Original Article

Characterization of Human Gene Expression Changes after Olive Oil Ingestion: an Exploratory Approach

(olive oil / gene expression / microarray / atherosclerosis / cancer)

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Abstract. Olive oil consumption is protective against risk factors for cardiovascular and cancer diseases. A nutrigenomic approach was performed to assess whether changes in gene expression could occur in human peripheral blood mononuclear cells after olive oil ingestion at postprandial state. Six healthy male volunteers ingested, at fasting state, 50 ml of olive oil. Prior to intervention a 1-week washout period with a controlled diet and sunflower oil as the only source of fat was followed. During the 3 days before and on the intervention day, a very low-phenolic compound diet was followed. At baseline (0 h) and at post-ingestion (6 h), total RNA was isolated and gene expression (29,082 genes) was evaluated by microarray. From microarray data, nutrient-gene interactions were observed in genes related to metabolism, cellular processes, cancer, and atherosclerosis (e.g. *USP48* by 2.16; *OGT* by 1.68-fold change) and associated processes such as inflammation (e.g. *AKAP13* by 2.30; *IL–10* by 1.66-fold change) and DNA damage (e.g. *DCLRE1C* by 1.47; *POLK* by 1.44-fold change). When results obtained by microarray were verified by qRT-PCR in nine genes, full concordance was achieved only in the case of up-regulated genes. Changes were observed at a real-life dose of olive oil, as it is daily consumed in some Mediterranean areas. Our results support the hypothesis that postprandial protective changes related to olive oil consumption could be mediated through gene expression changes.

Introduction

There is growing evidence that the Mediterranean diet, in which olive oil is the main source of fat, has a beneficial effect on diseases associated with oxidative damage such as cardiovascular (CVD), cancer, or neurodegenerative diseases, and also on ageing (Covas et al., 2007). Oxidation of low-density lipoproteins (LDL) is a hallmark for atherosclerosis and CVD development (Witztum, 1994), and oxidative DNA damage has been shown to be predictive for cancer development (Poulsen, 2005). In human intervention studies, sustained olive oil consumption has been shown to be able to reduce the *in vivo* lipid and DNA oxidative damage, as well as the inflammatory status (Covas et al., 2007; Fitó et al., 2007).

Nutrients can regulate gene expression at various stages, including transcription, mRNA processing and stability, and trans- and post-translational modifications. In experimental studies, olive oil has been shown to be able to influence: stages of carcinogenesis, cell membrane composition, signal transduction pathways, transcription factors, and tumour suppressor genes (Menendez et al., 2006). In some previous studies the ingestion

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Abbreviations: BMI – body mass index, GADPH – glyceraldehyde-3-phosphate dehydrogenase, HDL – high-density lipoprotein, IL – interleukin, LDL – low-density lipoprotein, PBMNC – peripheral blood mononuclear cells.

of a 25 ml dose of virgin olive oil did not promote postprandial oxidative stress (Weinbrenner et al., 2004), whereas doses greater than or equal to 40 ml did (Fitó et al., 2007). However, virgin olive oil, rich in phenolic compounds, reduced postprandial oxidative damage to lipids, endothelial dysfunction, and the pro-thrombotic profile both in healthy and hypercholesterolaemic individuals (Covas et al., 2007).

A lack of data exists on the *in vivo* effect of diet on human gene expression. The aim of this work was to explore the changes in gene expression after olive oil ingestion (50 ml) at the end of the postprandial time, particularly the changes related to atherosclerosis and cancer processes, in peripheral blood mononuclear cells (PBMNC) of healthy individuals.

Material and Methods

Subjects, study design and sample collection

Six healthy male volunteers, aged 22 to 28, were recruited. The ethical committee (CEIC-IMAS) approved the protocol and participants signed an informed consent. All volunteers were healthy on the basis of a physical examination and standard biochemical and haematological tests. Subjects had an average weight of 74.1 ± 11.7 kg, and a body mass index (BMI) of 24.5 ± 3.55 kg/m^2.

Prior to intervention, volunteers followed a one-week washout period in which sunflower oil was provided as the only source of fat for all purposes and participants followed an antioxidant-controlled diet. During the last three days of the washout and on the intervention day, volunteers followed a strict low-phenolic compound diet. A nutritionist gave instructions on excluding several foods rich in phenolic compounds from their diet (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cacao, marmalade, olive oil, and olives). Meals were served at the Centre during the intervention day. At 8 a.m., at fasting state, 50 ml (44 g) of olive oil were administered to the volunteers in a single dose. During the first six post-intervention hours, subjects abstained from food and drinks with the exception of caffeine-free, low-energy drinks and water. PBMNC were isolated from peripheral blood collected in cell preparation tubes (CPT™ tubes, Beckton Dickinson, Franklin Lakes, NJ) at baseline (0 h, pre-intervention) and at 6 h post-intervention. Whole blood was centrifuged at 1690 *g* for 30 min, and cells were washed with buffer phosphate (AMBION, Foster City, CA), centrifuged at 970 *g* for 15 min, re-suspended in Ultraspec® (Biotecx Laboratories, Houston, TX), and stored at -80 ºC until RNA isolation.

Olive oil characteristics

The olive oil used was virgin olive oil, Hojiblanca variety from Andalucía, Spain. Its fatty acid composition was: 1) monounsaturated fatty acids: 75 %; 2) polyunsaturated fatty acids: 18.6 %; and 3) saturated fatty acids: 6.4 %. Minor components were: α-tocopherol (1.47 mg/kg); β-carotene tocopherol (0.43 mg/kg), sterols (15.6 mg/kg); and phenolic compounds (316 mg/kg). The olive oil was stored in the dark, avoiding exposure to air, light and high room temperature in order to be protected against oxidative stress damage.

RNA extraction and microarray sample preparation

Total RNA was extracted from PBMNC by the Ultraspec® RNA isolation procedure (Khymenets et al., 2005). RNA concentration and purity were measured by a NanoDrop spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, DE). Total RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples at 0 h and 6 h were pooled and concentrated using the RNeasy Mini Elute Cleanup system (Qiagen, Barcelona, Spain); checked for total RNA quantity and quality; and concentration adjusted to meet ABI Microarray criteria. Samples were stored in aliquots at -80 ºC prior to use. All reagents, plastic ware, and supplies used were sterile, nuclease free, and of molecular biology grade.

Microarray analysis and q-PCR verification

Gene expression profiles were generated using the Human Genome Survey Microarray v2.0 (Applied Biosystems, Foster City, CA). Each microarray contains 32,878 60-mer oligonucleotide probes representing 29,098 individual human genes. Samples were processed in triplicate. Microarray hybridization, processing, chemiluminescence detection, imaging, auto gridding, and image analysis were performed according to Applied Biosystems protocols and using the 1700 Chemiluminescent Microarray Analyzer Software v.1.0.3. Quantile normalization was applied for inter-array normalization (Bolstad et al., 2003). Genes were excluded when their expression levels were below the detection threshold (signal to noise values < 3 and/or flags > 5,000). The resulting 15,308 genes from the filtering (from the initial 32,878 probe set) were then subjected to further gene selection and typified using PANTHER™ Protein Classification System analysis (Thomas et al., 2003). The microarray dataset is available under GSE 19590 Accession Number of GEO Database.

The identification of genes that were regulated by olive oil ingestion was done by comparing gene expressions in PBMNC at pre-intervention (0 h) with those at post-intervention (6 h). The cut off to consider a gene differentially expressed, on the basis of the pre- and post-intervention variability, was set at a signal $\log_2$ ratio higher than 0.5 (up-regulation, fold-change > 1.41), or lower than -0.5 (down-regulation, fold change < 1.41).

Real-time RT-PCR

The reverse transcription reaction was performed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA). The expression of nine genes (five up-regulated

and four down-regulated), with an expression range from low to high, was verified by quantitative TaqMan Real-Time PCR (TaqMan® Low Density Array by Design and ABI Prism 7900HT Sequence Detection System, Perkin-Elmer, Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was used as a housekeeping gene. Data obtained were analysed by the SDS 2.1 software.

Results

The general characteristics of the healthy volunteers at baseline are shown in Table 1. Total RNA obtained was of high quality and purity (A_{260}/A_{280} and $A_{260}/A_{230} \geq 1.8$; and RNA integrity number in the range from 8.5 to 9.5). The mean coefficient of variation of the log-signal probe values was lower than 0.1 for the triplicates.

Differential gene expression in PBMNC

From the 15,308 high-quality probes selected, 259 known genes were up-regulated and 246 down-regulated in human PBMNC after 50 ml of olive oil ingestion. The differentially expressed genes belonged to a wide range of gene ontology biological processes including metabolism, signal transduction and signalling, cancer, metabolic disorders, and cellular processes (Fig. 1). The highest up-regulation, from 1.0 to 1.29 units of $\log_2$ ratio, corresponding to a fold-change from 2 to 2.44, was observed in genes related to: 1) cancer, such as the A-kinase anchoring protein 13 (*AKAP13*) and *IKAROS* (*ZNFSF1A*); and 2) cellular processes, such as *CDC14* and ubiquitin protease *USP48*. The highest down-regulation, from -1.79 to -1.02, corresponding to a fold-change from -3.48 to -2.03, respectively, was observed in genes related to: 1) DNA damage, such as the DNA-damage-inducible transcript 4 (*DDIT4*) or the DNA-repair protein *XRCC4*; and 2) carcinogenesis, such as the cyclin-dependent kinase inhibitor 2B (*CDKN2B*), or the v-*akt* murine thymoma viral oncogene (*AKT3*). Due to the fact that phenotypic changes in markers related to atherosclerosis and DNA oxidative damage occur after olive consumption (Poulsen et al., 2005; Covas et al., 2007), genes differentially expressed related to these processes were identified using public databases (Tables 2 and 3). The verification of the microarray gene expression in a set of nine genes by quantitative real-time qRT-PCR showed that, in general, only the features of the gene expression changes for the up-regulated genes were similar (Fig. 2). NIH-DAVID software (version 2.1b) (Dennis et al., 2003) was used to search for Gene Ontology terms (Ashburner et al., 2000) and KEGG pathways (Kanehisa et al., 2000).

Table 1. General characteristics of volunteers at baseline

	Volunteers (N = 6)
Age (years)	24.8 (2.3)
BMI (kg/m^2)	24.5 (3.5)
Glucose (mmol/l)	4.93 (0.3)
Total cholesterol (mmol/l)	4.24 (0.45)
LDL cholesterol (mmol/l)	2.39 (0.64)
HDL cholesterol (mmol/l)	1.48 (0.42)
Triglycerides (mmol/l)	0.66 (0.35–1.20)

Values are presented as mean (SD) with the exception of triglycerides, which are presented as median (25^{th}–75^{th} percentile).

Discussion

The aim of this work was to assess the gene expression changes in PBMNC of healthy volunteers at the end of postprandial time (Axelsen et al., 1999), after 6 h of fat ingestion, virgin olive oil (50 ml). To our knowledge this is the first exploratory report assessing the human *in vivo* gene expression changes after food ingestion. From microarray data, the highest up-regulation was observed in genes related to metabolism, cellular processes, and cancer. The highest down-regulation was observed in genes related to environmental information processing.

Epidemiological studies suggest a protective effect of olive oil consumption on cardiovascular disease and certain types of cancer (Trichopoulou et al., 2000; Covas et al., 2007). After consumption of olive oil a decrease in the urinary concentration of 8-oxo-deoxygyuanosine, considered being a systemic marker of DNA oxidation, has been reported (Fitó et al., 2007). We observed an increase in DNA-repair genes: DNA cross-link repair 1C (*DCLRE1C*) (also known as *ARTEMIS*) and DNA polymerase κ (*POLK*), which were up-regulated at 6 h post-intervention. A recent study provides evidence for a possible protective role for POLK in mammalian nucleotide excision repair (Ogi et al., 2006).

Consumption of olive oil has been reported to increase plasma high-density lipoprotein (HDL) cholesterol levels (Covas et al., 2007; Fitó et al., 2007). In agreement with this, an increase in the *ABCA7* [ATP-binding cassette, sub-family A (ABC1), member 7] gene expression was observed after olive oil ingestion. ABCA7, together with ABCA1, mediates the apolipoprotein-dependent formation of the HDL (Takahashi et al., 2005). Besides increasing the HDL cholesterol, the ingestion of a virgin olive oil-based breakfast has shown to decrease the postprandial glucose and insulin concentrations, and to increase glucagon-like peptide-1 concentrations as compared with a carbohydrate-rich diet (Paniagua et al. 2007). The chain length of the fatty acid is considered to be a key factor for glucagon-like peptide-1 secretion, long chain monounsaturated fatty acids being the most effective ones stimulating Langerghans cells *in vitro* (Rocca et al., 2001). In agreement with this, we observed an up-regulation of some insulin-related genes such as a disintegrin and metalloproteinase domain 17 (*ADAM17*) (Togashi, 2002) and *OGT* (Whelan, 2008) after olive oil ingestion.

Olive oil consumption has also been reported to reduce inflammatory markers (Fitó et al., 2007). We observed an up-regulation of the interleukin 10 (*IL-10*) gene at 6 h after olive oil ingestion. IL-10 is an anti-in-

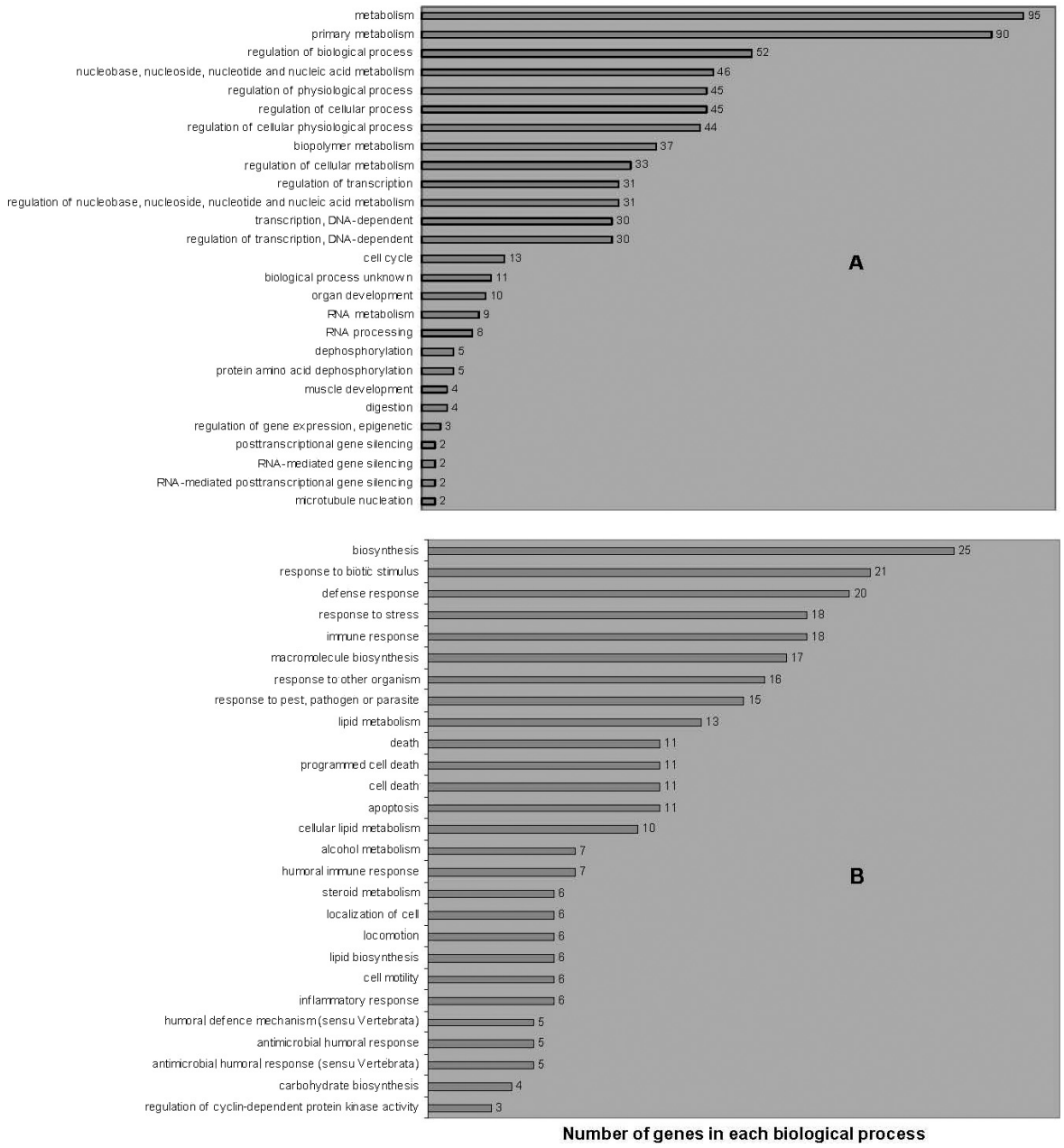

Fig. 1. Functional annotation of differentially expressed genes after olive oil ingestion (50 ml). A: up-regulated genes; B: down-regulated genes.

flammatory cytokine which inhibits production of interleukin 6 (Tedqui et al., 2006), considered as the most important inflammatory mediator. A pro-inflammatory cytokine down-regulated after olive oil ingestion was interferon γ (IFN-γ). IFN-γ is a strong pro-inflammatory cytokine that orchestrates several cellular programmes through transcriptional regulation of immunologically relevant genes, and recent studies suggest that reducing IFN-γ synthesis may lead to new therapies for graft arteriosclerosis (Tellides et al., 2007). USP48, a human ubiquitin-specific protease, is a deubiquitinating enzyme implicated in the regulation of NF-κB activation by members of the tumour necrosis factor receptor superfamily (Tzimas et al., 2006). Also AKAP13 (anchoring protein 13) plays a role in NF-κB activation, mediated by Toll-like receptors 2 (Shibolet et al., 2007).

The comparison of data obtained in pooled samples from microarray and qPCR experiments showed that there was some inconsistency between the results obtained using the different methods. The lack of concordance between methods was observed only in down-regulated genes measured by microarrays.

In this study, an approach was performed to assess whether changes in gene expression in human PBMNC could be detected after olive oil ingestion at postprandial state. Although the subjects' baseline data had served as a within-subject control, a limitation of the study is the lack of a control group for the intervention itself, which does not permit us to specify the contribu-

Table 2. Genes related with atherosclerosis and DNA damage processes up-regulated after olive oil ingestion (50 ml)

Gene ID	Gene Symbol	Gene Name	Change in Log_2 ratio	Fold Change
Oxidative stress				
8473	*OGT*	O-UDP-N-acetylglucosamine (polypeptide-N-acetylglucosaminyl transferase)	0.75	1.68
10539	*TXNL2*	thioredoxin-like 2	0.70	1.62
137872	*ADHFE1*	alcohol dehydrogenase, iron-containing, 1	0.70	1.62
4891	*SLC11A2*	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.65	1.57
1850	*DUSP8*	dual-specificity phosphatase 8	0.63	1.55
6095	*RORA*	RAR-related orphan receptor A	0.57	1.48
Inflammation				
3586	*IL-10*	interleukin 10	0.73	1.66
6654	*SOS1*	son of sevenless homologue 1 (Drosophila)	0.72	1.65
1286	*COL4A4*	collagen, type IV, α 4	0.68	1.60
6775	*STAT4*	signal transducer and activator of transcription 4	0.65	1.57
DNA repair				
64421	*DCLRE1C*	DNA cross-link repair 1C (PSO2 homologue, *S. cerevisiae*)	0.56	1.47
51426	*POLK*	polymerase (DNA directed) κ	0.53	1.44
Apoptosis				
11016	*ATF7*	activating transcription factor 7	0.97	1.96
389840	*MAP3K15*	FLJ16518	0.70	1.62
22861	*NALP1*	NACHT, leucine-rich repeat and PYD-containing 1	0.59	1.51
54739	*XAF1*	XIAP-associated factor 1	0.55	1.46
Lipid metabolism				
51422	*PRKAG2*	protein kinase, AMP-activated, γ 2 non-catalytic subunit	1.06	2.09
114881	*OSBPL7*	oxysterol binding protein-like 7	0.82	1.76
84129	*ACAD11*	putative acyl-CoA dehydrogenase	0.66	1.58
22848	*AAK1*	AP2-associated kinase 1	0.63	1.55
8398	*PLA2G6*	phospholipase A2, group VI (cytosolic, calcium-independent)	0.60	1.52
22876	*INPP5F*	inositol polyphosphate-5-phosphatase F	0.58	1.49
10347	*ABCA7*	ATP-binding cassette, sub-family A (ABC1), member 7	0.56	1.47
9517	*SPTLC2*	serine palmitoyl transferase, long-chain base subunit 2	0.54	1.46
5243	*ABCB1*	ATP-binding cassette, sub-family B (MDR/TAP), member 1	0.54	1.46
Tissue remodelling				
11214	*AKAP13*	A kinase (PRKA) anchor protein 13	1.20	2.30
84196	*USP48*	ubiquitin-specific protease 48	1.11	2.16
9924	*USP52*	ubiquitin-specific peptidase 52	0.57	1.48
6868	*ADAM17*	a disintegrin and metalloproteinase domain 17 (TNF, α, converting enzyme)	0.51	1.42
9098	*USP6*	ubiquitin-specific protease 6 (Tre-2 oncogene)	0.51	1.42

tion of the fasting state nor the oil's special characteristics on the observed changes. Due to this, the effects observed on gene expression could be secondary, not only to the virgin olive oil ingestion, but also to a time-course effect on a circadian regulated genes (Khymenets, 2008) and to physiological changes following any fat meal intake. Also, we could not distinguish between the effects promoted by the minor components of olive oil and those promoted by the fat content of the olive oil. However, an advantage of the study was the *in vivo* evaluation of the gene expression in PBMNC after a real-life dose of virgin olive oil, as is used to be consumed in some Mediterranean areas (Helsing, 1995). PBMNC were selected to explore changes in gene expression because they are: 1) critically involved in the atherosclerotic plaque formation; 2) easily available from volunteers considering the feasibility of collection plus deontological reasons; and 3) their collection can be directly done from BD Vacutainer® CPT™ tubes, thus ensuring rapid PBMNC isolation and avoiding *ex vivo* gene activation.

In summary, changes in several genes related with oxidative stress-associated diseases, such as cancer and atherosclerosis, occur in human PBMNC of healthy volunteers at 6 h postprandial after 50 ml olive oil ingestion. Changes were observed at a real-life dose of olive oil, as is daily consumed in some Mediterranean areas. Our results point out that the protective effect observed in primary and secondary markers for CVD or cancer, related to virgin olive oil consumption at postprandial state, could be mediated through gene expression changes.

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Table 3. Genes related to atherosclerosis and DNA damage processes down-regulated after olive oil ingestion

Gene ID	Gene Symbol	Gene Name	Change in Log_2 ratio	Fold Change
Oxidative stress				
1728	*NQO1*	NAD(P)H dehydrogenase, quinone 1	-0.94	-1.92
4698	*NDUFA5*	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 5, 13 kDa	-0.78	-1.72
51167	*NCB5OR*	cytochrome b5 reductase 4	-0.62	-1.54
Inflammation				
969	*CD69*	CD69 molecule	-0.94	-1.92
3576	*IL-8*	interleukin 8	-0.85	-1.80
6361	*CCL17*	chemokine (C-C motif) ligand 17	-0.73	-1.66
7852	*CXCR4*	chemokine (C-X-C motif) receptor 4	-0.62	-1.54
3458	*IFN-γ*	interferon, γ	-0.61	-1.53
1178	*CLC*	Charcot-Leyden crystal protein	-0.54	-1.45
51176	*LEF1*	lymphoid enhancer-binding factor 1	-0.53	-1.44
2357	*FPR1*	formyl peptide receptor 1	-0.51	-1.42
DNA repair				
54541	*DDIT4*	DNA-damage-inducible transcript 4	-1.55	-2.93
7518	*XRCC4*	X-ray repair complementing defective repair in Chinese hamster cells 4	-1.17	-2.25
Apoptosis				
4000	*LMNA*	lamin A/C	-0.82	-1.76
11235	*PDCD10*	programmed cell death 10	-0.78	-1.72
950	*SCARB2*	scavenger receptor class B, member 2	-0.77	-1.71
54205	*CYCS*	cytochrome c, somatic	-0.74	-1.67
27242	*TNFRSF21*	tumour necrosis factor receptor superfamily, member 21	-0.69	-1.61
23421	*ITGB3BP*	integrin β 3 binding protein (β3-endonexin)	-0.54	-1.45
122953	*JDP2*	jun dimerization protein 2	-0.50	-1.41
3553	*IL1B*	interleukin 1, β	-0.50	-1.41
Lipid metabolism				
27284	*SULT1B1*	sulphotransferase family, cytosolic, 1B, member 1	-0.95	-1.93
7941	*PLA2G7*	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	-0.71	-1.64
6309	*SC5DL*	sterol-C5-desaturase (ERG3 δ-5-desaturase homologue, *S. cerevisiae*)-like	-0.64	-1.56
3422	*IDI1*	isopentenyl-diphosphate δ isomerase 1	-0.59	-1.51
6342	*SCP2*	sterol carrier protein 2	-0.54	-1.45
56994	*CHPT1*	choline phosphotransferase 1	-0.52	-1.43
8310	*ACOX3*	acyl-coenzyme A oxidase 3, pristanoyl	-0.52	-1.43
Coagulation				
7056	*THBD*	thrombomodulin	-0.86	-1.82

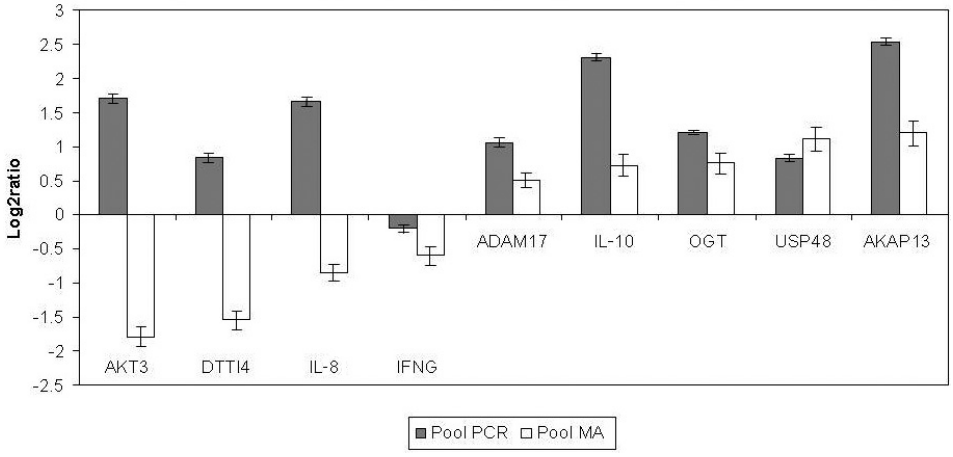

Fig. 2. Assessment of gene expression levels by real-time PCR. Log_2 ratio expresses the gene expression changes in human mononuclear cells according to RT-PCR (black bars) and microarray (white bars). *AKT3*, v-*akt* murine thymoma viral oncogene; *DDIT4*, DNA-damage-inducible transcript 4; *IFNG*, interferon γ; *IL-8*, interleukin 8; *ADAM17*, a disintegrin and metalloproteinase domain 17; *USP48*, ubiquitin specific protease 48; *OGT*, O-linked N-acetylglucosamine transferase; *IL-10*, interleukin 10; *AKAP13*, A-kinase anchoring protein 13.

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Author Disclosure Statement

* These authors contributed equally to this work, no competing financial interests exist.

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