylation level of ERA subjects at the first visit (T0) and after 6 months (T6) by paired students' t-test; whereas, for all others test for variables with two categories, the unpaired students' t-test was used.

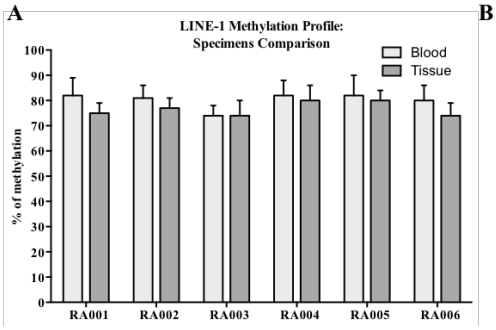
EULAR Response Criteria			
Value of composite measure at endpoint	Improvement in composite measure from baseline		
	> 1.2†	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2*	Good		
> 3.2 and ≤ 5.1		Moderate	
> 5.1			None

Table 7.1 *EULAR Response Criteria considering DAS28 variation.*

7.3 Results

First of all, in order to assess if the methylation score in PMBCs gDNA obtained from blood, could be comparable to tissue sample, analysis have been performed on RA cases, of which both specimens were available (6 cases). LINE-1 intra-individual global methylation has been reported in Figure 7.4 below and paired t-student tests have been carried out, to assess eventual statistical differences.

A



B

Case	|Δ| blood-tissue	± mean SD	paired t-student test	p-value
RA001	0.070	0.371	1.457	0.891
RA002	0.044	0.061	1.275	0.864
RA003	0.002	0.020	0.035	0.513
RA004	0.246	0.006	0.515	0.683
RA005	0.023	0.034	0.447	0.661
RA006	0.056	0.011	1.308	0.870

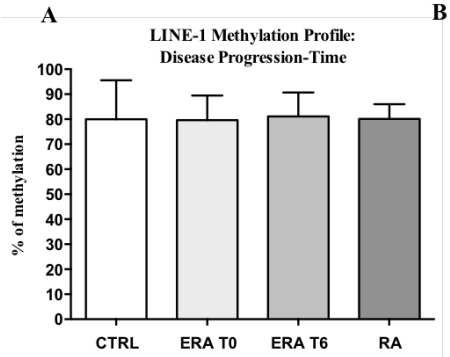
Figure 7.4 *LINE-1 Methylation Profile: inter-patient comparison between blood and biopsy specimens.* ***A.*** *Histogram;* ***B.*** *Paired t-student test between intra-individual samples.*

Results noticed the absence of statistical significant values between blood sample and tissue specimen within the same case and the pattern was widespread in all available subjects. Hence, it was possible to assume that blood samples broadly and appropriately reflected the outline of methylation profile in RA patients.

7.3.1 LINE-1: Global Methylation Analysis

Epigenetics analyses on LINE-1 global methylation have been carried out to investigate the methylation pattern according to the disease status. Patients studied were 30 subjects with RA diagnosis and 50 Early Rheumatoid Arthritis individuals, considered during the first visit (ERA T0) and after 6 months (ERA T6). Statistical analysis has been performed, firstly comparing all the cohorts with a matched healthy group and then between patients' subsets (Figure 7.5).

A



B

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

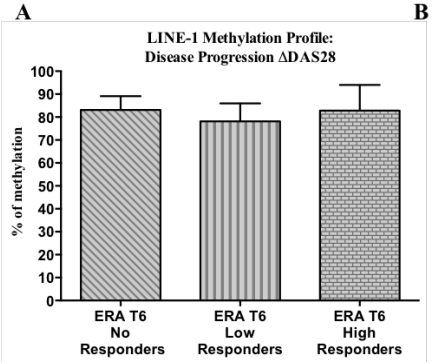
[a] mean of differences
[b] SEM of differences
[c] paired t-student test

Figure 7.5 *LINE-1 Methylation Profile: Disease Progression.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

Overall, results did not observe differences in the global methylation scores among the four different cohorts analyzed. More precisely, LINE-1 methylation level was 80.0±15.63% for controls, 79.6±9.91% for ERA T0, 81.1±9.60% for ERA T6, 80.1±5.93% for RA patients.
All the p values of comparison between groups were not significant.

Subsequently, LINE-1 methylation scores were explored stratifying ERA T6 subjects according to their DAS28 improvement (Figure 7.6). The underlying hypothesis concerned the methylation variation over the 6-months period, dependently on contribution of environmental factors, as treatment assumed.

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
ERA T6 No Responders vs ERA T6 Low Responders	0.050	0.051	0.983	0.3423
ERA T6 No Responders vs ERA T6 High Responders	0.005	0.056	0.082	0.9355
ERA T6 Low Responders vs ERA T6 High Responders	0.045	0.031	1.453	0.1546

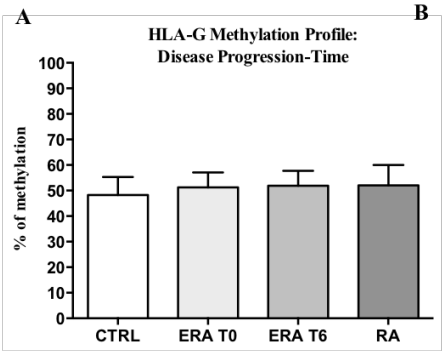
Figure 7.6 *LINE-1 Methylation Profile: Disease Progression according to DAS28 variation.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

As depicted in the Figure 7.6 A, LINE-1 methylation level was roughly similar; it was respectively 83.1±5.96% for ERA T6 No Responders, 78.1±7.87% for ERA T6 Low Responders, 82.8±11.20% for ERA T6 High Responders. Results revealed absence of difference among the three groups and all the p values of comparison were not significant.

7.3.2 HLA-G: Specific Gene Methylation Analysis

Following the same thread of LINE-1 global score, the methylation pattern has been investigated for HLA-G in the four subjects' groups (Figure 7.7).

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
CTRL vs ERA T0	0.030	0.018	1.704	0.0936
CTRL vs ERA T6	0.037	0.020	1.894	0.0630
CTRL vs RA	0.040	0.029	1.402	0.1780
ERA T0 vs ERA T6	0.007[a]	0.009[b]	0.785[c]	0.4365
ERA T0 vs RA	0.011	0.023	0.046	0.6470
ERA T6 vs RA	0.004	0.027	0.123	0.9024

[a] mean of differences
[b] SEM of differences
[c] paired t-student test

Figure 7.7 *HLA-G Methylation Profile: Disease Progression.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

Overall, results did not reveal differences concerning HLA-G methylation level. All the values were roughly around 50%, suggesting a balance of HLA-G methylation in the healthy population and even in subjects with RA or early RA condition.

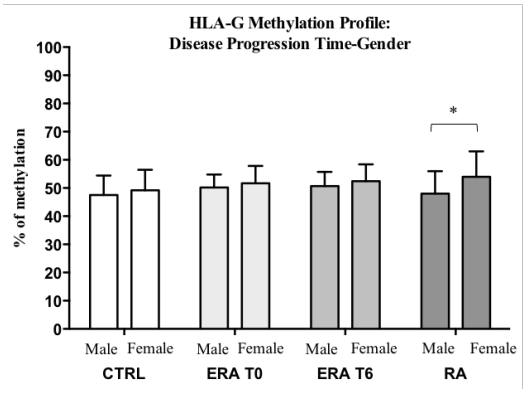
More precisely, HLA-G methylation level was 48.2±7.03% for controls, 51.2±5.81% for ERA T0, 51.9±5.78% for ERA T6, 52.3±8.35% for RA patients. All the p values of comparison between groups were not significant. To notice, it was possible observing a very light trend toward hypermethylation over the time and disease ascertainment.

Furthermore, considering the relation between HLA-G gene and female sex, especially due to its expression during pregnancy, subjects were been stratified according to sex.

Firstly, statistical analysis has been performed through one-way ANOVA to evaluate differences in each male cohort (ERA T0, ERA T6, RA) respect to healthy control males; and subsequently in each female cohort (ERA T0, ERA T6, RA) respect to healthy control females. Bonferroni correction did not reveal statistically significant values.

Secondly, statistical analysis has been performed comparing female and male subjects within each subset (Figure 7.8).

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
CTRL male vs CTRL female	0.016	0.037	0.435	0.6711
ERA T0 male vs ERA T0 female	0.015	0.018	0.828	0.4117
ERA T6 male vs ERA T6 female	0.013	0.021	0.610	0.5448
RA male vs RA female	0.056	0.013	4.561	**0.0103**

Figure 7.8 *HLA-G Methylation Profile: Time-Gender.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

As reported in Figure 7.8, only results on RA case-series showed statistically significant data.

HLA-G methylation level was 48.4±7.52% in RA men, whereas it was 51.2±8.76% in RA women, reaching the p value=0.0103.

This data showed HLA-G methylation difference between sex according to long-lasting RA disease assessment, suggesting a link between established autoimmune disease and sex, probably due to other environmental contributing and sex-related factors.

To investigate deeper the HLA-G methylation results according to sex, we have taken advantage of the small RA case-series of blood/tissue specimens (6 cases) to stratify the pattern of methylation/unmethylation levels sample by sample (Figure 7.9).

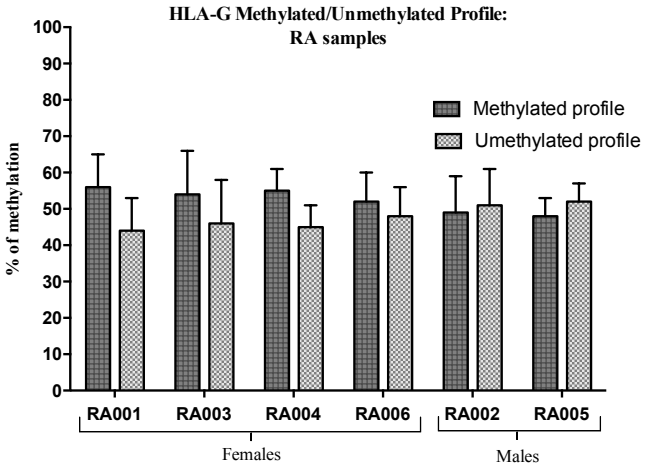
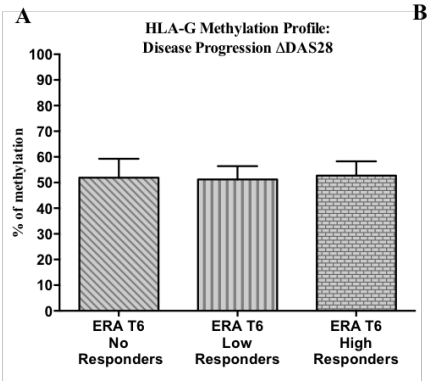


Figure 7.9 *HLA-G Methylated/Unmethylated Profile: RA samples.*

Figure 7.9 revealed the complete opposite trend in males respect to females, showing hypermethylated HLA-G profile in women, whereas in men HLA-G score was slightly toward hypomethylation. Although the small sample size, this result underlined the different condition between sex, focusing to the unlike treats and giving the prospective to tailored separated future interventions.

Subsequently, HLA-G methylation scores were explored stratifying ERA T6 subjects according to their DAS28 improvement (Figure 7.10).

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
ERA T6 No Responders vs ERA T6 Low Responders	0.007	0.037	0.182	0.8578
ERA T6 No Responders vs ERA T6 High Responders	0.008	0.039	0.194	0.8479
ERA T6 Low Responders vs ERA T6 High Responders	0.014	0.021	0.693	0.4925

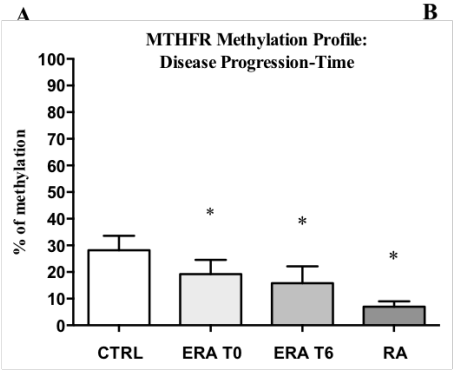
Figure 7.10 *HLA-G Methylation Profile: Disease Progression according to DAS28 variation.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

HLA-G methylation level was almost superimposable; it was respectively 51.9±7.42% for ERA T6 No Responders, 51.2±5.24% for ERA T6 Low Responders, 52.7±5.63% for ERA T6 High Responders. Results revealed absence of difference among the three groups and all the p values of comparison were not significant.

7.3.3 MTHFR: Specific Gene Methylation Analysis

The methylation pattern has been investigated for MTHFR in the four subjects' groups (Figure 7.11).

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
CTRL vs ERA T0	0.089	0.040	2.217	**0.0304**
CTRL vs ERA T6	0.123	0.033	3.673	**0.0005**
CTRL vs RA	0.209	0.068	3.057	**0.0068**
ERA T0 vs ERA T6	0.033[a]	0.023[b]	1.452[c]	0.1531
ERA T0 vs RA	0.119	0.051	2.357	**0.0222**
ERA T6 vs RA	0.086	0.037	2.303	**0.0253**

[a] mean of differences
[b] SEM of differences
[c] paired t-student test

C

	Ordinary one-way ANOVA Multiple comparisons	A Data Set-A Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y	E Data Set-E Y
2	Number of comparisons per family	3				
3	Alpha	0.05				
4						
5	Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
6						
7	CTRL vs. ERA T0	0.08931	0.005604 to 0.1730	Yes	*	0.0323
8	CTRL vs. ERA T6	0.1229	0.03923 to 0.2066	Yes	**	0.0016
9	CTRL vs. RA	0.2087	0.07422 to 0.3432	Yes	***	0.0008

Figure 7.11 *MTHFR Methylation Profile: Disease Progression.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.* ***C.*** *One-way ANOVA for multiple comparisons.*

Results showed MTHFR methylation level 29.8±5.57% for controls, 19.2±5.42% for ERA T0, 15.8±6.33% for ERA T6, 7.3±2.11% for RA patients, with a progressive significant ipomethylation to the increase of disease establishment.

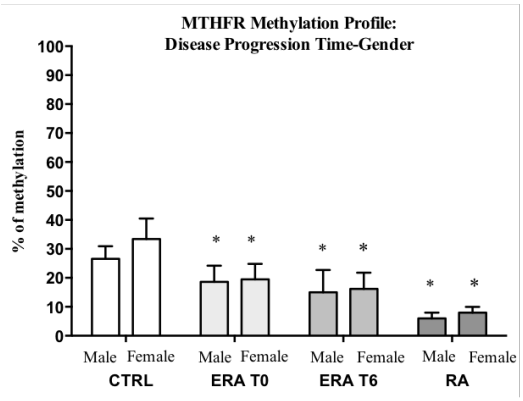
As depicted in the Figure 7.11, the two-sided t-student tests for the comparison between each patients' subset and control group, reported statistically significant differences in the MTHFR methylation scores, with p-values respectively: 0.0304 for CTRL vs ERA T0, 0.0005 for CTRL vs ERA T6, 0.0068 for CTRL vs RA.

The one-way ANOVA for multiple comparison (Figure 7.11 C) with the adjusted p values, confirmed all the significant results.

Following the same thread of HLA-G hypothesis, the gender dependency has been investigated to explore the eventual link between MTHFR methylation pattern and sex.

Thus, subjects belonging to each group (CTRL, ERA T0, ERA T6, RA) were stratified according to gender and statistical analyses have been performed (Figure 7.12).

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
CTRL male vs CTRL female	0.067	0.090	0.748	0.4700
ERA T0 male vs ERA T0 female	0.009	0.041	0.222	0.8257
ERA T6 male vs ERA T6 female	0.012	0.030	0.392	0.6966
RA male vs RA female	0.013	0.027	0.490	0.6497

Figure 7.12 *MTHFR Methylation Profile: Disease Progression Time-Gender.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.* ***C.*** *One-way ANOVA for multiple comparisons.*

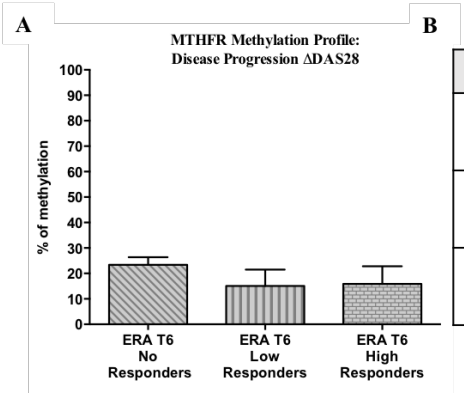
Firstly, analysis has been carried out through one-way ANOVA to evaluate differences in each male cohort (ERA T0, ERA T6, RA) respect to healthy control males; and subsequently in each female cohort (ERA T0, ERA T6, RA) respect to healthy control females. As depicted in Figure 7.12 A, all the comparison with controls were statistically significant.

Then, analysis has been performed comparing female and male subjects within each subset.

Figure 7.12 B revealed absence of significant difference values, suggesting the MTHFR methylation scores regardless gender.

Furthermore, MTHFR methylation levels were explored stratifying ERA T6 subjects according to their DAS28 variation (Figure 7.13). The underlying hypothesis concerned the methylation modulation over the 6-months period, dependently on contribution of environmental factors, as treatment assumed.

A



B

Comparison	|Δ| means	± ΔSEM	unpaired t-student test	p-value
ERA T6 No Responders vs ERA T6 Low Responders	0.084	0.006	0.201	0.2006
ERA T6 No Responders vs ERA T6 High Responders	0.074	0.051	0.158	0.1582
ERA T6 Low Responders vs ERA T6 High Responders	0.009	0.024	0.704	0.7040

Figure 7.13 *MTHFR Methylation Profile: Disease Progression according to DAS28 variation.* ***A.*** *Histogram;* ***B.*** *t-student tests between subjects' subsets.*

MTHFR methylation level was respectively 23.4±2.96% for ERA T6 No Responders, 15.1±6.38% for ERA T6 Low Responders, 15.9±6.85% for ERA T6 High Responders. Results observed absence of difference among the three groups and all the p values of comparison were not significant.

7.5 Discussion and Conclusion

Given the complex nature of Rheumatoid Arthritis (RA) etiology, epigenetic analysis make an effort to provide new insight into the disease and lead to novel therapeutic strategies. Chromatin structure and DNA methylation patterns are both influenced by the inherited DNA sequence and by environmental exposures (Svendsen AJ et al., 2016). Several studies have been appreciated the importance of DNA methylation in RA, following different approaches: a genome-wide DNA methylation profiling study in peripheral blood mononuclear cells reported differentially methylated regions in the major histocompatibility complex loci (Liu Y et al., 2013); a study analyzed chromatin-modifying enzymes in B and T cell from arthritic mice and peripheral blood mononuclear cells from patients with RA (Glant TT et al., 2013); other studies focused on RA synovial fibroblast, founding widespread DNA hypomethylation of the promoter of CXCL12 gene (Karouzakins E et al., 2011) and the LINE-1 retrotransposons (Neidhart M et al., 2000). All these finding observed DNA methylation as a potential mediator of genetic risk and it could make a significant contribution to the susceptibility of developing RA. Moreover, RA is an ideal test case for analyzing the relationships between genes, methylation and disease pathogenesis due to the readily available leucocytes, one of the main classes of cells involved in the disease (Liu Y et al., 2013). Leucocytes in the blood sample provided easily DNA and they reproducibility determined the disease status over the time.
Here, we report a comprehensive study from healthy subjects to RA disease establishment, passing through several phases of progression, including early symptoms recognized in the first visit (ERA T0) and after 6 months (ERA T6), hypothesizing the epigenetic contribution on disease condition and a compositional remodeling of DNA methylation patterns.

Initial analysis concerned LINE-1 to assess the global methylation status. Results did not observed differences among the four different cohorts analyzed: more precisely, LINE-1 methylation level was 80.0±15.63% for controls, 79.6±9.91% for ERA T0, 81.1±9.60% for ERA T6, 80.1±5.93% for RA patients ($p>0.05$). Our finding was in contrast with other studies, reporting global hypomethylation in peripheral blood mononuclear cells of patients with RA (Liu CC et al., 2011); but it was consistent whit what has previous observed by Nakano K. and colleagues, using exactly the same technique of pyrosequencing to quantify global methylation status (Nakano K et al., 2013). In addition, Richardson et al, demonstrated that there was no significant difference in the DNA methyltransferase activities between RA patients and controls (Richardson B et al., 1990).
The discrepancy among studies probably might be related to different techniques or methods used and it reflected the complexity of methylation mechanism, which could be link to feedback pathways or indirect response to others environmental factors.
Furthermore, in this study, the LINE-1 methylation level was not significantly associated with the disease activity of ERA T6 subjects. This data could be explained considering that the limited period of time (6 months), may be restricted or insufficient to evaluate methylation variation.

Subsequently, the methylation score of HLA-G gene has been investigated, due to its role in the autoimmune mechanism of RA disease. Our results did not find statistically differences in the methylation pattern of patients groups respect to controls: more precisely, methylation scores were 48.2±7.03% for controls, 51.2±5.81% for ERA T0, 51.9±5.78% for ERA T6, 52.3±8.35% for RA patients and the HLA-G methylated/unmethylated ratio was almost balanced even in healthy subjects. Although HLA-G gene methylation involvement has not been explored so far, our results noticed the very light trend toward hypermethylation according to the disease establishment, that probably might be link to the increment of immune system activation. Not last, the heterogeneity of RA phenotype strengthened the challenge to find the exact mechanism of environmental contribution (Knevel R et al., 2017). In addition, when RA patients have been stratified by sex, HLA-G methylation score was 48.4±7.52% in RA men, whereas it was 51.2±8.76% in RA women, reaching the significant p value=0.0103. This opposite pattern has been confirmed observing the methylated/unmethylated profile within samples.

To our knowledge, no previous studies reported this finding, but in the *CHAPTER 4 "PHARMACOGENETICS AND TREATMENT RESPONSIVENESS IN RHEUMATOID ARTHRITIS PATIENTS"* of this dissertation, we have observed the HLA-G genotype effect according to sex, and we focused the research direction investigating women and the particular pregnancy condition. Further analysis could include HLA-G methylation scores and specific females' parameters of RA diseased women.

Furthermore, MTHFR methylation levels have been explored, especially with regard to the csDMARD Methotrexate (MTX), the first line and key-treatment of RA. MTX is an inhibitor of dihydrofolate reductase (DHFR), enzyme which converts dihydrofolic acid (DHF) in tetrahydrofolic acid (THF). This molecule turns into 5,10-methylTHF, the substrate of MTHFR enzyme, encoded by MTHFR gene (Goyette P et al., 1994), which catalyzed the conversion to 5-methylTHF, a cosubstrate for homocysteine remethylation to methionine.

Studies observed that, although folate is a mediator in the transfer of methyl groups for DNA methylation, moderate folate depletion did not result in a significant reduction in global DNA methylation (Liu CC et al., 2011) and this has been confirmed in our results on LINE-1. As previous described, we did not report hypomethylated profile. Since past several years, studies examined the effect of folate deficiency on genomic DNA methylation (Balaghi M et al., 1993; Kim YI et al., 1995), however, no one has explored the effect on MTHFR methylation in RA patients.

Our data revealed significant difference of MTHFR methylation score in the patients subsets respect to control group: more precisely, methylation level was 29.8±5.57% for controls, 19.2±5.42% for ERA T0, 15.8±6.33% for ERA T6, 7.3±2.11% for RA patients, showing a progressive significant ipomethylation to the increase of disease establishment (p-values were respectively: 0.0304 for CTRL vs ERA T0, 0.0005 for CTRL vs ERA T6, 0.0068 for CTRL vs RA).

This very interesting finding could be explained through the concept that loss of the DNA methylation signal results in higher gene expression. MTHFR enzyme increases its activity cause the need to convert all the little amount of substrate available. In fact, it has been reported that MTX assumption induces the folate deficiency and perturbed folate metabolism (Kim YI et al., 1995).

Concerning results on ERA patients according to DAS28 improvement after 6 months, MTHFR methylation pattern did not reveal significant differences; it might be due to the limited time elapsed from the beginning of therapy.

However, this absence of methylation differences regarding DAS28 variation after 6 months in the MTHFR status and even in global LINE-1 pattern, open the view to the suggestion that the MTHFR variation over the time could be mainly ascribable to the RA disease progression and severity, which induce epigenomes changes in cells population, regardless the treatment assumed. This hypothesis is emphasized by the absence in our study of DAS28 stratification according to the subtypes of medication assumed; but it was not possible to evaluate due to the low sample size available. Hence, the MTX effect remains to be clearly depicted and it could be further analyzed following this research strategy.

This study provides evidences that epigenetic changes occur in early RA disease and they persist over the time; the methylation mediation implies additional complementary mechanisms for RA, as basal levels of gene expression, expression in response to antigen provocation, or alternative splicing, since both gene expression and splicing are regulated by DNA methylation. Therefore, defining the methylome offers a new way to explore RA susceptibility risk and to identify novel therapeutic targets.

References

Abecasis, G.R. et al. A map of human genome variation from population-scale sequencing. Nature 467, 1061-73 (2010).

Absher DM et al. Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations. PLoS Genet. 2013;9(8):e1003678.

Aletaha D et al., 2010. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis.,* 69(9):1580-8.

Babushok, D.V. & Kazazian, H.H., Jr. Progress in understanding the biology of the human mutagen LINE-1. Hum Mutat 28, 527-39 (2007).

Balaghi M, Wagner C. DNA methylation in folate deficiency: use of CpG methylase. Biochem Biophys Res Commun 1993; 193:1184-90.

Catamo E, Addobbati C, Segat L, Sotero Fragoso T, Domingues Barbosa A, Tavares Dantas A et al. HLA-G gene polymorphisms associated with susceptibility to rheumatoid arthritis disease and its severity in Brazilian patients. Tissue Antigens. 2014 Sep;84(3):308-15.

Cheung P., Lau P., Epigenetic regulation by histone methylation and histone variants. Mol. Endocrinol. 19:563-573. (2005).

Coppedé F, Denaro M, Tannorella P, Migliore L, increased MTHFR promoter methylation in mothers of Down syndome individuals, Mutation Research, 2016;787:1-6.

Dupont C, Armant DR, Brenner CA., Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med. 2009. 27(5):351-7.

Ge J, Wang J, Zhang F, Diao B, Song ZF, Shan LL, Wang W, Cao HJ, Li XQ, Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical significance, Genetics and Molecular Research, 2015;14(3):8021-8028.

Geraghty AA, et al., Nutrition During Pregnancy Impacts Offspring's Epigenetic Status—Evidence from Human and Animal Studies. Nutrition and Metabolic Insights 2015:8(S1) 41–47.

Ghattas M, El-Shaarawy F, Mesbha N, Abo-Elmatty D, DNA methylation of the methylenetetrahydrofolate reductase gene peripheral blood of end-stage renal disease patients, Mol. Biol. Rep., 2014;41(2):683-688.

Glant TT, Besenyei T, Kadar A, Kurko J, Tryniszewska B, Gal J, Soos G, Szekanecz Z, Hoffmann G, Block JA, Katz RS, Mikecz K, Rauch TA. Differentially expressed epigenome modifiers, including aurora kinases a and B, in immune cells in rheumatoid arthritis in humans and mouse models. Arthritis Rheum. 2013;65:1725–1735.

Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, Rozen R. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nature Genetics. Jun 2004, 7 (2): 195–200.

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

Hashemi M, Mokhtari M, Khazaeian S, Bahar G, Rezaei M, Nakhaee A, Taheri M. Evaluation of HLA-G 14-bp ins/del and +3142G>C polymorphisms with susceptibility to recurrent spontaneous abortion. Taiwan J Obtet Gynecol. 2017 Jun;56(3):276-280.

Hedrich CM, Tsokos GC. Epigenetic mechanisms in systemic lupus erythematosus and other autoimmune diseases. Trends Mol Med 2011 Dec;17(12):714-24.

Holliday R., Epigenetics: a historical overview. Epigenetics. 2006. 1(2):76-80.

Jaenisch R, Bird A. Epigenetic regulation of gene expression how the genome integrates intrinsic and environmental signals. Nat Genet. 2003, 33 Suppl:245-54

Karouzakis E, Rengel Y, Jungel A, Kolling C, Gay RE, Michel BA, Tak PP, Gay S, Neidhart M, Ospelt C. DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. Genes Immun. 2011;12:643–652.

Kim YI, Christman JK, Fleet JC, et al. Moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or c-myc specific hypomethylation of co- lonic DNA in rats. Am J Clin Nutr 1995;61:1083-90.

Knevel R, Huizinga TWJ, Kurreman F. Genomic Influences on Susceptibility and Severity of Rheumatoid Arthritis. Rheum Dis Clin North Am. 2017 Aug;43(3):347-361.

Lander, E.S. et al. Initial sequencing and analysis of the human genome. Nature 409, 860-921 (2001).

Li H, Lu H, Tang W, Zuo J. Targeting methionine cycle as a potential therapeutic strategy for immune disorders. Expert Opin Ther Targets. 2017 Aug 23:1-17.

Liu CC, Fang TJ, Ou TT, Wu CC, Li RN, Lin YC, Lin CH, Tsai WC, Liu HW, Yen JH. Global DNA methylation, DNMT1, and MBD2 in patients with rheumatoid arthritis. Immunol Lett. 2011 Mar 30; 135(1-2):96-9.

Liu H., et al. DNA methylation dynamics: identification and functional annotation, Briefings in Functional Genomics, 2016, 1–15.

Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, Reinius L, Acevedo N, Taub M, Ronninger M, Shchetynsky K, Scheynius A, Kere J, Alfredsson L, Klareskog L, Ekstrom TJ, Feinberg AP. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol. 2013;31:142–147.

Marques-Rocha JL., et al. LINE-1 methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals, Epigenetics 2016, VOL. 11, NO. 1, 49–60.

Moran, J.V. et al. High frequency retrotransposition in cultured mammalian cells. Cell 87, 917-27 (1996).

Nakano K, Whitaker JW, Boyle DL, Wang W, Firestein GS. DNA methylome signature in rheumatoid arthritis. Ann Rheum Dis. 2013;72:110–117.

Neidhart M, Rethage J, Kuchen S, Kunzler P, Crowl RM, Billingham ME, Gay RE, Gay S. Retrotransposable L1 elements expressed in rheumatoid arthritis synovial tissue: association with genomic DNA hypomethylation and influence on gene expression. Arthritis Rheum. 2000;43:2634–2647.

Oyama K, Kawakami K, Maeda K, Ishiguro K, Watanabe G, The association between methylenetetrahydrofolate reductase polymorphism and promoter methylation in proximal colon cancer. Anticancer. Res, 2004; 24(2B):649-654.

Patterson, K., Molloy, L., Qu, W., Clark, S. DNA Methylation: Bisulphite Modification and Analysis. J. Vis. Exp. (56), e3170, (2011).

Pérez-Razo JC, Cano-Martínez LJ, Vargas Alarcón G, Canizales-Quinteros S, Martínez-Rodríguez N, Canto P, Roque-Ramírez B, Palma-Flores C, Estaban-Martínez R, López-Hernández LB, Rojano-Mejía D, Coral-Vázquez RM, Functioinal Polymorphism rs133065560 of the MTHFR Gene Is Associated With Essential Hypertension in a Messican-Mestizo Population, Circ. Cardiovasc. Genet., 2015;8(4):603-60.

Qiu Q, Huang J, Shu X, Fan H, Zhou Y, Xiao C. Polymorphisms and Pharmacogenomics for the Clinical Efficacy of Methotrexate in Patients with Rheumatoid Arthritis: A Systematic Review and Meta-analysis. Sci Rep. 2017 Mar 7;7:44015.

Razin A., and Cedar H., DNA methylation and gene expression. Microbiol. Rev. 55: 451-58. (1991).

Richardson B, Scheinbart L, Strahler J, Gross L, Hanash S, Johnson M. Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. Arthritis Rheum, 33 (1990), pp.1665-1673.

Rotondo JC, Bosi S, Bazzan E, Di Domenico M, De Mattei M, Selvatici R, Patella A, Marci R, Tognon M, Martini F, Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of

infertile couples correlates with recurrent spontaneous abortion, Human Reproduction, 2012;27(12):3632.3638.

Saffery R, Novakovic B. Epigenetics as the mediator of fetal programming of adult onset disease: what is the evidence?. Acta Obstet Gynecol Scand 2014; DOI: 10.1111/aogs.12431.

Shao W, Yuan Y, Li Y. Association Between MTHFR C677T Polymorphism and Methotrexate Treatment Outcome in Rheumatoid Arthritis Patients: A Systematic Review and Meta-Analysis. Genet Test Mol Biomarkers. 2017 May;21(5):275-285.

Song F., Smith JF., Kimura MT., Morrow AD., Matsuyama T., Nagase H., Association of tissue-specific differentially methylated regions (TDMs) with different gene expression. Proc. Natl. Acad. Sci. U.S.A. 102:3336-3341. (2005).

Svendsen AJ, Gervin K, Lyle R, Christiansen L, Kyvik K, Junker P, Nielsen C, Houen G, Tan Q. Differentially Methylated DNA Regions in Monozygotic Twin Pairs Discordant for Rheumatoid Arthritis: An Epigenome-Wide Study. Front Immunol 2016; 7: 510.

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

Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, Schübeler D, Distribution, silencing, potential and evolutionary impact of promoter DNA methylation in the human genome, Nature Genetics, 2007,39:457-466.

Wu W, Shen O, Qin Y, Niu X, Lu C, Xia Y, Song L, Wang S, Wang X, Idiopathic Male Infertility Is Strongly Associated with Aberrant Promoter Methylation of Methylenetetrahydrofolate Reductase (MTHFR), PLoS One, 2010.

Yamada L. Chong S. Epigenetic studies in Developmental Origins of Health and Disease: pitfalls and key considerations for study design and interpretation, Journal of Developmental Origins of Health and Disease, 2017, 8(1), 30–43.

CHAPTER 8

GENERAL CONCLUSION
AND
FUTURE DIRECTIONS

GENERAL CONCLUSION

Genetic occurrence risk of RA in the case-control study reported HLA-DQA2 gene as the strongest associated of developing the disease. Nonetheless HLA complex has been identified as the main contributing factor in RA susceptibility in conventional GWAS and in several studies, HLA-DQA2 gene is not extensively studied as a single concurring element. This evidence the uniqueness of our result, especially in consideration of the specific Italian population analyzed, highlighting the importance of selected pool of patients. Additionally, the synergic interaction between variants belonging to HLA complex, underlined the concerted action between different MHC molecules in immune activation and in exacerbating the arthritis inflammation.

Our study on Undifferentiated and Early Rheumatoid Arthritis patients revealed that combination among different biomarkers, including serological factors, was determinant even as a predictive role to treatment efficacy. Although it has been known the complex interplay between genetics, serology and disease activity score variation, our study provided the formulation of predicting algorithm for *Best responder* patients to the Methotrexate treatment, the first-line therapy commonly used in the management of RA disease. Our finding remarked the importance of a promptly and effective therapeutic choice and it might be the key in the individuation of a specific profile responsiveness to a specific treatment, strengthening the precision and predictive medicine approaches.

Moreover, our present research was able to capture the substantial variation across gender, in genetic occurrence RA risk, including single SNP analysis and SNP-SNP combination, and in terms of Methotrexate pharmacogenetics. Sexual dimorphism was assumed to be a relevant environmental factor and gene-sex interaction could exert an important role in both, RA etiology and in treatment responsiveness. RA is claimed to be more common and frequent in female population exhibiting a F/M ratio of about 3-4/1 and specific characteristics of women, as previous pregnancy and miscarriage events, allowed to identify a specific group of female RA patients, suggesting the new frontier of gender-pharmacogenetics.

The outcome on male microchimerism (fMC) detection analyzed in RA and in SLE women blood samples, seemed to support the "repair" hypothesis, affirming the chimeric cells would be able to provide a tissue injury and flushing resolutions associated to the pathology, acting as stem cells. This observation was consistent to our different findings on fMC detection in SLE and RA women, showing higher presence of fMC in SLE than which has detected in RA patients' bloodstream, due to the widespread symptoms of SLE respect more localized in RA. The investigation was carried out in synovial biopsies of female RA subjects, the closest possible tissue which is directly affected in RA. Results did not reveal significant levels of fMC manifestation, corroborating the repairing hypothesis of male fetal cells in case of maternal injures, like joint inflammation. Hence, due to fMC action as stem cells, male fetal cells positive detection could be revealed in RA women with

moderate, stable or remitted condition. Besides, histological analysis concerning CD4+ T-lymphocytes, $CD20^+$ B-lymphocytes and $CD68^+$ macrophages, supplemented better exploration of the immune cells infiltration in RA synovium. Results showed different features among cases, identifying a connection between the particular immune cell subgroup detected and the treatment assumed.

The epigenetic investigation is a unique study, which is the first to gain insight methylation regulation of RA etiology and disease progression, exploring the methylation status from healthy subjects, passing through early symptoms, to established RA diseased patients. To the best of my knowledge, this is the first study to explore methylation status over the time. Results highlighted the role of sexual dimorphism and revealed that epigenetic state persists beyond RA stages. Overall, outcomes allow to open new research frontiers in the context of environmental non-inherited contributing factors, able to modulate the onset and the development of RA.

FUTURE DIRECTIONS

Considering our findings, further and in-depth investigation on epigenetics modulation could be potentially involved in therapeutic decisions, contributing to clinical responses and good prognosis. In this view, subsequent researches in this area may focus on in-vitro PBMCs study, obtained from RA and even in Early Rheumatoid Arthritis patients, evaluating the specific epigenetics modification in subjects before and after Methotrexate (MTX) treatment. PBMCs are easily collected through a normal blood sample and they grow in sufficient quantities, thereby facilitating a periodically epigenome investigation, without influencing and impacting on the clinical RA management.

Presuming the outcome of the epigenetic study, it is plausible to implement its potentiality exploring the effect of folates supplementation on in-vitro PBMCs. Considering Folic Acid as normally used in clinical practice for the rescue role after MTX administration, it could be investigated in the view of epigenetics modification, thinking about its capacity to restore the methylation status before MTX therapy. In addition, the research strategy may include other folates, as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, due to their effect bypassing MTHFR enzyme.

One other area still needs to be explored is the relationship in our understanding between the influence of genetics variants on DNA methylation profiles, which could provide the ultimate link between inherited and modifiable factors leading to RA disease.

Lastly, a consideration regards to the complexity of DNA methylation regulation in different cell types needs to be made. PBMCs include several cells subtypes, therefore it should be useful to employ strategies that use cell sorting techniques to isolate cell to homogeneity and use technologies that allow analysis with small cell numbers. Thus, in the context of technological advances contribution, more precisely questions on epigenome modulation, will be adequately addressed.

SUPPLEMENTARY MATERIAL

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

Table 1
Classification of HLA-DRB1 alleles according to the third hypervariable region of the DRβ chain.

HLA DRB1 alleles	HLA-DRB1 amino-acid position								
	67	...	70	71	72	73	74	... 85	86
HLA DRB1*0101	L	...	Q	R	R	A	A	... V	G
HLA DRB1*0102	L	...	Q	R	R	A	A	... A	V
HLA DRB1*0103	I	...	D	E	R	A	A	... V	G
HLA DRB1*03	L	...	Q	K	R	G	R	... V	V
HLA DRB1*0401	L	...	Q	K	R	A	A	... V	G
HLA DRB1*0402	I	...	D	E	R	A	A	... V	V
HLA DRB1*0403	L	...	Q	R	R	A	E	... V	V
HLA DRB1*0404	L	...	Q	R	R	A	A	... V	V
HLA DRB1*0405	L	...	Q	R	R	A	A	... V	G
HLA DRB1*0407	L	...	Q	R	R	A	E	... V	G
HLA DRB1*0408	L	...	Q	R	R	A	A	... V	G
HLA DRB1*0411	L	...	Q	R	R	A	E	... V	V
HLA DRB1*07	I	...	D	R	R	G	Q	... V	G
HLA DRB1*08	F	...	D	R	R	A	L	... V	G
HLA DRB1*0901	F	...	R	R	R	A	E	... V	G
HLA DRB1*1001	L	...	Q	R	R	A	A	... V	G
HLA DRB1*1101	F	...	D	R	R	A	A	... V	G
HLA DRB1*1102	I	...	D	E	R	A	A	... V	V
HLA DRB1*1103	F	...	D	E	R	A	A	... V	V
HLA DRB1*1104	F	...	D	R	R	A	A	... V	V
HLA DRB1*12	I	...	D	R	R	A	A	... A	V
HLA DRB1*1301	I	...	D	E	R	A	A	... V	V
HLA DRB1*1302	I	...	D	E	R	A	A	... V	G
HLA DRB1*1303	I	...	D	K	R	A	A	... V	G
HLA DRB1*1323	I	...	D	E	R	A	A	... V	G
HLA DRB1*1401	L	...	R	R	R	A	E	... V	V
HLA DRB1*1402	L	...	Q	R	R	A	A	... V	G
HLA DRB1*1404	L	...	R	R	R	A	E	... V	V
HLA DRB1*15	L	...	Q	A	R	A	A	... V	V
HLA DRB1*16	F	...	D	R	R	A	A	... V	G

	Gregersen (×)	Gao (×)		De Vries (×)		Mattey (×)		Reviron (×)		Tezenas du Montcel (×)	
Predisposing	*0401 *0404 *0405 *0408	*0401 *0404 *0405 *0408	E_3 E_2 E_1	*0401 *0404 *0405 *0408	SE^+	*0401 *0404 *0405 *0408	SE	*0401 *0404 *0405 *0408	SE	*0401 *1303 *1310	S_2
	*0101 *0102 *1402 *1001	*0101 *1402 *1001 *0102	E_1 E_1 E_2	*0101 *0102 *1001	L_{67}	*0101 *0102 *1402 *1001		*0101 *0102 *1402 *1001		*0101 *0102 *1402 *1001 *10	S_{3P}
	*1303 *1310 *1419 *1421	*1303 *1310 *1419 *1421	$E_\times$	*03 *0403 *0406 *0407 *08 *09 *11 *14 *16	N	*03 *0403 *0406 *0407 *09 *14 *15	N D_{70-}	*03 *0403 *0406 *0407 *0901 *1107 *14 *15 *16	N	*1101 *1104 *12 *1305 *1306 *1325 *1422 *16	S_{3D}
Neutral	*0403 *0406 *0407 *0901 *1107 *14 *15 *16	*0403 *0406 *0407 *0901 *1107 *14 *15 *16	$E_\times$							*0103 *0402 *1102 *1103 *1301 *1302 *1304 *1323	S_1
										*03 *0403 *0407 *0411 *07 *08 *0901 *1401 *1404	×
Protective	*07 *08 *11 *12 *13	*07 *08 *11 *12 *13	$E_\times$								
				*07 *08 *11 *12 *13 *15	P	*07 *08 *11 *12 *13 *16	P	*07 *08 *11 *12 *13	P		
	*0103 *0402 *1102 *1103 *1301 *1302 RAP (DERAA)	*0103 *0402 *1102 *1103 *1301 *1302	$E_\times$	*0103 *0402	I_{67}	*0103 *0402	D_{70+}	*0103 *0402			