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Research paper

Transcriptome modification of white blood cells after dietary administration of curcumin and non-steroidal anti-inflammatory drug in osteoarthritic affected dogs

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ABSTRACT

The dietary effect of non-steroidal anti-inflammatory drug (NSAID) or curcumin on the gene expression of peripheral white blood cells in osteoarthritis (OA) affected dogs was investigated using a 44K oligo microarray. Two groups of OA dogs and one group of healthy dogs (6 dogs each) were clinically evaluated and blood was sampled before (T0) and after 20 days (T20) of dietary administration of NSAID (NSAID group) or curcumin (CURCUMIN group). Differentially expressed genes ($P < 0.05$) in comparison to the control group were identified with MeV software and were functional annotated and monitored for signaling pathways and candidate biomarkers using the Ingenuity Pathways Analysis (IPA). After 20 days of treatment, the differentially expressed transcripts significantly ($P < 0.05$) decreased from 475 to 173 in NSAID group and from 498 to 141 in CURCUMIN group. Genes involved in "inflammatory response" and in "connective tissue development and function" dramatically decreased at T20. Other genes, included in "cellular movement", "cellular compromise" and "immune cell trafficking", were differentially expressed at T0 but not at T20 in both groups. Specific molecular targets of CURCUMIN, not observed for NSAID, were the IkB up regulation in the "TNRF1 signaling pathway" and IL18 down regulation in the "role of cytokines in mediating communication between immune cells". The activity of CURCUMIN was also evidenced from the inhibition of macrophages proliferation (HBEGF), related to a strong down regulation of TNFα and to activation of fibrinolysis (SERPINE1). The results would suggest that curcumin offers a complementary antinflammatory support for OA treatment in dogs.

1. Introduction

Osteoarthritis (OA) is one of the most common chronic musculoskeletal diseases and causes of lameness and disability in dogs (Bennet, 1990; Innes, 1995). This degenerative disease is caused by a progressive condrocyte phenotypic modification and extracellular changes that result in a progressive degradation and eventual loss of arthritic cartilage and in the damage of subchondral bone, frequently accompanied by secondary mild synovitis (Creamer and Hochberg, 1997; Felson and Zhang, 1998).

The therapeutic management of OA in dogs is dominated by non-steroidal anti-inflammatory drugs (NSAIDs), which only treat the symptoms of OA by decreasing the pain and inflammation (Innes et al., 2003). However, the use of NSAIDs is often associated with gastrointestinal side effects (Buttgereit et al., 2001). For these reasons alternative treatments of canine osteoarthritis would be desirable

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Table 1
Composition of experimental groups, biological replicates (pools) and microarray setup (array_code) of the clinical study.

ID	Group	Age (years)	Sex	Breed	Body weight (kg)	Pool
1	CTRL	6	M	Italian Hound	19	1
2	CTRL	5	F	Crossbreed	11	1
3	CTRL	7	F	Labrador Retriever	32	2
4	CTRL	4	M	Crossbreed	15	2
5	CTRL	9	M	Crossbreed	10	3
6	CTRL	6	F	Cirneco dell'Etna	9	3
7	NSAID	13	M	German Shepherd	30	4
8	NSAID	13	F	German Bracco	20	4
9	NSAID	14	F	Italian Pointer	16	5
10	NSAID	9	F	Crossbreed	22	5
11	NSAID	4	M	Crossbreed	17	6
12	NSAID	6	M	German Shepherd	32	6
13	CURCUMIN	15	F	Italian Pointer	18	7
14	CURCUMIN	12	M	Italian Hound	21	7
15	CURCUMIN	11	M	German Shepherd	36	8
16	CURCUMIN	5	F	Italian Hound	22	8
17	CURCUMIN	13	M	Crossbreed	8	9
18	CURCUMIN	6	M	Irish Setter	28	9

M, male; F, female.

and recently nutraceuticals, such as curcumin, have been proposed for this purpose (Henrotin et al., 2005; Innes et al., 2003).

Curcumin is a polyphenolic compound (Stansby et al., 1993; Kagan and Tyurina, 1998), commonly found in the dietary spice turmeric known to be a potent antioxidant, anti-inflammatory, antiseptic and anticancer agent (Commandeur and Vermeulen, 1996; Surh et al., 2001; Miller et al., 2001). Curcumin has been shown to exhibit therapeutic potential in various chronic illnesses where inflammation is known to play a major role, like neurodegenerative, cardiovascular, neoplastic, pulmonary, metabolic and autoimmune diseases (Aggarwal and Harikumar, 2009).

From *in vitro* studies it has been reported that curcumin is similar to NSAIDs in suppressing the production and catabolic action of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), that are known to play a key role in the pathogenesis of OA (Shakibaei et al., 2007). Curcumin has also been shown to be an effective scavenger of reactive oxygen species (ROS) and reactive nitrogen species *in vitro* (Sreejayan and Rao, 1996, 1997), peculiarity of primary importance considering that the degradation of cartilage results from the combination of mechanical stress and mainly increase of metalloproteinases and ROS (Henrotin and Kurz, 2007).

In previous studies conducted on mares affected from OA and osteochondritis (Farinacci et al., 2009a) we have measured the expression of single target genes by real time PCR. The study confirmed that curcumin possesses an anti-inflammatory activity through the suppression of pro-inflammatory cytokines and catabolic enzymes.

Considering these results, we evaluated the systemic effect that NSAIDs and curcumin exert at the transcriptome level by using DNA microarray technique in dogs in white blood cells.

This study analyzed the expression of the whole *Canis familiaris* genome before and after the treatment with curcumin and NSAID of dogs affected by OA. The aim of the research was to investigate the molecular activity of curcumin in OA affected dogs and to find new biomarkers to depict this pathological condition.

2. Methods

2.1. Animals and treatments

Eighteen dogs were recruited in a kennel; at their first visit dogs were examined for signs of OA or other diseases and a case history reporting the status of body systems and a punctual evaluation of muscoskeletal apparatus were done. Elbow and hip joints were manipulated and the degree of lameness was graded with a score from 1 to 5 (where 5 indicate the worst condition). In this way, 12 dogs resulted affected by OA and 6 were healthy.

The 12 affected dogs were randomly assigned to two groups of 6 animals each and the characteristics of subjects are reported in Table 1. Between the affected dogs, a first group of 6 randomly selected animals (test group – CURCUMIN) was treated twice a day with curcumin (4 mg/kg body weight), in the form of phytosome (CURCUVET®, Indena Spa, Milan, Italy), for 20 days. A second group of 6 dogs (positive control – NSAID) received oral Firocoxib (Previcox®, Merial Italia Spa, Milan, Italy) administration for the same period (5 mg/kg per day). A third group (negative control – CTRL) was constituted by 6 healthy and untreated dogs.

Blood sampling were performed on the 3 groups at the beginning of the trial (T0) and after 20 days (T20), that corresponded to the end of administration of NSAID or CURCUVET® in the experimental groups. Protocol was under ethical approval and informed consent was obtained from kennel before the beginning of study.

2.2. Blood sampling and isolation of RNA

Blood was sampled from the cephalic vein with the PAXgene Blood RNA System (PreAnalytiX, Switzerland) and RNA was extracted following the manufacturer's instructions for genes expression evaluation. The concentration

of the extracted RNA was quantified with a spectrophotometer (NanoDrop 1000 ThermoScientific, Wilmington, DE, USA). RNA integrity was evaluated by the observation of 18S and 28S ribosomal RNA bands after electrophoresis on 1% agarose gel, in the presence of ethidium bromide. The RNA integrity was checked again using the Agilent Bioanalyser and the RNA nano Lab chip kit (Bioanalyser Nanochip QC, USA).

2.3. Oligo-microarray

Transcriptome analysis was performed using a canine oligo microarray (Agilent Technologies Italia S.p.A., Milan, Italy) containing 42034 genes of the domestic dog (*C. familiaris*). Platform information is available at http://www.ncbi.nlm.nih.gov/geo at accession GPL11214. Before hybridization, three pools for each experimental group were formed; these contained an equal aliquot of RNA of 2 randomly selected individuals (3 biological replicates for each group).

Total RNA (500–1000 ng) was labelled prior to microarray hybridization using the Quick Amp Labeling Kit one color (Agilent #5190-0442). Labeling was performed on pool of samples produced to have three biological replications within each group.

Agilent 4x44K Canine Expression Microarray slides (AMADID 15607) were hybridized, washed and then scanned on an Agilent Microarray Scanner. Images from the scanner were processed using Agilent Feature Extraction Software v9.1. A quantile normalization algorithm was applied as described in Bolstad et al. (2003). Afterwards, an in-slide replicates analysis from Midas (Saeed et al., 2006) was used to reduce data redundancy. To evaluate the significant over and under expressed genes between experimental groups, the ratios of NSAID group to CTRL group and of CURCUMIN group to CTRL group at T0 and at T20 were separately calculated for each gene and data were log(2) transformed to ensure normal distribution.

Statistical analysis was performed with MeV open-source software (v 4.6 – http://www.tm4.org/mev/; 55) using one-way ANOVA model with group as fixed factor (Saeed et al., 2006). *P*-values were based on permutation test, with 1000 permutations, and Bonferroni correction was applied setting alpha (critical *P*-value) at 0.05.

2.4. Ingenuity® Pathways Analysis (IPA)

Ingenuity® Pathways Analysis program version 9.0 (Ingenuity Systems, Mountain View, CA, USA) was used to search for biological processes, pathways and networks involved. This web-based entry tool allows the mapping of gene expression data into relevant pathways based on the gene's functional annotation and known molecular interactions (Calvano et al., 2005; Li et al., 2007). This information, which comes from published, peer reviewed scientific publications, is stored in the Ingenuity Knowledge Base, which is continuously updated. A molecular network of direct or indirect physical, transcriptional and enzymatic interactions between mammalian orthologs was computed from this knowledge base. By comparing the imported microarray data with the Ingenuity Knowledge Base, the list of genes was transformed into a set of relevant networks, focus genes and canonical pathways were identified, and functional annotation was performed (Wognum et al., 2009). A detailed description of IPA can be found at www.Ingenuity.com. The differentially expressed genes identified with Mev software were used for network analysis, using a cutoff of the log(2) ratio of ±0.6. The gene products were categorized based on location, cellular components and reported or suggested biochemical, biological, and molecular functions.

Gene network analysis was carried out by using a global molecular network developed from information contained in the Ingenuity Knowledge Base. Identified gene networks were ranked according to scores provided by IPA.

Canonical pathway analysis revealed molecular pathways in the IPA library of canonical pathways (part of the Ingenuity Knowledge Base) that were the most significant for the data set. Genes from the data set that were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis.

2.5. Validation data by real time-PCR analysis

Primer3 Input software (Rozen and Skaletsky, 2000) was used to design the primer sequences encoding for: beta-actin (ACTB), mitochondrial ribosomal protein S7 (MRPS7), interleukin 8 (IL8), toll-like receptor 4 (TLR4), mitogen-activated protein kinase 14 (MAPK14), interleukin 18 (IL18), prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) (PTGS1), prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2) and TNFα (tumor necrosis factor alpha). Primers, product lengths and specific amplification conditions for each gene are listed in Table 2.

Reverse transcriptions were performed for each biological replicate with 400 ng of extracted total RNA by using Improm-II Reverse Transcriptase (Promega, Milan, Italy). Total RNA with 1 μl oligo$(dT)_{18}$ primers (0.5 μg/μl MBI Fermentas, Italy) and nuclease free water to a final volume of 20 μl, were incubated at 70 °C for 5 min in a PTC-100 thermocycler (MJ Research Inc. Waltham, MA, USA). Then a mix was prepared with 4 μl of Improm-II Reverse Transcriptase buffer (5× Promega, Milan, Italy), 1.2 μl $MgCl_2$ (50 mM), 1 μl of Improm-II Reverse Transcriptase and 1 μl of dNTP (10 mM) and was added to the reaction and incubated at 37 °C for 90 min and finally at 94 °C for 5 min. The final concentration of cDNA was assumed as 20 ng/μl.

For each gene, an aliquot of cDNA samples were pooled and standard curves with serial dilution of pool were used to optimize PCR conditions and to calculate the efficiency, fluorescence baseline and threshold.

Real time PCRs were performed for each sample in triplicate form using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Milan Italy) for IL8, IL18, PTGS1, PTGS2, TLR4, MAPK14 and TNFα. For these reactions, a master mix with the following components was prepared to the indicated end concentration: 1 μl of cDNA, 9.5 μl water, 1 μl of each primer and 12.5 μl of 2× Platinum SYBR Green qPCR SuperMix-UDG for a total volume of 25 μl. cDNA concentrations and primers molarities were

Table 2
Primer pair sets and parameters used in the real time PCR analysis.

Gene	Genbank accession number	Sequence (5′ → 3′)	Product length (bp)	cDNA (ng)	Primer (nM)	Ta (°C)	*E*
BACT	5597004	For: ACTGGGACGACATGGAGAAG Rev: AAGGCGTACCCCTCGTAGAT	280	1	200	59	1.91
MRPS7	73965035	For: CGCAAC CCCTATGTCATCTT Rev: CAACTTCTCTGGCATCAGCA	201	10	300	57	1.95
IL8	415265	For: TCTTGGCAGCTTTTGTCCTT Rev: GGGCCACTGTCAATCACTCT	151	10	600	57	1.90
TLR4	50950158	For: TTTAGGAATGCCACCCTGTC Rev: TCCTCACCCAGTCTTCATCC	214	10	600	58	1.98
MAPK14	50978967	For: CCAAATTCTCCGAGGTCTCA Rev: TATGCATCCCACTGACCAAA	241	5	600	56	1.95
IL18	50978897	For: GAGGATATGCCC GATTCTGA Rev: ATCATGGCCTGGAACACTTC	240	2.5	600	57	1.90
COX1	23452498	For: TGCTCATGCGTCTGGTACTC Rev: GTCTGGCAACTGCTTCTTCC	188	10	600	57	1.90
COX2	13641174	For: ATCCCCTTCCTGCGAAATAC Rev: CATCAGGTACAGGGGGAAGA	160	10	600	57	1.88
TNF	167765472	For: ACCACACTCTTCTGCCTGCT Rev: CTGGTTGTCTGTCAGCTCCA	219	10	600	57	1.96

For, forward; Rev, reverse; Ta, annealing temperature; *E*, PCR amplification efficiency.

different for each gene and determined with standard curves analyses performed before real time PCR reactions.

PCR amplification was conducted applying 45 cycles (1 s at 95 °C, 30 s at the specific annealing temperature, 30 s at 72 °C) in a 96-well spectrofluorometric thermal cycler (DNA Engine Opticon 2; MJ Research, Inc. Waltham, MA USA). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression of target genes was normalized using the ACTB and MRPS7 genes and ΔCts were calculated by the difference between Ct of genes target and the geometric mean of the two housekeeping genes.

The expression level of a given target gene in each experimental group was analyzed by the $2^{-\Delta\Delta Ct}$ method (Bustin et al., 2009; Livak and Schmittgen, 2001) where $2^{-\Delta\Delta Ct}$ represents the difference of a given target gene between each treated group (CURCUMIN and NSAID) at T0 *vs.* CTRL group at T0, the same analysis was done at T20. More precisely, ΔΔCt was calculated for each group as $\Delta\Delta Ct = \Delta Ct_j - \Delta Ct_0$, where "*j*" is the pool value of treated group at T0 and "0" is the pool value of CTRL group at T0. The *n-fold* expression of a given target gene was calculated as log(2) (ratio).

3. Results

3.1. Clinical examination

The trial was designed to assess the transcriptome modification of circulating leukocytes in OA affected dogs after pharmacological treatment with NSAID or dietary administration of extract of *Curcuma longa*. A limited number of dogs was used for the study, to accomplish with the ethical rule of minimum animal for *in vivo* trials. Moreover, to reduce the experimental residual error related to the synchronicity of sampling and treatment between groups, only few contemporary OA affected dogs were available in the kennel. During the experimental period, all the animals received regular clinical control and were daily monitored by the veterinarians. At the end of the treatment, the OA dogs showed a reduction of pain and a partial recovery of articular functions even though at a variable degree. However, due to the limited number of dogs, no clinical conclusions were drawn.

3.2. Gene expression

Data analysis was conducted comparing gene expression of the positive control (NSAIDs) and test (CURCUMIN) groups *vs.* negative control groups (CTRL at T0 and at T20). Therefore, gene expression analysis at T0 permits to describe the OA conditions of the animals before starting the experimental trial, and data obtained at T20 are consistent with the activity of NSAID and CURCUMIN.

3.3. Analysis of differences in gene expression in the positive control and test groups

The transcripts differentially expressed identified from the ANOVA statistical analysis ($P < 0.05$) and from the use of filter and general setting database of IPA software were 2804. The annotated transcripts eligible decreased to 771 after the application of an expression value cut-off of ±0.6 log ratio. The differentially expressed transcripts before treatments (T0) in NSAID group and in CURCUMIN group were 475 and 498, respectively (Table 3).

Table 3
Total and annotated transcripts which were differentially expressed ($P < 0.05$) above the ±0.6 log ratio filter according to IPA analysis at T0 and T20. The table also reports the number of up and down regulated transcripts in NSAID group and CURCUMIN group.

	T0		T20	
	NSAID	CURCUMIN	NSAID	CURCUMIN
Total genes	475	498	173	141
Up regulated	290	271	52	31
Down regulated	185	228	121	110

Table 4
Number of genes and significance (*P* value) for the common ontology categories selected from IPA analysis for their involvement in OA disease in NSAID group and in CURCUMIN group at T0 and T20.

	NSAID		CURCUMIN	
	P-value	Gene *n*	*P*-value	Gene *n*
T0				
Diseases and disorders				
Inflammatory response	0.0000589	32	0.0000523	66
Molecular and cellular functions				
Cellular movement	0.0000589	28	0.0000740	70
Cellular compromise	0.0015600	20	0.000152	10
Physiological system development and function				
Hematological system development and function	0.0000589	37	0.000198	62
Hematopoiesis	0.0000589	21	0.000257	23
Immune cell trafficking	0.0000589	21	0.000274	42
Connective tissue development and function	0.0002710	26	0.000814	36
T20				
Diseases and disorders				
Cancer	0.0008140	57	0.000124	36
Inflammatory response	0.0026600	12	0.000194	15
Molecular and cellular functions				
Cellular growth and proliferation	0.0002070	57	0.000125	32
Cell death	0.0002740	29	0.000182	27
Cell cycle	0.0003510	25	0.0000238	21
Physiological system development and function				
Hematological system development and function	0.000198	31	0.000194	23
Tumor morphology	0.0002571	13	0.00000920	15
Cardiovascular system development and function	0.0002741	11	0.000542	19
Connective tissue development and function	0.000814	16	0.000125	16

After the treatment, the differentially expressed transcripts decreased dramatically to 173 and 141 in NSAID group and in CURCUMIN group, respectively. This was mainly related to a reduction of up regulated genes that decreased from 290 to 52 in NSAID group and from 271 to 31 in CURCUMIN group (Table 3).

3.4. Function and canonical pathway analysis

The differentially expressed genes were annotated in functionally categories using gene ontology (GO) terms. In Table 4, the most interesting categories related to OA and common to NSAID group and to CURCUMIN group with a *P*-value lower that 0.001 were reported.

Between these categories, "inflammatory response", "haematological system development and function" and "connective tissue development and function", were present at T0 and T20, but the number of genes involved in "inflammatory response" and in "connective tissue development and function" dramatically decreased at T20. The categories within molecular and cellular functions shifted from cell response to external stimuli ("cellular movement" and "cellular compromise") at T0 to categories involved in cell fate (cellular growth and proliferation, cell death and cell cycle) at T20. Moreover, categories as "cellular movement", "cellular compromise" and "immune cell trafficking", which were affected by OA at T0, were not present after the treatments (Table 4).

In NSAID group at T0, functions annotation of the category were "chemokinesis of neutrophils", "transmigration of granulocytes", "degranulation of neutrophils" and "transmigration of neutrophils" and in CURCUMIN group at T0 also "phagocitosis of macrophages", "migration of lymphatic system cells" and "migration of bone marrow cells" were involved (Table 5). After treatments, functions annotations were dramatically reduced both for NSAID and CURCUMIN groups, especially with regard to the activity of neutrophils (Table 5).

The IPA software identified 25 networks active at T0 and 14 networks at T20 in both groups. These networks are involved in wide variety of physiological and pathophysiological processes, including "cell death", "connective tissue disorders", "genetic disorder", "cardiovascular system development and function", "cell-to-cell signaling and interaction", "hematological system development and function", "infectious disease", "lipid metabolism" and "free radical scavenging".

Relationships between networks generated by the IPA system and known pathways were further investigated by canonical pathway analysis. We found that numerous pathways were affected by treatments and among the pathways identified, we focused on the "TNRF1 signaling pathway" (Fig. 1) and on the "role of cytokines in mediating communication between immune cells" (Fig. 2). Before treatments (T0) the OA dogs showed the same significant genes differentially expressed compared to the CTRL group. Conversely, at T20 a different gene modulation was shown after NSAID and CURCUMIN administration. In particular, in the "TNFR1 signaling" pathway CURCUMIN down regulated TNFα and up regulated IkB, whilst NSAID modulated the expression of genes involved, without affecting the IkB (Fig. 1). In the "role of cytokines in mediating communication between immune cells" CURCUMIN treatment modulated the expression of TNFα, IL8 and IL18, NSAID treatment up regulated the same genes, but did not changed the IL18 expression (Fig. 2).

Table 5
Effect of NSAID and curcumin administrations to dogs with chronic osteoarthritis on the expression levels of the most relevant genes, reported as log(2) ratios, included in the categories of functional annotations (Table 4). The role of genes as candidate biomarkers for diagnosis, efficacy, prognosis, response to therapy, and safety according to IPA analysis was also indicated.

Gene	T0		T20		Categories					Candidate biomarkers
	NSAID	CURCUMIN	NSAID	CURCUMIN						
CAST	0.742	0.853	0.632		IR	CM		ICT	CTDF	
IL8	0.963	0.933			IR	CM	CC	ICT		Efficacy
S100A9	1.404	1.843			IR	CM	CC	ICT		Diagnosis
TNF	0.662	0.867		−0.647	IR	CM	CC	ICT		Diagnosis, efficacy, prognosis, safety
ANXA1	0.782	1.047			IR	CM	CC	ICT		
CD47	0.974	1.140			IR	CM	CC	ICT		
TLR4	0.746	1.007			IR	CM	CC	ICT		
IL18	0.709	0.793	0.618		IR	CM		ICT	CTDF	Diagnosis, efficacy, prognosis, response to therapy, safety
S100A8	1.199	1.610			IR	CM		ICT		Diagnosis, efficacy, response to therapy
JAK2	0.723	0.783				CM		ICT		
SAT1	0.819	0.953							CTDF	
UCHL3	0.676	0.613							CTDF	
BCL6	0.756	1.283								Diagnosis, efficacy, prognosis
HMGB2	1.271	1.553								
LDHA	0.766	0.987								Diagnosis, efficacy, prognosis
LYZ	0.843	0.780								
USP13			−0.866	−0.650						
SMTN			−0.657							
HBEGF				−0.642					CTDF	Diagnosis, efficacy,
SERPINE1				−0.603	IR				CTDF	Diagnosis, efficacy, response to therapy, safety
CTLA4			−0.689	−0.601	IR					Diagnosis, efficacy, prognosis
MYLK3	−0.884	−1.043			IR	CM		ICT		
APOA2	−0.697	−0.717			IR					
DRD2	−1.569	−1.943							CTDF	
GCGR	−0.802	−1.250							CTDF	
BGLAP	−0.846	−0.733								Diagnosis, efficacy, safety

IR, inflammatory response; CM, cellular movement; CC, cellular compromise; ICT, immune cell trafficking; CTDF, connective tissue development and function.

3.5. Identification of candidate biomarkers

The IPA analysis of biomarker identified the best candidates based on biological characteristics most relevant to the discovery study and elucidated the mechanisms linking potential markers to the disease or biological process of interest. Biomarkers and their relations with the inflammatory perturbations were evidenced by pattern recognition analyses among the genes that displayed common expression patterns between T0 and T20 in the NSAID and CURCUMIN-treated groups (Table 5). Between genes up regulated at T0 in both groups IL18, IL8, S100A8, S100A9 and TNFα were found. Between these genes only TNFα was down regulated by CURCUMIN treatment, the expression of other genes was within the ±0.6 log ratio and IPA tool excluded these values from the biomarker analysis.

3.6. Validation of gene expression with real-time PCR

In order to validate the results obtained from IPA, we measured the expression of IL8, IL18, TLR4, MAPK14 and TNFα genes included in function categories and biomarkers with real time PCR. Moreover, PTGS1 (aliase Cox-1) and PTGS2 (aliase Cox-2), genes were also analyzed independently from IPA analysis as they are often considered relevant targets of inflammatory conditions and drugs.

The expression values resulted by real-time PCR analysis confirmed the findings obtained with microarray and IPA analysis (Fig. 3).

4. Discussion

Osteoarthritis is one of the most common causes of lameness in dogs. It is caused by a deterioration of joint cartilage, followed by pain, inflammation and loss of range of motion of the joint. Pharmacological treatments for OA are restricted to the use of NSAIDs. However, the use of NSAIDs is associated with numerous side effects, which can be quite adverse (Innes et al., 2010).

Curcumin (diferuloylmethane) is a naturally occurring polyphenol derived from the rhizome of *C. longa* Linn, with the potential for treatment of various diseases acting *via* NF-κB signaling pathway (Bharti et al., 2004; Buhrmann et al., 2011). This polyphenol has antioxidant and anti-inflammatory activity and has been found to suppress tumor initiation, promotion, and metastasis (Aggarwal and Harikumar, 2009). The positive effect of curcumin administration *per os* was also described in chronic OA affected mares (Farinacci et al., 2009a). Although Innes et al. (2003) reported a remittance of pain and a recovery of articular movements after curcumin treatment in dogs, the investigations of molecular mechanisms with a full trascriptome analysis in this specie is still lacking. These studies on dogs and horses used a dose of 4 mg curcumin/kg of live

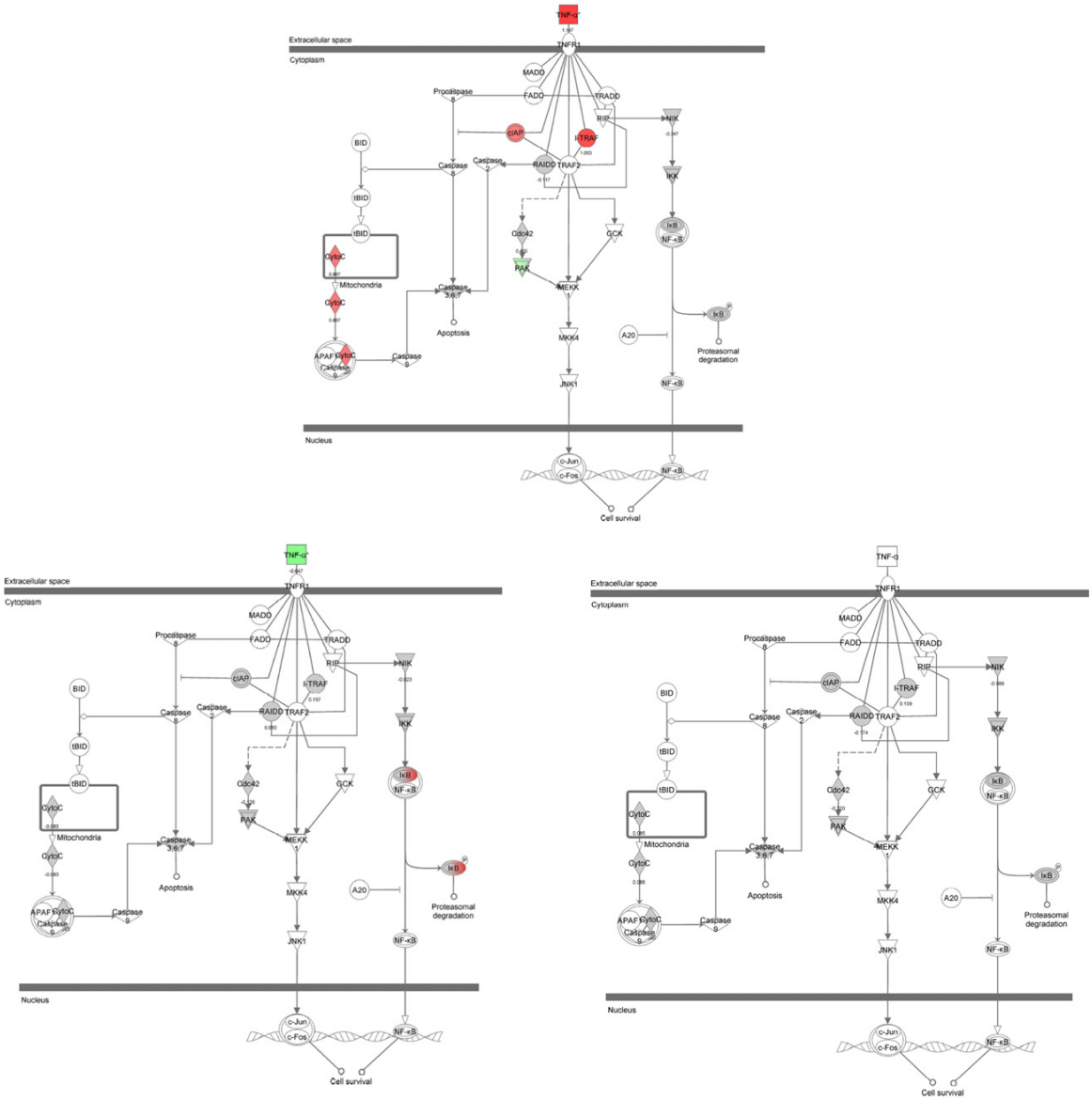

Fig. 1. "TNRF-1 signaling pathway" at T0 (above) and T20 (below) in CURCUMIN group (left) and NSAID group (right). Green and red colors mean respectively genes down- and up-regulated.

weight, which was the same amount chosen in the present research.

Blood samples were used in this study considering that the circulating cells of peripheral blood are a surrogate tissue that contains transcriptional profiles correlating with disease (Rockett and Burczynski, 2006), avoiding more invasive clinical practices as the collection of synovial fluid or tissue biopsies. The comprehensive microarray-based pathway analysis used in this study provided an appealing approach. Using this tool, we effectively identified candidate genes and pathways involved in the response of OA to NSAID and to CURCUMIN treatments. In our study we succeeded in identifying a comparable number of differentially expressed genes in OA affected dogs at T0 and T20. These results indicate that curcumin could mimic the antinflammatory activity of NSAID (Shakibaei et al., 2007). To further refine the significance of the differentially expressed genes we next investigate gene ontology categories, function annotations and potential biomarkers in the two groups. In particular, the highlight of this study was the identification of the inflammatory and immune response pathways.

NSAID and CURCUMIN treatments reduced TNF-α expression a pro-inflammatory cytokine produced by

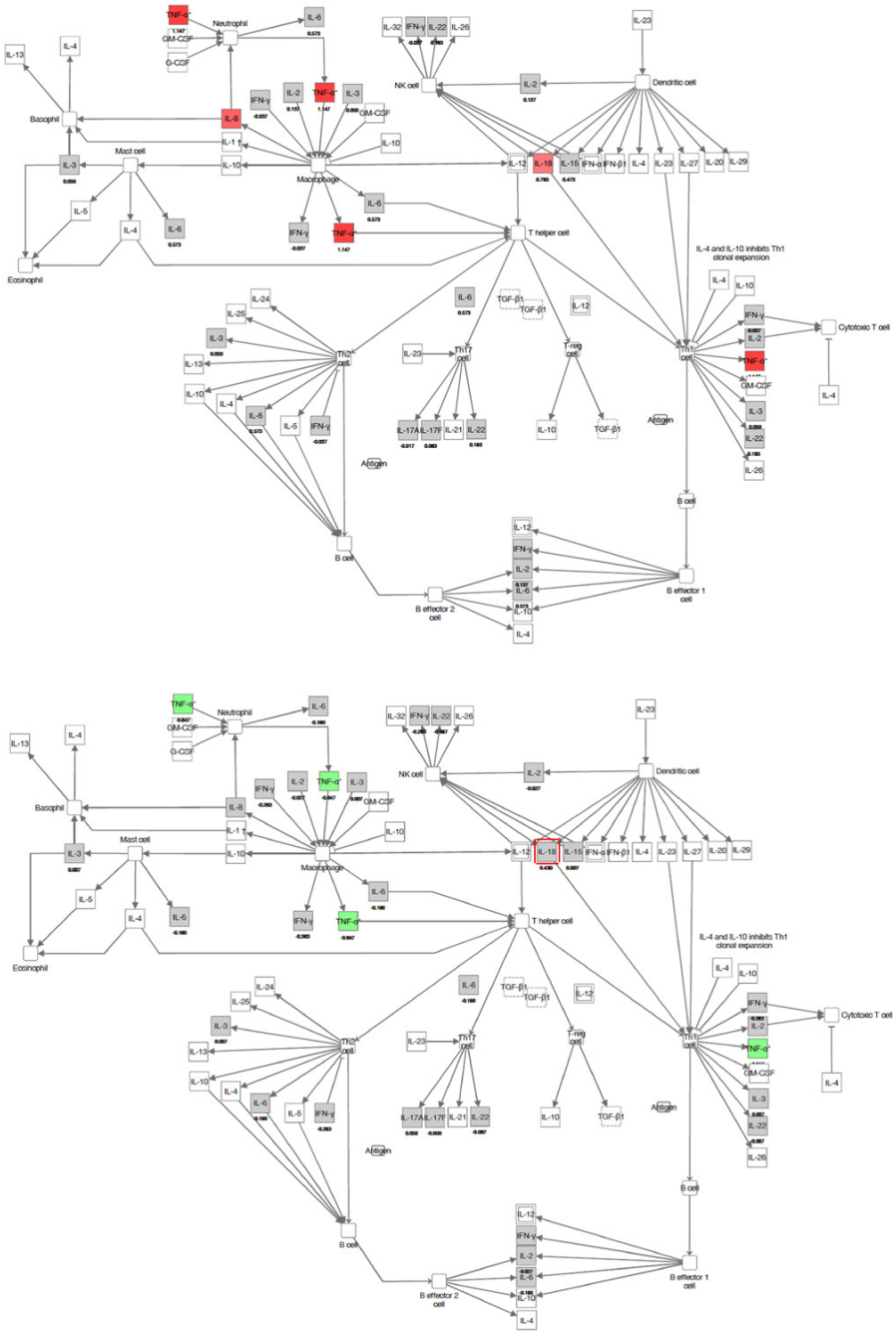

Fig. 2. "Role of cytokines in mediating communication between immune cells" at T0 (above) and T20 (below) in CURCUMIN group. The only difference with NSAID group was the up regulation of IL18 at T20, highlighted in red square.

activated synoviocytes, macrophages and chondrocytes (Fernandes et al., 2002). TNF-α is well known to activate the ubiquitous transcription factor NF-κB, which leads to further production and up regulation of pro-inflammatory cytokines and enzymes such as PTGS2. Curcumin has previously been shown to antagonize some catabolic effects of TNFα and IL-1β *via* inhibition of NF-κB in different cell types (Singh and Aggarwal, 1995; Aggarwal et al., 2004). At T20 curcumin up regulated IkB, which inactivates NF-κB by trapping it in the cytoplasm, without affecting

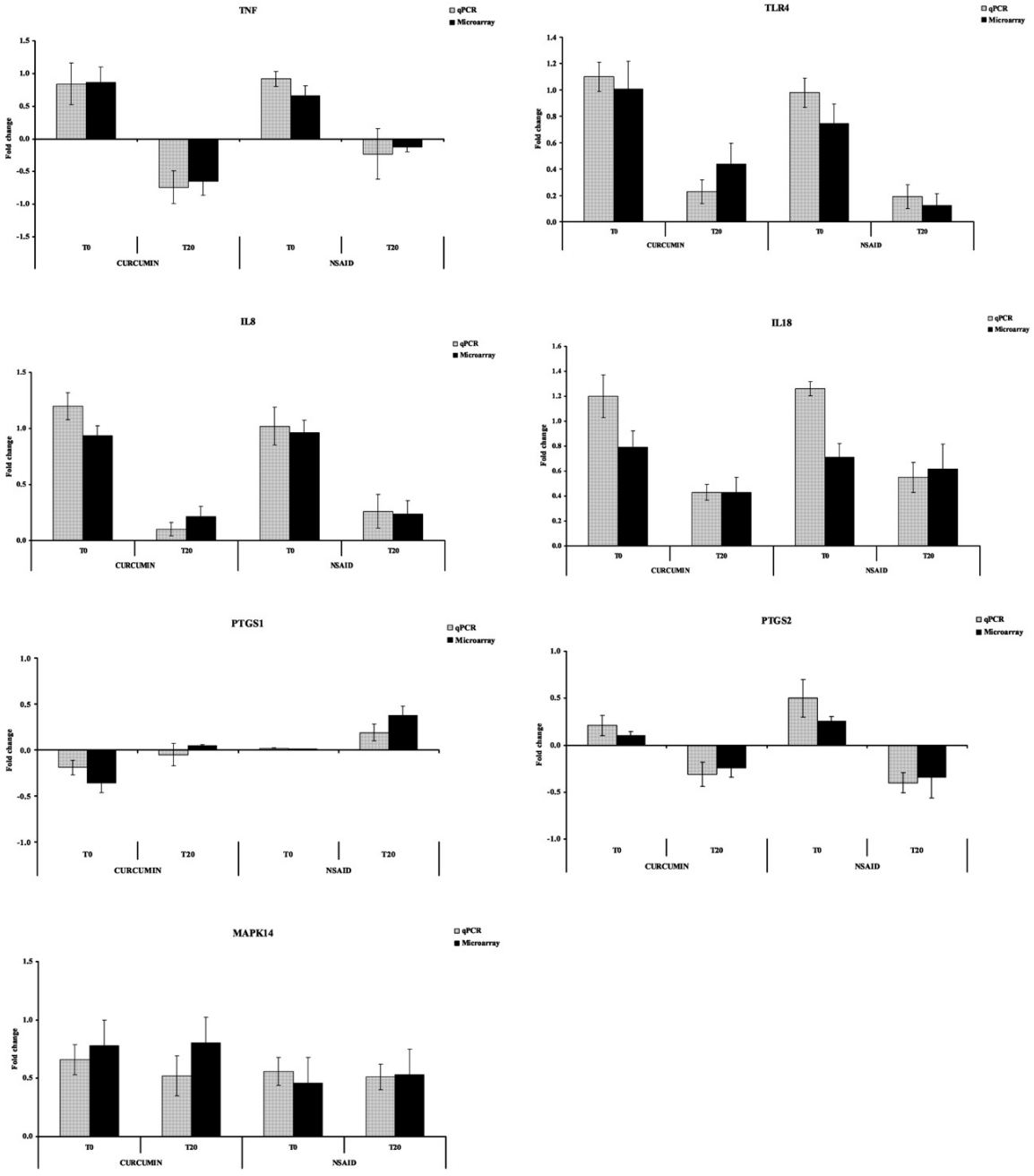

Fig. 3. mRNA expression of selected genes as determined by real time PCR and microarray analyses. The gene expression level determined by real time PCR was normalized to geometric mean of BACT and MRPS7. Error bars indicate ±SD.

its phosphorylation. The antinflammatory efficacy of curcumin and resveratrol was reported in primary human chondrocytes and tenocytes *in vitro* (Csaki et al., 2009; Buhrmann et al., 2011). It has been shown that resveratrol and curcumin inhibit the NF-κB signal transduction pathway in human articular chondrocytes, but in different ways (Csaki et al., 2009). While resveratrol inhibits ubiquitination of phosphorylated IkBα and blocks translocation of the activated NF-κB to the nucleus, curcumin only inhibits translocation of the activated NF-κB to the nucleus without affecting phosphorylation. Another specific target of activity of curcumin in human cancer cell lines is the inhibition of Ikk activity (Anand et al., 2011), a serine/threonine protein kinase that phosphorylates the inhibitors of NF-κB thus leading to the dissociation of the inhibitor/NF-κB complex and ultimately the degradation of the inhibitor. In this study we only observed an increase of IkB expression level. It is likely that the complex mechanism of NF-κB regulation and the cross talking among white cells of blood stream could have mask this specific activity of curcumin *in vivo*.

The down regulation of TNF-α observed after curcumin and NSAID administrations played also a role in the control of the expression of IL8 and IL18. López-Armada et al. (2002) observed a significant reduction of IL8 in rabbits with antigen-induced arthritis after treatment with meloxicam and clofenac (NSAIDs). Moreover, Farinacci et al. (2009b) reported a strong anti-inflammatory activity of curcumin on sheep neutrophils and the inhibition of IL8.

IL18 is present at increased levels in serum and in the rheumatoid synovium (Cho et al., 2005; Boraschi and Dinarello, 2006). In the present study, IL18 was down regulated ($P < 0.05$) only after curcumin treatment and this result is in agreement with findings of Okunieff et al. (2006), which observed that curcumin, administered before or after radiation in mice, significantly decreased mRNA expression of early responding cytokines including IL18.

IPA analysis provided also a set of genes either included in functional categories or in candidate biomarkers. S100A9 and S100A8 play a role as pro-inflammatory mediators in acute and chronic inflammation and up regulate the release of IL8 and cell-surface expression of ICAM1 (Ryckman et al., 2003). The reduction of their up regulation after treatments is coherent with the antinflammatory activity of these compounds (Schäfer and Heizmann, 1996).

Toll-like receptor-4 (TLR4) plays a key role in sensing microbial components and inducing innate immune responses (Youn et al., 2006). TLRs mediate the activation of NFkB, resulting in the production of various proinflammatory cytokines, including arthritogenic TNF-α, IL6, IL12, IL18, and interferon-γ (IFNγ) (Guha and Mackman, 2001; Takeda et al., 2003). The activity of curcumin and NSAID on the regulation of TLR4 is consistent with data of Youn et al. (2006), which demonstrated that the daily consumption of curcumin modulates the activation of TLRs and subsequent immune/inflammatory responses, inhibiting both ligand-induced and ligand-independent dimerization of TLR4. Other genes were differentially expressed from the healthy dogs only after the administration of antinflammatory compounds. If NSAID activated the immune response of T cells through CTLA4 gene deactivation, curcumin also acted as a suppressor of genes related to macrophages proliferation as HBEGF (Triantafyllopoulou et al., 2010) and inhibition of fibrinolysis, as SERPINE1 (Hu et al., 2010).

The data obtained by real time PCR confirmed the microarray analysis for some the most relevant genes, TNFα, TLR4, IL8, IL18 and MAPK14. The NSAID used, Firocoxib, is known to selectively inhibit PTGS2 and a similar activity has been also reported for curcumin (Vane and Botting, 1996; Anand et al., 2011). However, PTGS2 was not found among the eligible genes in IPA analysis lower than the IPA threshold (±0.6) and therefore cyclooxygenase expressions of the inducible and constitutive forms were measured by real time PCR. Our data confirmed the selective inhibitory function of curcumin and NSAID on PTGS2 expression.

5. Conclusions

This study was carried out on dogs affected by a chronic pathology, OA, and indicated that curcumin regulated molecular target of inflammatory response, as observed in human. Specific molecular targets of curcumin, not observed for NSAID, were those related to inhibition of macrophages proliferation, also related to a strong down regulation of TNFα, and to activation of fibrinolysis, thus offering a good complementary support for OA treatment. However, the limited number of subjects does not allow to draw definitive conclusion for what the clinical remittance is concerned and a large number of cases is required to validate the use of curcumin for the treatments of OA in dogs.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2012.04.001.

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