Identification and quantification of selected inflammatory genes modulated by leflunomide and prednisone treatment in early rheumatoid arthritis patients

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Abstract

Objective

The present study evaluates the effects of combined leflunomide (LEF) and low dose of prednisone therapy, on selected inflammatory gene expression in peripheral blood mononuclear cells (PBMCs) of early rheumatoid arthritis (ERA) patients by gene microarray analysis and quantitative real time-polymerase chain reaction (qRT-PCR).

Methods

Ten ERA patients (mean age 53±10 years) were assigned as untreated (group 1) or pre-treated (group 2) with prednisone (5 mg/day for 3 months) after informed consent and ethics committee approval. Five sex- and age-matched healthy subjects were used as controls (CNT). RNA was extracted by PBMCs, amplified, labelled and hybridised on inflammation DualChip microarray. The expression ratio of 282 inflammatory genes between CNT and ERA patients, before (T0) and after 12 weeks (T1) of combined therapy was detected. qRT-PCR was performed on 7 selected inflammatory RA-related genes (STAT4, MAPK9, HIF1A, MIF, STAT6, NFKB1, TNFRSF1B).

Results

At T0, microarray analysis showed 34 altered genes in both ERA groups when compared to CNT (vs. CNT). Seven RA-related genes, investigated in further details, were found up-regulated in group 1 and down-regulated or unchanged in group 2 vs. CNT. At T1, combined therapy induced the down-regulation of these genes in both groups vs. CNT as also confirmed by qRT-PCR performed on selected genes.

Conclusion

Untreated ERA patients seem characterised by up-regulation of specific genes involved both in the resistance/inhibition to apoptosis and in the stimulation of pro-inflammatory cytokine production by immune inflammatory cells. Combined LEF and low dose of prednisone therapy seems to play synergistic effects on down-regulation of these genes.

Key words

rheumatoid arthritis, leflunomide, glucocorticoids, gene microarray, gene profile expression, inflammation

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Introduction

Rheumatoid arthritis (RA) is a chronic, immune-mediated and inflammatory syndrome mainly characterised by recruitment and activation of inflammatory cells, synovial tissue hyperplasia, and joint integrity destruction (1, 2). Predominant cell types involved in the synovial inflammation include T and B cells, activated monocytes/macrophages and neutrophils (3-5). In RA the initial activation of the immune system leads to an inflammatory cascade followed by perpetuation and diffusion of the inflammatory reaction determining organ damage (6).

In recent years, it has become clear that RA treatment should aim at suppressing the inflammatory process early and effectively, in order to relieve patient symptoms and achieve better long-term functional and structural outcomes (7). In the management of early RA (ERA), the combination of disease modifying anti-rheumatic drugs (DMARDs) and low dose of prednisone represents the most common and standard therapeutic intervention (8).

Leflunomide (LEF) is a DMARD inhibiting dihydro-orotate dehydrogenase enzyme, a key molecule involved in *de novo* synthesis of pyrimidines at the level of activated inflammatory cells (9). This cellular anti-proliferative action blocks important steps in the pathogenesis of RA and reduces joint inflammation. In addiction, the combined therapy of LEF with glucocorticoids seems to exert a synergistic effect on immunosuppressive and anti-inflammatory activities (10).

Microarray analysis is a technology used to discriminate differences in gene expression profile and to identify predictive biomarkers in peripheral blood mononuclear cells (PBMCs) that may act as a reporter of the ongoing chronic process (11, 5).

Synovial and peripheral blood cells from RA patients have been examined using a comprehensive analysis of mRNA expression profiles (12-14). Although many studies have shown that gene expression profile has been associated with both a variable natural course of RA and heterogeneous response to different drugs – as anti-TNF biological – no studies have attempted to identify genetic markers for LEF efficacy in RA (15-17).

In this pivotal study we employed the microarray analysis and quantitative real time-polymerase chain reaction (qRT-PCR) assay to examine the effects of combined LEF and low dose of prednisone therapy on a selected profile of inflammatory genes in ERA naive patients.

In particular, the aim of the study was to evaluate if a specific gene expression profile might supervise the effects of the LEF and low dose of prednisone therapy.

In addition, it was analysed if the pre-treatment with prednisone might increase the efficacy of the subsequent LEF therapy, further acting on the expression of selected inflammatory genes in ERA patients.

Materials and methods

Patients and controls

A total of 10 patients (9 female and 1 male, mean age 53±10 years), fulfilling the American College of Rheumatology (ACR) criteria for RA, and 5 sex and age matched healthy subjects (CNT, mean age 50±5 years) cured at the Academic Unit of Clinical Rheumatology at the University of Genova were enrolled in the study. The criteria of eligibility included early RA (ERA) with disease duration less then 2 years (mean 12±3 months). The Ethics Committee of the University approved the protocol and all patients were asked to sign the informed consent.

As described in our recent study (18), selected ERA patients were further assigned in two groups according to their treatment regimen: the group 1 was constituted by 6 untreated RA patients and group 2 by 4 RA patients already pre-treated with prednisone (5 mg/day for 3 months) before starting with the study. No previous other treatments with biological drugs or DMARDs were allowed during the recruitment. No previous steroid administration was accepted before the study entry.

Therefore, at the basal time (T0), patients of both groups were given a constant dose of prednisone (5mg/day) and LEF (100 mg/day) for the first 3 days,

then 20 mg/day for 12 weeks (T1). Disease activity score (DAS-28) was recorded at T0 and T1 (Table I).

Table I. Baseline clinical and demographics parameters of RA patients enrolled in the study.

Demographic parameters	Group 1		Group 2	
Age (years±SD)	49±12		59±10	
No. patients	6		4	
RA duration (months)	12.3		12	
Patients with RF	2		0	
Patients anti-CCP Ab positive	0		1	
Clinical parameter time	Basal time T0	12 weeks T1	Basal time T0	12 weeks T1
DAS-28	5.75±0.98	4.26±1.64	3.54±1.29	3.12±1.45

Baseline clinical and demographics characteristics of the two groups of RA patients enrolled into the study. RF: Rheumatoid factor; Anti-CCP Ab: anti-cyclic citrullinated peptide antibodies; DAS-28: disease activity score.

PBMC isolation and mRNA extraction and labelling

The PBMCs were isolated from venous blood by Ficoll-Hystopaque density gradient (Sigma, St Louis, MO). Total RNA was extracted by RNeasy Midy Kit (Qiagen, Milan, Italy) and the quality controlled on Agilent Bioanalyzed (Agilent Technology, Palo Alto, USA). The RNA was amplified by MessageAmp II-Biotin enhanced kit (Ambion, Austin, USA) to obtained labelled amplified RNA (aRNA).

Hybridisation and data acquisition procedure

Labelled aRNAs were hybridised with the inflammation DualChip microarray kit, (Eppendorf, Hamburg, Germany) according to the DualChip microarray extended procedure. The DualChip microarray consisted of 282 genes involved in the inflammatory process, 12 internal standard (IS) and 14 housekeeping genes (HKG). The IS, which were synthetic RNA of *Leucopersicum esculentum* at different concentrations, and HKG genes were necessary for the normalisation steps. The relative IS and HKG probes were spotted on the DualChip microarray. The detection of hybridisation reaction was obtained by SilverQuant system and it was carrier out with a laser scanner (ScanArray 4000XL, Eppendorf). Finally, the analysis of signal quantification was performed by SilverQuant software (Eppendorf).

Normalisation procedure

The DualChip microarray have been designed to efficiently integrate two normalisation steps. The first step starts adding 1 μg of IS mix-biotinate to the reverse transcription in order to control the reaction and to normalise the results. The local normalisation factor is calculated from the acceptable intensity of the IS of experimental (each RA patient) and referee samples (CNT).

To verify the purity and the quantity of the RNA, a second normalisation step is performed, comparing the expression levels of HKG between RA patients and CNT samples. The variance of the normalised set of HKG is used to generate a confidence interval to test the significance of the gene expression ratios obtained. Ratios outside the 95% confidence interval were considered significantly different.

Real time-polymerase chain reaction (PCR) assay

To perform the quantitative real time-PCR (qRT-PCR) analysis, cDNA was synthesised from RNA using SuperScript II reverse transcriptase system (Invitrogen, Paisley, UK), and oligo(dt) primer (Invitrogen). The qRT-PCR was performed on a Real plex 4 Master cycler and liquid-handling EP-motion 5070 (Eppendorf) and the reaction was obtained using Real MasterMix SYBR Green detection system (Eppendorf). Briefly, for each sample 2μl cDNA was loaded in triplicate with 1x SYBR green mix (Eppendorf) and the following 10mM primer mix by PrimerDesign (PrimerDesign, UK): for beta-actin (Accession Number (AN) NM_001101), HIF1A (AN: NM_001530), STAT4 (AN: NM_003151), MIF (AN: NM_002415), MAPK9 (AN: NM_002752), STAT6 (AN: NM_003153), NFKB1 (AN: NM_003998), TNFRSF1B (AN: NM_001066).

The melting curve obtained by each qRT-PCR amplification confirmed the specificity of the SYBR green assay. The qRT-PCR was performed for all patients enrolled into the study.

Data mining and statistical analysis

Due to limited number of replicates, definitive conclusions on the quantitative level of differential gene expression between groups of patients were voluntarily omitted. On the contrary, keeping into account the exploratory nature of the study, only significant labelled data produced by SilverQuant software as "qualitative" were considered for statistical evaluation in the analysis of microarray gene expression profiles. In our analysis, the definition of ratio category – which represents the categorical level of expression of a gene within a patient – and the gene status – which represents the majority category of a gene within a group of patients – play an important role in the gene selection procedure.

All gene expression ratios of RA patients that significantly differed with respect to CNT, were labelled as category 1 (up-regulated) if the sign of the log-ratio was positive, otherwise they were indicated as category -1 (down-regulated), if otherwise. Unchanged ratios were labelled as category 0. Consequently, a vector of -1, 0, 1 numbers is produced for each patient. This procedure was performed using scripts implemented in R language (19) on data generated by SilverQuant software.

At T0, the status of every gene in the two groups was defined according to a simple majority criterion, based on the distribution within study patients of the above defined ratio categories. Genes with a different status between groups and CNT at T0 were selected for further analysis. On such genes, clustering analysis was used to detect similarities in gene expression profile among patients and among genes. Agglomerative hierarchical clusters were computed using the Manhattan distance (*i.e.* absolute distance) be-

tween single categorical vectors and the Ward method (20). Ratio categories and status were assigned to genes at T1, using the same simple majority criterion on gene expression data observed at that time. Fisher's exact test (21) was eased to test the null hypothesis of independence between the probability distributions of ratio categories in the two groups of patients at T0 and T1. *P*-values less than 0.05 were considered statistically significant. All statistics were produced with the R software.

Quantitative real time-PCR data were obtained by a relative quantification analysis comparing the expression levels of a target gene among the studied samples by realplex analysis software (Eppendorf). These expression level values were calculated using the comparative threshold ($\Delta\Delta C_t$) method (22) and represented the fold expression of the target gene compared to CNT. The expression level values were expressed as a mean±SD. The statistical analysis was performed by ANOVA system using Tukey-Kramer method. *P*-values less than 0.05 were considered significant.

Results

Expression profile of the inflammatory genes between RA patients and healthy subjects before combined LEF and prednisone therapy

The ERA patients were selected according to no treatment (group 1) or pre-treatment with prednisone (group 2) before starting with the combined LEF and low dose of prednisone therapy, as also been described in our recent study (18). Table I provides the recruitment parameters of these ERA patients at T0 and T1. The DAS-28 average score was 5.75±0.98 in the group 1 and 3.54±1.29 in the group 2, indicating that all patients had an active disease. The lower DAS-28 value in RA patients of group 2 was related to the pre-treatment with prednisone (Table I).

At T0, the expression profile analysis of 282 genes involved in the inflammatory process showed that 34 genes were altered in ERA patients when compared to the CNT (*vs*. CNT), with an opposite expression category in the two groups, as showed in our previous study (18)

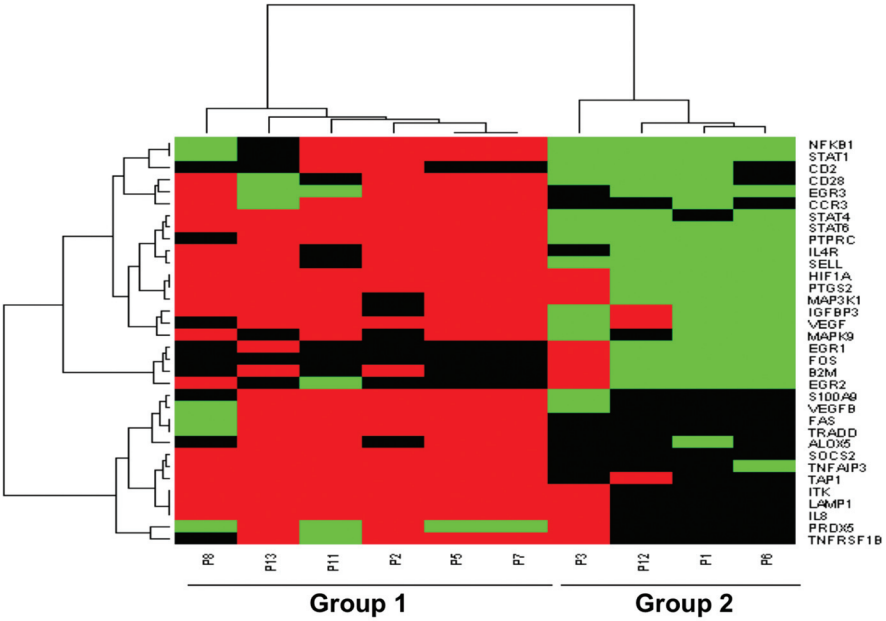

Fig. 1. Hierarchical cluster of inflammatory genes in RA patients enrolled into the study at basal time. Agglomerative hierarchical cluster of inflammatory gene expression in the RA patients enrolled into the study. Hierarchical cluster was performed on 282 inflammatory genes and the data shown 34 genes that differ between the two groups. All gene expression ratios of RA patients that significantly differed with respect to CNT, were labelled as category 1 (up-regulated = red) if the sign of the log-ratio was positive, and category -1 (down-regulated = green), if the sign of the log-ratio was negative. Unchanged ratios were labelled as category 0 (black). Consequently, a vector of -1, 0, 1 numbers was produced for each patient.

Table II. Official symbol name and chromosomal location of genes evaluated by microarray analysis.

Official gene symbol	Gene name (according to HUGO database and Entrez Gene)	Chromosome Location
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	22q11.2
FAS	Fas (TNF receptor superfamily, member 6)	10q24.1
IL4R	interleukin 4 receptor	16p12.1-p11.2
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	4q23-q24
STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	12q13
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3	6q23
TNFRSF1B	Tumour necrosis factor receptor superfamily, member 1B	1p36.3-p36.2

Genes involved in the resistance or inhibition of apoptosis analysed by microarray in the two groups of RA patients enrolled in the study at T0 and T1.

(Fig. 1). Six genes (FAS, IL4R, NFKB1, STAT6, TNFAIP3 and TNFRSF1B), having a suspected link with the pathological events of RA and playing a role in the resistance or inhibition of apoptotic process, were here investigated in further details. Moreover, the gene MIF, which is known to contribute to the major pathogenic events in RA, was also included in the successive analysis (23) (Table II, Fig. 2). The molecules codified by these seven genes are involved in the RA inflammatory process (24-25).

Microarray analysis showed that these genes had an opposite expression profile in the two groups. In group 1 all genes were up-regulated *vs*. CNT, whereas in group 2, MIF, IL4R, NFKB1 and STAT6 were down-regulated and FAS, TNFAIP3 and TNFRSF1B were unchanged *vs*. CNT (Fig. 2A).

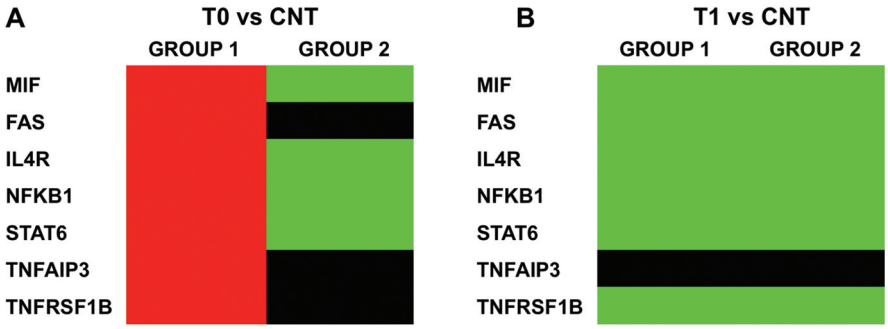

Fig. 2. Expression profile of genes in ERA patient group 1 and 2 before and after LEF and low dose of prednisone therapy evaluated by microarray analysis.
(**A**) Expression profile of genes involved in the resistance or inhibition of apoptotic process at basal time (T0) in the untreated (group 1) and pre-treated RA patients (group 2) before starting with the study. Gene expression ratios of RA patients that were significantly different with respect to CNT were labelled as category 1 if the sign of the log-ratio was positive (Up-regulated = red); as category -1 if the sign of the log-ratio was negative (down-regulated =green). Unchanged ratios were labelled as category 0 (black). Consequently, a vector of -1, 0, 1 numbers was produced for each patient.
(**B**) Expression profile of genes involved in the resistance or inhibition of the apoptotic process after combined LEF and prednisone therapy (T1) in the untreated (group 1) and pre-treated RA patients (group 2) before starting with the study. Gene expression ratios of RA patients that were significantly different with respect to CNT were labelled as category 1 if the sign of the log-ratio was positive (Up-regulated = red); as category -1 if the sign of the log-ratio was negative (green = light grey). Unchanged ratios were labelled as category 0 (black). Consequently, a vector of -1, 0, 1 numbers was produced for each patient.

Expression profile of the inflammatory genes after LEF and prednisone therapy (comparison T1 vs. CNT)

The expression profile of 7 RA-related genes was analysed in both groups at T1 and these results were normalised *vs*. CNT.

The expression profile analysis showed that in group 1, MIF, FAS, IL4R, STAT6, TNFAIP3, NFKB1, TNFRSF1B, which were up-regulated at T0, were down-regulated, and TNFAIP3 was unchanged *vs*. CNT (Fig. 2B) by combined LEF and low dose of prednisone therapy. In this group the Fisher's exact test showed a significant difference in the expression profile of these genes at T1 *vs*. T0 ($p<0.01$).

In group 2, the same 4 genes (MIF, IL4R, STAT6, NFKB1) already found down-regulated at T0 *vs*. CNT, were found down-regulated also at T1 (Fig. 2B) by combined LEF and low dose of prednisone therapy. Moreover, among the 3 genes unchanged at T0 *vs*. CNT, TNFAIP3 was unchanged, whereas FAS and TNFRSF1B were found to be down-regulated at T1 *vs*. CNT (Fig. 2B) by combined therapy.

Fisher's exact test in group 2 showed that the expression profile of this genes was not significantly different at T1 *vs*. T0 ($p>0.05$).

In conclusion, at T1 the results showed that between the two groups there were not statistically significant differences in the genes analysed in this study.

Transcription levels of RA-selected genes during combination therapy

In order to quantify the effect of com-

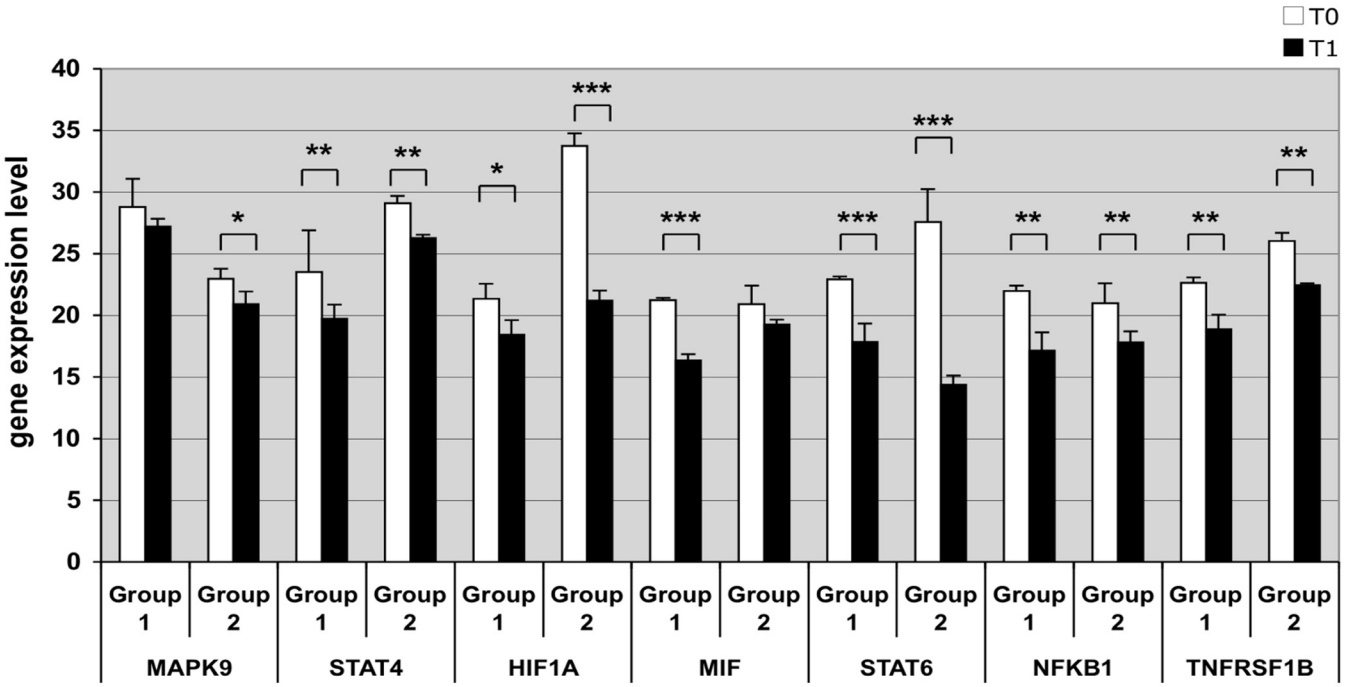

Fig. 3. Expression level of selected genes in ERA patient group 1 and 2 before and after LEF and low dose of prednisone therapy evaluated by qRT-PCR. Expression level of STAT4, MAPK9, HIF1A, MIF, STAT6, NFKB1 and TNFSFR1B genes in group 1 and group 2 at basal time (T0) and after 12 weeks (T1) of combined LEF and low dose of prednisone therapy analysed by quantitative real-time PCR. Samples were loaded in triplicate and results were reported as mean ± standard deviation (SD) gene expression for each RA patient. $^{*}p<0.05$; $^{**}p< 0.01$, $^{***}p<0.001$.

bined LEF and low dose of prednisone therapy on the gene expression level in both groups, qRT-PCR was performed for all ERA patients enrolled into the study. The expression level between T1 and T0 was evaluated on 7 selected genes involved in the pro-inflammatory (MAPK9, HIF1A) or anti-apoptotic (MIF, STAT6, NFKB1, TNFRSF1B) signalling pathways and in genetic susceptibility (STAT4) to the disease. In particular, the 7 selected genes are considered to have a link with the pathogenesis of RA since they codify for transcription factors and other molecules involved in the signalling pathways of important cytokines in rheumatoid inflammation (24-26).

The qRT-PCR showed that the expression level of the genes involved in the resistance and/or inhibition of apoptosis (MIF, STAT6, NFKB1 and TNFRSF1B) were decreased during the combined LEF and low dose of prednisone therapy in both groups of ERA patients and the gene expression changes were statistically significant at T1 *vs*. T0, except for MIF gene in group 2 (Fig. 3, Table III).

Similar results were observed for STAT4, HIF1A and MAPK9, which showed a decreased expression level in both groups of ERA patients at T1 *vs*. T0 (Fig. 3, Table III). Gene expression decreases were statistically significant except for MAPK9 in group 1 (Fig. 3, Table III). The modulations in gene expression level were found to be similar between the two groups, except for MIF, which showed higher decrease in group 1 than group 2 (fold change <4.86 *vs*. <1.68) as well as STAT6 and HIF1A that were found to be more decreased in group 2 compared to group 1 (fold change <13.16 *vs*. <5.11; fold change <12.56 *vs*. <2.92, respectively) (Table III).

These data confirm the differences already observed for these genes in the global expression by microarray analysis.

Table III. Differences in expression levels of selected genes in ERA patient group 1 and 2 before and after LEF and low dose of prednisone therapy by qRT-PCR.

	Group 1 T1 vs T0		Group 2 T1 vs T0	
Genes	Fold change	*p*-value	Fold change	*p*-value
STAT4	<3.76	<0.01	<2.04	<0.01
MAPK9	<1.54	n.s.	<2.04	<0.05
HIF1A	<2.92	<0.01	<12.56	<0.001
MIF	<4.86	<0.001	<1.68	n.s.
STAT6	<5.11	<0.001	<13.16	<0.001
NFKB1	<4.87	<0.01	<3.17	<0.01
TNFRSF1B	<3.79	<0.01	<3.55	<0.01

Differences in expression levels (indicated as fold change) of STAT4, MAPK9, HIF1A MIF, STAT6, NFKB1 and TNFSFR1B genes in group 1 and group 2 both at basal time (T0) and after 12 weeks (T1) of combined LEF and low dose of prednisone therapy. Relative *p*-value was indicated for each gene. n.s.: not significant.

Discussion

In recent years, microarray and qRT-PCR have been representing the most powerful technologies applied in the research both to compare gene expression in a defined status of a disease and to investigate pharmacological mechanisms or molecular patterns relevant for treatment (27). These aspects are very important since rheumatic disease can be rapidly modified by targeted therapeutic interventions and can serve as a model for other inflammatory disorders (6).

Moreover, in the RA, the study of gene expression profile has been used to evaluate the differences in transcription levels measured at basal time versus a specific treatment (25). Recent studies based on gene ontology have observed that some up-regulated genes during ERA were more specifically involved in stress responses, defense mechanisms and apoptosis (28).

Trying to analyse the every single gene evaluated in this study, MIF (macrophage migration inhibitory factor), which plays an important role in the inflammatory process of RA, systemic lupus erythematosus and other rheumatic diseases, was an important gene found up-regulated in untreated ERA patients. The MIF protein is a pro-inflammatory cytokine produced by macrophages; it is able to induce the synthesis of a large number of proinflammatory molecules, such as TNF-α, IFN-γ, IL-1β, IL-6, IL-8, nitric oxide, and cyclo-oxygenase 2 (COX2) and it inhibits the p53-dependent apoptosis (29, 30). Recent studies on ERA found an up-regulation of the genes involved in the hypoxia response via hypoxia-inducible factor (HIF) activation (28) and showed that MIF is not only induced by hypoxia but it is also necessary for maximal expression of hypoxia-inducible factor 1, alpha subunit (HIF-1A) (31).

In our study, the combined down-regulatory effects exerted by the LEF and low dose of prednisone therapy on these proinflammatory gene expressions might be important in the modulation of the ERA inflammatory process.

Untreated ERA patients were also characterised by an up-regulation of gene expression of mitogen-activated protein kinase JNK2 (c-Jun N terminal kinase 2). This protein – codified by MAPK9 gene – was detected in mononuclear cell infiltrates at the level of synovial lining area, suggesting a possible involvement in joint damage associated with proinflammatory cytokine production.

Interestingly, the down-regulation of this gene here observed in ERA patients after combined LEF and low dose of prednisone therapy, not only support the recent data showing the link between their inhibition and the reduction of inflammation, but also suggests that the activation of MAPKs signalling play a fundamental role in the ERA inflammatory process. (32).

An important cytokine involved in the pathogenesis of RA by inducing proinflammatory and adhesion molecules production is TNF-alpha (tumour necrosis factor-alpha) (33). The TNF-alpha signalling is partly mediated by NF-κB (nuclear factor κ-B), a transcription factor both regulator and coordinator of immune and inflammatory processes (34).

Therefore, recent investigations have shown that molecules such as TNFAIP3 (TNF-alpha induced protein 3,

also know as A20), Fas, TNFRSF1B (TNF-alpha receptor superfamily member 1B or TNF-alpha receptor II) and NF-κB play important roles in the TNF signalling.

Interestingly, TNFRSF1B is associated with the activation of the NF-κB1, produced by NFKB1 gene activation, and it is able to induce an anti-apoptotic cell-signalling, mediated by binding to cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/cIAP2) (24). c-IAP 1 and 2 proteins have been found also recruited by the interaction and heterodimer formation between TNFAIP3 and TNF-receptor 1 (35, 25). On the other hand, TNFAIP3 is located in region 6q23, characterised by the presence of RA genetic factors, and a recent study showed that single nucleotide polymorphisms in this gene were involved in the susceptibility to RA (36).

Finally, TNF also share a common pathway with Fas, produced by the gene FAS, that results in the activation of NFKB1. Interestingly, all these signal transduction pathways involved the NFKB1, which was shown be a very important protein in the anti-apoptotic process since their deregulation can lead to the constitutive over-production of pro-inflammatory cytokines and it plays a role in the regulation of the expression of anti-apoptotic proteins (37, 38).

The last two genes involved in the resistance and inhibition of apoptosis we found to be up-regulated in untreated ERA patients and then down-regulated by combined LEF and low dose of prednisone therapy were STAT6 (signal transducer and activator of transcription 6) and IL4R (interleukin-4 receptor).

In particular, STAT6 codify for a protein playing a central role in IL4-mediated biological responses. STAT6 was found to induce the expression of BCL2L1/BCL-X(L), which is responsible for the anti-apoptotic activity of IL-4 (39).

Activated STAT6 binds to the promoter of various genes and plays an important role in target cells through the modification of growth and the induction of resistance to apoptosis (38).

Last gene here discussed is STAT4. As largely known, this gene is associated to RA susceptibility and located in the highly polymorphic HLA region of chromosome 2q, which is a major contributor to the genetic risk of the disease (40, 41). Moreover, STAT4 has found to be activated by interleukin-12 *via* its receptor and involved in the development of a newly discovered subset of Th17 cells, which displays a dominant role in autoimmunity-associated inflammation (42).

In conclusion, the present study seems to contribute to a better understanding of the relationship between therapeutical modulation of specific gene expression and inflammation that characterise ERA patients. In fact recent cluster analyses permitted the separation of patients with ERA from those with long-standing disease (13).

Present results suggest that LEF might be more effective in combined therapy with low dose of prednisone. This therapy should be started as soon as possible since a significant down-regulation of crucial genes involved in the resistance or inhibition of the apoptotic process, in the stimulation of pro-inflammatory cytokine production and in RA susceptibility was here observed.

As matter of fact, the recent EULAR guidelines suggest the combination of low dose of prednisone and one antiproliferative DMARD (*i.e.* methotrexate or leflunomide) as treatment approach to ERA (8).

To better understand the efficacy of LEF therapy on the expression of other selected genes future studies in RA patients treated with LEF alone might be required.

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