Postprandial transcriptome associated with virgin olive oil intake in rat liver

Roberto Martinez-Beamonte[1, 3], Maria A. Navarro[1], Natalia Guillen[1, 3], Sergio Acin[1], Carmen Arnal[2, 3], Mario A Guzman[1] and Jesus Osada[1, 3]

[1]Departamento de Bioquimica y Biologia Molecular y Celular, Facultad de Veterinaria, Instituto Aragones de Ciencias de la Salud (Universidad de Zaragoza- Salud del Gobierno de Aragon), Spain. [2]Departamento de Patologia Animal, Facultad de Veterinaria, Universidad de Zaragoza, Spain. [3]CIBER de Fisiopatologia de la Obesidad y Nutricion, Instituto de Salud Carlos III, Spain

TABLE OF CONTENTS

1. ABSTRACT

Liver has been proposed as a gatekeeper that regulates postprandial lipemia and a potential target for regulation by acute intake of virgin olive oil. To characterize the hepatic gene expression response to a fat gavage, male rats were fed a bolus of 5 ml of extra-virgin olive oil and the hepatic mRNA expression analyzed 4 hours later using DNA microarrays. To provide an initial screening of candidate genes, only twenty one with remarkably modified expression between both conditions (signal $\log_2$ ratio > 2.5 or < -2.5) were considered and confirmed by quantitative real time PCR. Those that presented biological significance were also analyzed 8 hours after the experimental approach. Hepatic *A2m Slc13a5* and *Nrep* mRNA expressions were found significantly changed in both studied conditions and showed the highest significant associations with postprandial plasma triglycerides and lack of association with basal triglyceridemia. These results highlight new gene regulation in liver by postprandial triglyceridemia and will help to understand the complex human pathology providing the involvement of hepatic proteins and new strategies to cope with postprandial metabolism.

2. INTRODUCTION

The contribution of postprandial lipemia to atherosclerosis has been well established (1) and a large series of studies have found significant associations between impaired elimination of postprandial lipoproteins and cardiovascular diseases (2-4). Postprandial metabolism can be influenced by a lot of factors whose complete list is far behind to be known. Aspects such as genetic and hormonal influences, feeding schedule, composition of food with emphasis in cholesterol and dietary fat content**,** and saturation have been found to modulate postprandial responses (5-9). Cianflone *et al* have proposed intestine, adipose tissue, muscle and liver as the gatekeepers that regulate postprandial lipemia and potential targets for regulation (7). In this regard, Perona *et al* have observed that incubation of hepatocytes with different postprandial lipoproteins induces changes in the mRNA expression of receptors (10), confirming an important role of liver in regulation of the postprandial process susceptible to be tested using high-throughput analyses.

Rat is an animal model which most of its plasma cholesterol is transported into HDL**,** due to the

absence of cholesterol ester transfer protein (11)-an activity found to parallel postprandial triglyceride response- (12). Despite this difference with the human model, this animal represents an interesting approach to study changes in the postprandial state in the absence of the mentioned protein. Development of functional approaches in this animal is particularly relevant due to its recent incorporation to the transgenic and knock-out technology (13, 14), which will bring a renew interest and a demanding use. In addition, rat lipoprotein metabolism has been found to be sensitive to chronic dietary fat amount and composition (15). Also, chronic administration of a diet enriched in 10% (w/w) olive oil has been observed to reduce plasma TG and modify hepatic gene expression in apoE- deficient mice (16). Previous experiments in mice showed that a bolus of 16 mL olive oil/ kg was sufficient to induce their plasma postprandial response (17), suggesting that this dose may also be sufficient in rats. To test the hypotheses that this dose may change plasma triglycerides and induce important changes in hepatic gene expression, rats were administered a virgin olive oil gavage and sacrificed four hours later. Then, gene expression was determined by micro array analysis to systematically examine the expression of a large number of hepatic genes. The 21-gene expressions were confirmed by quantitative real time PCR. The relevance of observed changes was further tested in animals eight hours after the fat intake, the confirmation of these results shows the association of them with hepatic- specific mechanisms involved in the postprandial regulation induced by an acute fat intake.

3. MATERIAL AND METHODS

3.1. Rats

Male Wistar rats, weighing 250- 300 g (purchased from Charles River, Barcelona, Spain), were used for experiments. Rats, housed in sterile filter-top cages (3-4 per cage), were acclimatized in a room maintained at 20ºC with a 12-h light-dark cycle for 10 days, allowed *ad libitum* access to water and standard chow diet (Pascual S.A., Barcelona, Spain), and fasted for 18 h before experiments. Animals were handled and killed, always observing criteria from the European Union for care and use of animal laboratory in research, and the protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza.

3.2. Study design

Prior to the experiment, a baseline fasting blood sample was obtained cutting a piece of tail that had been anaesthetized by subcutaneous injection with 0.5 ml of 5% lidocaine HCl (Braun Medical SA, Barcelona, Spain). Tail blood samples were collected in heparin-coated capillary tubes and centrifuged at 2000 g for 5 min. Obtained plasma was kept at 4ºC for immediate triglyceride analysis. Rats were randomly allocated into 3 groups of 5 rats each. The control group did not receive any fat meal. The other two groups were fed 5 ml of extra virgin olive oil (Aceites Toledo, Spain) as a bolus and sacrificed 4 and 8 hours after the feeding, respectively. This amount represents the use of a dose of 16 mL olive oil/ kg, sufficient to induce a plasma postprandial response in mice (17). Olive oil was directly administered to stomach using a 1.1 mm diameter and 50 mm long flexible Abbocath connected to a sterile polypropylene syringe and delivered in 4 seconds. At the moment of sacrifice, rats were anesthetized with 1 ml of 8% Avertine (Aldrich Chemical Co., Madrid, Spain) in 0.1 M phosphate, pH 7.2, and blood drawn from hearts. Blood was collected in tubes containing 1 g/l sodium EDTA. Liver was removed and quickly frozen in liquid N_2 until total RNA was extracted.

3.3. Plasma triglycerides

Total plasma triglyceride concentrations were enzymatically quantified in a microtiter assay using commercial kits from Sigma Chemical Co. (Madrid, Spain). Cardiolipid (Sigma) was used as quality control.

3.4. Histological analysis

A sample of liver from each rat was stored in neutral formaldehyde and embedded in paraffin wax. Sections (4 μm) were stained with hematoxylin and eosin and observed using a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section (18).

3.5. Hepatic lipid analysis

Tissues (10 mg) were homogenized in 1 ml of PBS. An aliquot was saved to determine protein concentration by the BioRad dye binding assay (BioRad, Madrid, Spain). One volume of homogenate was twice extracted with two volumes of chloroform: methanol (2:1). The separated organic phases of each animal were combined and evaporated under N_2 stream. Extracts were dissolved in 100 μL of isopropanol to estimate cholesterol and triglyceride concentrations using commercial kits as above described.

3.6. RNA isolation, Affymetrix oligonucleotide array hybridization, and data analysis

RNA from each liver was isolated using Trigent reagent (Sigma). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was greater than 1.75). The integrity of the 28 S and 18 S ribosomal RNAs was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining and the 28S/18S ratio was greater than 2. Twenty μg aliquots of total liver RNA from each rat of each group (n= 5 for the control and n= 5 for 4h group) were pooled and purified using the RNeasy system (Quiagen, Barcelona, Spain). Eight micrograms of total liver RNA were then used for biotin labeling. Hybridization, washing, scanning and analysis with the Affymetrix GeneChip Rat Expression Array 230 version 2.0 (Affymetrix, Santa Clara, CA) were performed according to the standard Affymetrix protocols. Fluorometric data were generated by Affymetrix software, and the fluorimetric signal adjusted so that all the probe sets provided intensities within a manageable range. The data obtained in the microarray hybridizations were processed with Affymetrix® GeneChip® Command Console® 1.1 and Expression Console™ 1.1 (Affymetrix)

Table 1. Nucleotide sequence of primers used for RT-PCR

Gene	Forward 5'-3'	Reverse 5'-3'
BE113272	ATTGAAAAGCCACCGTTCCAC	TCTGTGCTCCTGGATGAATCG
Gadd45b	TGATGAATGTGGACCCCGA	CAGCAGAACGATTGGATCAGG
Cxcl1	CCCAAACCGAAGTCATAGCCA	CGCCATCGGTGCAATCTATCT
A2m	GGAAGCCGTTCTGAATCCAAC	TGTTCGGCTCACATGCACA
Slc13a5	TGCTGAATTGGAAAGTCACCC	GAGCCCTGAAGTCTCACATCCT
Sds	TCACCATTGAGCGGCTGAA	GGTTGTTCTTTTCCAGAGCCTT
Zfp354a	GGCAGGATTCCTGTTTACCAAA	TGCACTTCAATCCGAGAGAGAA
C8orf4a	CGATGAAAGCAAAACCAAGCC	TTTCTTGCGAGCAGCTGTGTC
Rell1	AGAAGGAGCGCAGAAGCTTGAT	AAGGTGCTGCCATCTACTCCGT
Ces2	TGACCATGCTGATGAGGTTCCT	CCGTGTCTTGCAAAATTGGC
AI236188	ATACCTACGTTGGTCAAACCCG	AAGGTGCCAGCTTACCCCATA
Ccrn4lb	AGCCCTGGTCACCATGTCTAGA	TGCAATGGCCACCTGATTG
Fam134b	TCCAAGCCAGATGAAAGACCC	AGACCAGGAGGCAAAACTTGC
Coq10b	CCACATTTGGTAAAGGCCTCTT	CATGTTCTTGGATAGCCAGGAA
Fabp4	AAATCACCCCAGATGACAGGA	CTTTATGGTGGTCGACTTTCCA
AI144796	CAGATGCAGTGTGATTAGCCCA	CACTGATCATGCAATGGAATGG
Nrep	CTGTTGGTCTCCCTGGACTGAA	AGGCACAGGAAGTCTTCCCTT
Inhba	ATAAGGCCAAAGAAGCTATCGG	TCCATTCCAGCAGATACTCCG
Slc34a2	AATCATCGTCAGCATGGTTGC	TCATTTCTGTCTCCTGCCTGC
Trim47	TGAGCCAGATGTTTGCCGA	GAGAAAGCTGACTGAATCCGCC
RGD1309362	AGGACCAGCATTCTATTGCCAG	TCTGTGTGCCTAGTCCTTGTGG
Ppib	TCGGAGCGCAATATGAAGGT	CTTCTTCTTATCGTTGGCCACG

software. Annotation was confirmed by checking the MGI Gene Ontology Browser at the Jackson's website http://www.informatics.jax.org/searches/GO.cgi?id=GO:0042416 and the Rat Genome Browser at http://rgd.mcw.edu/tools/genes/genes_view.cgi?id=631411. An initial approach of selecting genes that were up- or down-regulated in the postprandial state was carried out by first considering only those whose signal $\log_2$ ratio between control and 4 h after feeding was higher than 2.5 (up-regulated genes) or lower than –2.5 (down-regulated genes). Transcripts whose signal intensities were lower than the noise of the matrix ± 3 standard deviations were not taken into account either. The complete datasets were deposited in the GEO database (accession number GSE17859).

3.7. Quantification of mRNA

The difference in mRNA expression observed with the microarrays was confirmed by quantitative real-time RT-PCR (qRT-PCR) analysis of individual samples. Equal amounts of DNA-free RNA from each sample of each animal were used in qRT-PCR analyses. First-strand cDNA synthesis and the PCR reactions were performed using the SuperScript II Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Madrid, Spain), according to the manufacturer's instructions and as previously described (19). Primers were designed by Primer Express® (Applied Biosystems, Foster City, CA) and checked by BLAST analysis (NCBI) to verify specificity and selective amplification of the target gene as well as to get amplification of the cDNA and not of genomic DNA. The sequences are shown in Table 1. Real time PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) following the standard procedure. The specificity of the PCR reaction was confirmed by observing a single dissociation curve. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Cyclophilin B (*Ppib*) mRNA levels were used as the invariant control.

3.8. Statistical analysis

The results are expressed as means ± SD. Comparisons were made using one-way ANOVA and the Tukey-Kramer multiple comparison test (*post hoc*) when the distribution of the variables was normal. When the variables did not show such a distribution (according to the Shapiro-Wilk's test), or failed to show homology of variance, comparisons were made using the Mann-Whitney U test. Correlations between variables were sought using the Pearson's or Spearman's correlation tests. All calculations were performed using SPSS version 15.0 software (SPSS Inc, Chicago, IL). Significance was set at $P \leq 0.05$.

4. RESULTS

4.1. Plasma and hepatic triglycerides

Baseline and postprandial plasma TG concentrations of the three experimental groups are presented in Figure 1. As shown in Panel A, the basal triglyceridemia of rats showed no significant difference among the three established groups. Postprandial plasma triglycerides (Panel B) significantly increased either 4 or 8 hours after feeding compared to the control group, and the values reached at the latter time point were also significantly higher than those after 4 hours. '

Analysis of hepatic TG content, shown in panel C, indicated that this parameter increased significantly four hours after the fat meal and its levels remained significantly elevated at the eight-hour-time point. No significant change was observed between both time periods. A similar trend was observed for hepatic cholesterol (Fig 1D). Therefore, this experimental design constitutes an interesting approach to study the presence of triglyceridemia and the transitory postprandial steatotic liver as well as the potential involved mechanisms.

4.2. Gene expression in livers of rats 4 hours after a fat meal

Prior to the RNA isolation, a liver histological analysis was undertaken to discard any pathological feature

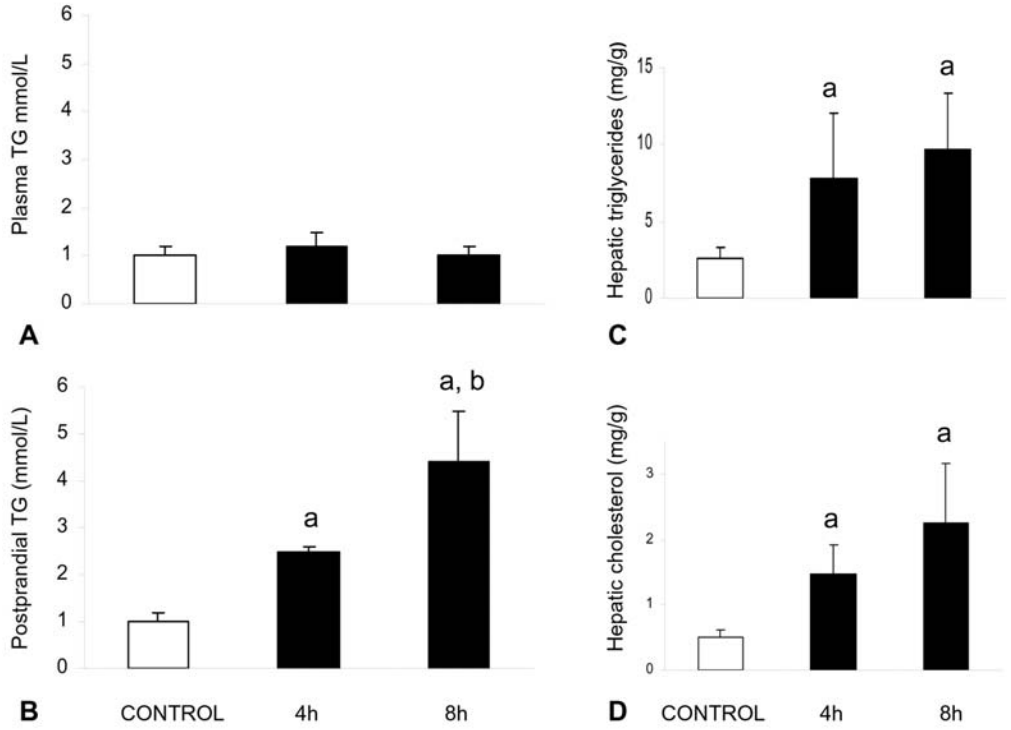

Figure 1. Plasma and hepatic lipid concentrations. A) Plasma baseline triglycerides of all groups and B), postprandial triglyceridemia at different time points after receiving 5 ml of olive oil as a bolus. C) and D), hepatic triglyceride and cholesterol contents, respectively. Data are means ± SD for each group. Statistical analysis to evaluate dietary response was done using one-way ANOVA and the Mann Whitney's U as post hoc test. [a], $P< 0.05$ vs control and [b], $P< 0.05$ vs 4h.

in any of the rats. Once verified the previous criterion and to determine the changes in hepatic gene expression induced by the administration of a fat gavage, the expression of 31,100 transcripts represented on the Affymetrix GeneChip Rat Expression Array 230 version 2.0 was quantified in pooled liver samples of 5 fast rats as controls and 5 rats sacrificed 4 hours after having been fed them with a bolus of 5 ml extra-virgin olive oil. The livers of control group expressed 15,667 transcripts (identified as "present" by Affymetrix software), while those of the fed animals expressed 15,061. Using the Mann–Whitney ranking feature of the Affymetrix software to determine significant differences in gene expression ($P<0.01$), the increased expression of 1369 sequences plus the reduced expression of 1675 transcripts was identified in samples from the fed animals compared to those not fed, when no multiple test correction was applied. Based in previous works (20), a cutoff point of variation taken as signal $\log_2$ ratio higher and lower than 1.5 was introduced, showing this as the biological mRNA variation, we identified the increased expression of 170 sequences plus the reduced expression of 94 transcripts in samples from the fed animals compared to those not fed. Another source of false positives previously identified was the insensitivity of the scanner (21). For this reason, transcripts whose signal intensities were lower than the noise of the matrix ± 3 standard deviations were removed as well as equally redundant expressions. When both criteria were taken into consideration, the number of genes with increased and repressed expression was reduced to 114 and 70, respectively.

Genes, with a signal $\log_2$ ratio higher than 2.5 (for those genes up-regulated) or lower than –2.5 (for those down-regulated), were taken into account to select the most relevant differentially regulated ones. Table 2 lists the genes whose mRNAs reflected these expressions. Sixteen genes fulfilled the criterion of showing increased expression as a response to the olive oil bolus. Among these genes, 3 coded for cellular membrane proteins (*Rell1, Fam134b* and *Coq10b*), 2 were involved in immunity (*Cxcl1 and A2m*), 2 belonged to lipid metabolism (*Ces2* and *Fabp4*), 6 were proteins with miscellaneous functions (e.g., 1 was involved in signal transduction [*Gadd45b*], another was an enzyme involved in amino acid metabolism [*Sds*], 1 was a metabolite transport protein [*Slc13a5*], 1 was an apoptotic protein [*C8orf4a*], 1 coded for a protein involved in circadian rhythm [*Ccrn4lb*], and 1 was a transcription factor [*Zfp354a*]). Finally, 3 EST were also found (*BE113272, AI236188* and *AI144796*). Five genes met the criterion of showing a reduced expression as a response to the olive oil bolus (Tables 2). Of these, 2 were involved in signaling pathways (*Nrep* and *RGD1309362*), 1 coded for a hormone (*Inhba*), 1 was a metabolite transport protein (*Slc34a2*) and finally 1 coded for an ubiquitin ligase (*Trim47*).

Table 2. Hepatic genes differentially regulated 4 hours after the administration of a bolus of 5 ml of olive oil at the level of signal log_2 ratio >2.5 or <-2.5 in rats. Data represent intensity of signal for each condition with the Affymetrix chip

Biological process	Gene bank	Probe Set ID	Name	Gene symbol	Control	Postprandial	Signal log_2 ratio
Up-regulated							
EST	BE113272	1374908_at	---	---	9.6	291.2	**4.6**
Activation of MAPKK activity	BI287978	1372016_at	Growth arrest and DNA-damage-inducible, beta	*Gadd45b*	39.8	793.6	**4**
Immune response	NM_030845.1	1387316_at	Chemokine (C-X-C motif) ligand 1	*Cxcl1*	160.3	1208.3	**3.4**
Acute phase response	NM_012488.1	1367794_at	Alpha-2-macroglobulin	*A2m*	310.1	2964.6	**3.3**
Citrate transporter	BG381311	1383600_at	Solute carrier family 13, member 5	*Slc13a5*	105.9	843	**3.2**
Amino acid metabolism	NM_053962.1	1369864_a_at	Serine dehydratase	*Sds*	730.6	6795.7	**3.1**
Transcription factor	NM_052798	1368877_at	Zinc finger protein 354A	*Zfp354a*	568.7	4177.4	**2.9**
Apoptosis	AI230625	1373403_at	Protein C8orf4	*C8orf4a*	313.1	1904.9	**2.8**
Membrane protein	BF403558	1383357_a_at	RELT-like 1	*Rell1*	94.7	596.4	**2.8**
Lipid metabolism	NM_133586	1368905_at	Carboxylesterase 2	*Ces2*	323.4	1763.9	**2.7**
EST	AI236188	1394207_at	---	---	119.7	741.8	**2.7**
Circadian rhythm	BI284261	1377869_at	CCR4 carbon catabolite repression 4-like B	*Ccrn4lb*	54.2	431.1	**2.6**
Membrane protein	BE109520	1373011_at	Family with sequence similarity 134, member B	*Fam134b*	1057.7	6150.5	**2.5**
Mitochondrial protein	AI228596	1373866_at	Coenzyme Q10 homolog B	*Coq10b*	688	3850.4	**2.5**
Lipid-binding protein	NM_053365	1368271_a_at	Fatty acid binding protein 4	*Fabp4*	29.4	175.2	**2.5**
EST	AI144796	1372616_at	---	---	34.5	209.4	**2.5**
Down-regulated							
Transforming growth factor beta receptor signaling pathway	BE107450	1371412_a_at	Neuronal regeneration related protein	*Nrep*	1841.6	76.9	**-3.9**
Hormone	AA926109	1383486_at	Inhibin b	*Inhba*	861.4	87.2	**-3.5**
Phosphate transporter	NM_053380	1368168_at	Solute carrier family 34, member 2	*Slc34a2*	743.9	86.2	**-3.3**
Ubiquitin ligase	BE113215	1374228_at	Tripartite motif-containing 47	*Trim47*	122.4	21.8	**-3.2**
GTP binding	AA955213	1377950_at	Similar to interferon-inducible GTPase	RGD1309362	738.7	105.4	**-2.6**

Table 3. Hepatic genes differentially regulated 4 hours after the administration of a bolus of 5 ml of olive oil at the level of signal log_2 ratio >2.5 or < -2.5 in rats

Group\ gene	Control (n = 5)	Postprandial (n = 5)	SL_2R vs control
Up-regulated			
BE113272	1.1 ± 0.5	67.7 ± 51.1[a]	5.9
Gadd45b	1.1 ± 0.4	13.4 ± 8.7[a]	3.6
Cxcl1	1.2 ± 1.3	1.0 ± 0.9	-0.2
A2m	1.1 ± 0.5	12.2 ± 14.3[a]	3.4
Slc13a5	2.3 ± 1.7	26.9 ±17.2[a]	3.5
Sds	1.1 ± 0.7	21.4 ± 15.9[a]	4.3
Zfp354a	1.3 ± 1.1	12.5 ± 8.5[a]	3.2
C8orf4a	1.1 ± 0.5	6.6 ± 7.5	2.6
Rell1	1.1 ± 0.5	7.3 ± 6.0[a]	2.7
Ces2	1.2 ± 0.6	7.0 ± 5.7	2.5
AI236188	1.2 ± 0.7	11.7 ± 14.6[a]	3.3
Ccrn4lb	2.3 ± 2.0	7.6 ± 10.8	1.7
Fam134b	1.5 ± 1.8	11.3 ± 3.7[a]	2.9
Coq10b	1.3 ± 1.2	14.8 ± 12.4[a]	3.5
Fabp4	2.0 ± 2.4	7.7 ± 6.4	1.9
AI144796	1.2 ± 0.7	15.5 ± 17.1[a]	3.7
Down-regulated			
Nrep	1.2 ± 0.7	0.2 ± 0.2[a]	-2.5
Inhba	1.4 ± 1.2	0.9 ± 1.5	-0.6
Slc34a2	1.1 ± 1.0	1.1 ± 1.8	0
Trim47	1.2 ± 0.8	0.3 ± 0.2[a]	-2
RGD1309362	1.3 ± 0.8	0.1 ± 0.1[a]	-3.7

Values are means and their standard deviations. Data represent arbitrary units normalized to the Cyclophilin B (Ppib) expression for each condition with the qRT-PCR. Statistical analysis was done using non-parametric unpaired Mann-Whitney U-test to test pair-wise differences. a, $P< 0.05$ vs Control

To validate the results obtained with the microarray, the expressions of the 21 genes - *BE113272, Gadd45b, Cxcl1, A2m, Slc13a5, Sds, Zfp354a, C8orf4a, Rell1, Ces2, AI236188, Ccrn4lb, Fam134b, Coq10b, Fabp4, AI144796, Nrep, Inhba, Slc34a2, Trim47* and *RGD1309362*- that were up- or down-regulated (signal log_2 ratio > 2.5 or < -2.5) were individually studied by specific qRT- PCR assays. *Cyclophilin B* was used to normalize the results that are shown in Table 3. Among the sixteen up-regulated genes included in the validation analysis (Table 3), only eleven - *BE113272, Gadd45b, A2m, Slc13a5, Sds, Zfp354a, Rell1, AI236188, Fam134b, Coq10b and*

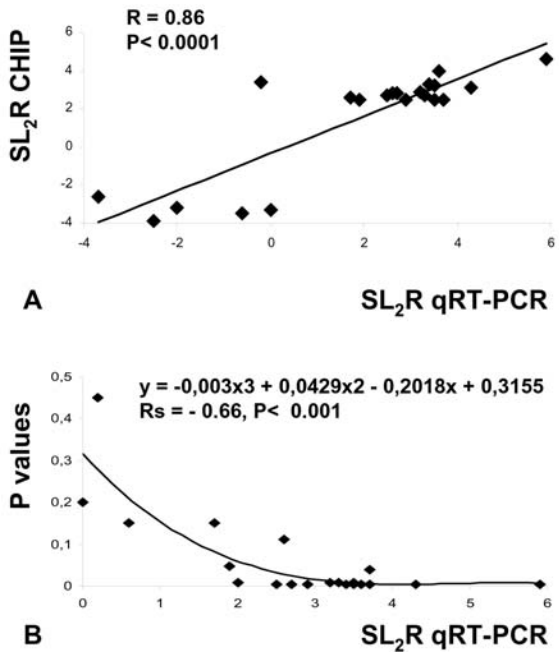

Figure 2. Quality and biological meaning of microarray data. A) Correlation analysis between microarray and qRT-PCR data. The expression of 21 genes - *BE113272, Gadd45b, Cxcl1, A2m, Slc13a5, Sds, Zfp354a, C8orf4a, Rell1, Ces2, AI236188, Ccrn4lb, Fam134b, Coq10b, Fabp4, AI144796, Nrep, Inhba, Slc34a2, Trim47* and *RGD1309362*- was individually studied by qRT- PCR and normalized to the invariant *Cyclophilin B* gene. The mean values obtained for signal $\log_2$ ratio from individual analyses (Table 3) were plotted against the microarray values which used pooled samples (Table 2). Good agreement between the procedures was seen (r= 0.86, P<0.0001). B) Correlation analysis between signal $\log_2$ ratio of qRT- PCR data and p values obtained in individual comparisons using nonparametric Mann-Whitney U-Test. Signal $\log_2$ ratios are taking in absolute values.

AI144796- appeared significantly increased in their expressions by the administration of the olive oil bolus. Three of the five down-regulated genes selected- *Nrep, Trim47* and *RGD1309362*- were significantly decreased in their expression after the fat gavage challenge.

Figure 2A shows the correlation between the mean values of signal $\log_2$ ratio for the above mentioned 21 genes with the microarray assay performed with pooled samples (Table 2), and the signal $\log_2$ ratio obtained by qRT- PCR of individual samples (Table 3). Good agreement between these procedures was obtained (r= 0.86, P<0.0001) and most of the samples**,** except three**,** were correctly classified, although the magnitude of the response differed between both methods. In an attempt to evaluate the accuracy of setting a cut-off point of a signal $\log_2$ ratio 2.5 in microarray analysis, the value of signal $\log_2$ ratio obtained in qRT-PCR analysis using individual samples was plotted against the probability value obtained for comparison of individual expressions for each gene (Figure 2B). A significant inverse relationship was observed where a signal $\log_2$ ratio of 2.3 was the minimum value required to obtain a P< 0.05. These results indicate that pooled samples can be successfully used to provide an initial screening of gene expression, with the attending economic and time savings although with the limitation of no information on biological variability. In addition, the high biological variation of mRNA indicates a certain threshold of change to evaluate significant changes by nutritional interventions.

4.3. Gene expression in livers of rats 8 hours after a fat meal.

To verify whether the hepatic expression changes observed after the postprandial were maintained for a longer period, a new group of rats receiving the fat meal were euthanized 8 hours after the food ingestion, and the fourteen previously selected genes were assayed. *Fabp4* was also included due to its role in fatty acid transport. All these data are shown in Table 4. Three groups of genes were observed: those whose hepatic expressions were not significantly increased or decreased at this time period versus control (*BE113272, Gadd45b, Sds, Zfp354a, Rell1, AI236188, Fam134b, Coq10b, AI144796, RGD1309362*), those whose change persisted (*Slc13a5, Nrep and Trim47*)**,** and those whose values were even more pronounced (*A2m* and *Fabp4*). As evidenced, the first category of genes was the most relevant and this suggests that the postprandial state induces a burst of gene expression at the mRNA level that is extinguished rapidly.

To verify whether the response of these gene expressions was linked to plasma triglycerides on each individual, an analysis of association between gene expressions (Tables 4) and the concentration of plasma triglycerides (Figure 1) was carried out. Figure 3 shows those associations found statistically significant. Interestingly, *A2m* and *Slc13a5* mRNA expressions showed the highest positive associations with postprandial triglyceridemia (r_s= 0.86, P<0.000 and r_s= 0.76, P<0.001, respectively)**,** and *Nrep* the lowest negative (r_s= -0.907, P<0.000). No significant association was observed with initial triglyceridemia (data not shown). These results indicate that these new selected genes are associated with postprandial triglyceridemia.

To analyze to which extent the response of these three gene expressions was associated with the hepatic triglyceride and cholesterol contents, an analysis of correlation between gene expressions (Table 4) and the content of these lipids (Figure 1) was carried out and shown in Figure 4. Both lipid parameters were significantly associated with gene expression, although the value of association was stronger for hepatic cholesterol than triglycerides. These results suggest that these compounds are associated with these gene expression changes.

5. DISCUSSION

Using a fat gavage intervention in rats and postprandial microarray expression profiling and its confirmation by qRT-PCR at two independent time points,

Table 4. Time follow-up of rat hepatic gene expression following the consumption of 5 ml of virgin olive olive as a bolus

Group\ gene	Control (n = 5)	4 h (n = 5)	8 h (n = 5)	SL_2R 4 h vs control	SL_2R 8 h vs control
Up- regulated					
BE113272	1.1 ± 0.5	67.7 ± 51.1[a]	11.3 ± 6.9[b]	5.9	3.3
Gadd45b	1.1 ± 0.4	13.4 ± 8.7[a]	4.4 ± 2.4[b]	3.6	2
A2m	1.1 ± 0.5	12.2 ± 14.3[a]	76.0 ± 65.5[a]	3.4	6.1
Slc13a5	2.3 ± 1.7	26.9 ±17.2[a]	27.7 ± 14.8[a]	3.5	3.6
Sds	1.1 ± 0.7	21.4 ± 15.9[a]	2.1 ± 1.2[b]	4.3	0.9
Zfp354a	1.3 ± 1.1	12.5 ± 8.5[a]	4.4 ± 4.4	3.2	1.7
Rell1	1.1 ± 0.5	7.3 ± 6.0[a]	2.1 ± 1.9	2.7	0.9
AI236188	1.2 ± 0.7	11.7 ± 14.6[a]	3.5 ± 1.8	3.3	1.5
Fam134b	1.5 ± 1.8	11.3 ± 3.7[a]	2.6 ± 1.0[b]	2.9	0.8
Coq10b	1.3 ± 1.2	14.8 ± 12.4[a]	2.4 ± 1.8[b]	3.5	0.9
Fabp4	2.0 ± 2.4	7.7 ± 6.4	15.6 ± 9.8[a]	1.9	2.9
AI144796	1.2 ± 0.7	15.5 ± 17.1[a]	4.5 ± 4.0	3.7	1.9
Down- regulated					
Nrep	1.2 ± 0.7	0.2 ± 0.2[a]	0.0 ± 0.1[a]	-2.5	-3.5
Trim47	1.2 ± 0.8	0.3 ± 0.2[a]	0.6 ± 0.2[a, b]	-2	-1
RGD1309362	1.3 ± 0.8	0.1 ± 0.1[a]	0.4 ± 0.7	-3.7	-1.7

Values are means ± standard deviations. Data represent arbitrary units normalized to the Cyclophilin B (*Ppib*) expression for each condition with the qRT-PCR. Statistical analysis was done using non-parametric one-way ANOVA according to Kruskal-Wallis test and unpaired Mann-Whitney U-test as post-hoc test. Different superscripts ([a] vs Control, [b] vs 4 h) are significantly different from each other at $P< 0.05$.

we have provided new panoply of genes whose hepatic expression is modified by an acute olive oil intake. Thereby, our work provides new resources whereby the liver may response to the handling of exogenous fat intake as well as an experimental approach to test the quality of oils *in vivo*.

In this work, the vast amount of information provided by microarrays has been handled by selecting only the genes with the highest expression changes and removing those with array signals within the signal noise of the matrix in agreement with our previous experience (20-22). With this criterion, only 16 genes were found to be remarkably upregulated and 5 notably downregulated. The modification of the expression of these genes was individually confirmed by qRT-PCR. Good agreement was observed between the Affymetrix chip and qRT-PCR data (Figure 2A). However, as shown in Figure 2A and Table 3, this approach might provide false positive variations regarding number of genes involved in a dietary response by the skewing effect of those highly responders. Furthermore in response to an acute intake of fat, results of Fig 2B indicate that the biological variation of mRNA when individually studied was high enough that only genes with a certain threshold of change (signal $\log_2$ ratio ± 2.3) would show biological significance. Despite these limitations, the present approach provides meaningful and manageable data and is appropriate to find new candidate genes in experimental conditions.

The validation analysis carried out by qRT-PCR in individual samples (Table 3) showed that eleven genes - *BE113272, Gadd45b, A2m, Slc13a5, Sds, Zfp354a, Rell1, AI236188, Fam134b, Coq10b and AI144796*- were found significantly increased and three - *Nrep, Trim47* and *RGD1309362*- were significantly decreased in their expression 4 hours after the olive oil challenge. In our experience (20-22), one single time point in RNA analysis may not be representative due to its high metabolic rate. For this reason, the variations of the previous fourteen genes and *Fabp4*, were further analyzed in an independent experiment where animals received the same amount of olive oil and were sacrificed eight hours after (Table 4). As indicated, 10 genes were not found increased at this time period. These results clearly indicate that RNA response following an acute olive oil intake is different at various time periods. However, five genes (*A2m*, *Fabp4, Slc13a5, Nrep and Trim47*) persisted in their significant changes at this time period as well. In an association analysis with plasma postprandial triglyceridemia (Fig 3), only four of these hepatic gene expressions were significantly associated (*A2m*, *Fabp4, Slc13a5 and Nrep*). When association analysis was carried out with hepatic cholesterol and triglycerides, only three of the previous genes (*A2m*, *Slc13a5 and Nrep*) showed significant associations (Fig 4). Using this biological procedure of selection, we have shown that expressions of these genes are highly associated with plasma triglyceridemia elicited after consuming olive oil gavage and at two time points in rats and with hepatic lipids. To our knowledge, this is the first report involving the hepatic changes of *A2m*, *Slc13a5 and Nrep* genes as a postprandial response.

Plasma postprandial levels of alpha-2 macroglobulin one hour after the meal intake in humans were not found significantly elevated (23), which would be in agreement with time dependence observed in our results (Table 4). An alpha-2 macroglobulin increase might modulate the levels of chylomicron remnants due to the fact that both compete for the low density receptor related protein (LRP) in their removal (24). Alpha-2 macroglobulin, as a major endoprotease inhibitor in mammalian blood, controls metalloproteinases and disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS-7 and ADAMTS-12) (25). The A2m-protein complex binds to the cell surface clearance receptor LRP and triggers rapid internalization of the complex by receptor- mediated endocytosis. Due to the fact that endoproteases are implicated in many diseases, including

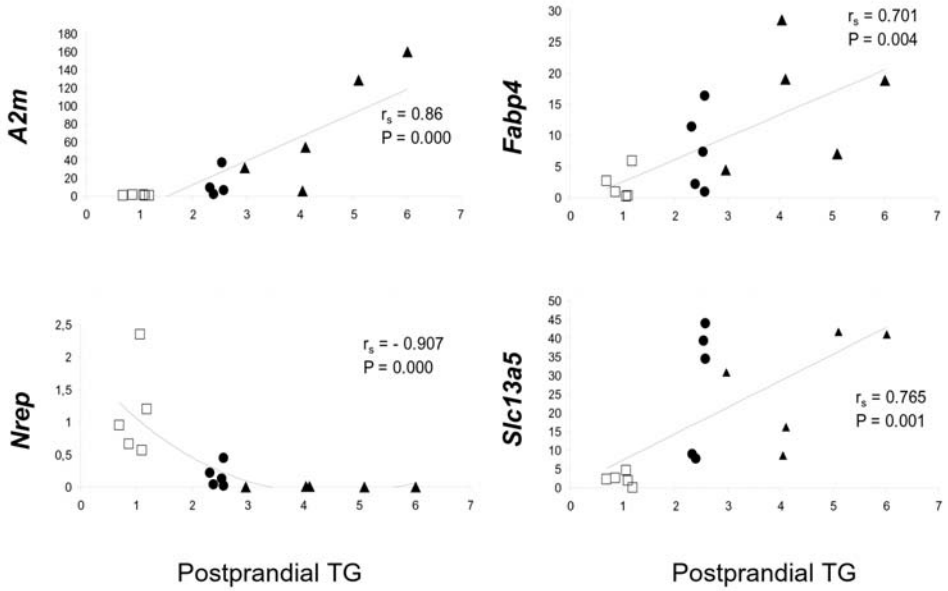

Figure 3. Relationships among plasma triglyceridemia and hepatic gene expressions in rats. Open squares correspond to control, black circles to 4 h- and black triangles to 8h- fed animals. Correlations were calculated according to Spearman's test.

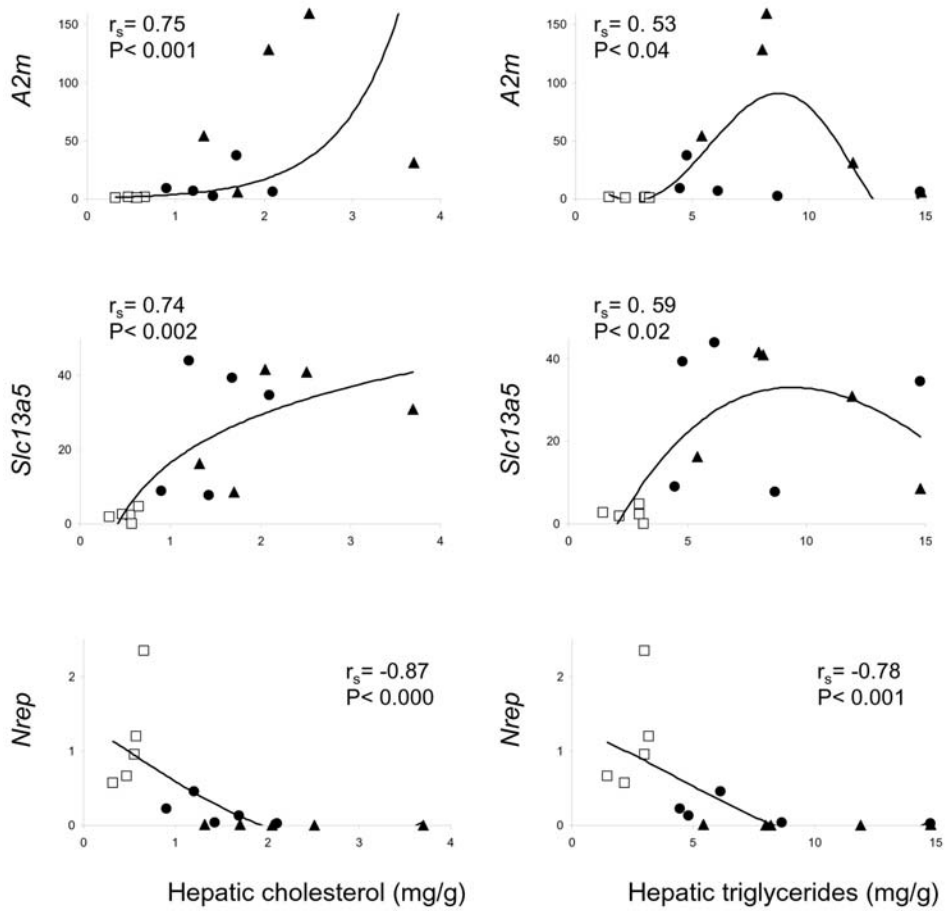

Figure 4. Relationships among hepatic lipids and gene expressions. Open squares correspond to control, black circles to 4 h- and black triangles to 8h- fed animals. Correlations were calculated according to Spearman's test.

those of the cardiovascular system, arthritis and cancer (26), the observed increase after the postprandial fat meal may provide a new insight into the pathophysiology of degradative diseases.

Slc13a5 or Na^+- coupled citrate transporter (NaCT) is a member of the vertebrate SLC13 gene family (27), mainly expressed in the liver and highly similar among mammalians (28). The expression of NaCT in human (HepG2 and Huh-7), rat liver primary hepatocytes and cell lines**,** showed robust activity for Na^+- coupled citrate uptake in the sinusoidal membrane of hepatocytes (29). In a postprandial experiment using stable isotopes of triolein, Metges *et al* (30) observed different metabolites from these fatty acids pointing out to beta-oxidation, citric acid cycle and the phosphoenolpyruvate carboxykinase reactions in peripheral tissues. Citrate is present in blood at 135 μM (28) and the stimulation of the hepatic expression of NaCT in this postprandial design raises interesting questions regarding the utilization of circulating citrate for the generation of metabolic energy or for the synthesis of fatty acids and cholesterol in liver.

Nrep (also known as chromosome 5 open reading frame 13, *C5orf13*, *D0H4S114,* NM31*,* P311 or PTZ17) is an 8-kDa, polypeptide highly expressed in late-stage embryonic brain and adult cerebellum, hippocampus and olfactory bulb (31). In growing pigs fed a diet- restricted (20% less protein and 7% less energy) and showing an increase in intramuscular lipids, there was a concomitant increase of P311 (32). P311 expression also increased intracellular cholesterol and triglyceride levels, and intracellular lipid droplets in fibroblasts. Therefore, P311 has been proposed as an alternative pathway of lipid-droplet accumulation particularly induced by retinoic acid (33). Likewise, the opposite was observed in skeletal muscle atrophy in different experimental models such as fasted mice, cachetic, diabetic and uremic rats (34). In this sense, the changes of expressions of the hepatic P311/Nrep mRNA levels associated with hepatic cholesterol and triglycerides indicate that some of the processes observed in other tissues could be also involved in lipid handling in hepatic cells.

As a whole, our results provide new evidences to advance in the knowledge of hepatic management of postprandial administration of virgin olive oil. Through microarray analysis, hepatic gene expressions not previously associated with this phenomenon have been found differentially expressed and significantly associated. In this respect, our findings in rats will help to understand the complex human pathology providing hepatic proteins involved in postprandial regulation and new markers to evaluate the quality of olive oils *in vivo*.

6. ACKNOWLEDGMENTS

We thank Drs. C. Junquera and L. Osaba of Progenika Biopharma for performing the microarray analyses and Dr. Martinez for critical reading of the manuscript. We also thank Jesus Cazo, Jesus Navarro, Carmen Navarro and Clara Tapia of the Unidad Mixta de Investigacion for their invaluable help in maintaining the animals and Rosario Puyo for her technical assistance. This research was funded by grants CIBER Fisiopatologia de la Obesidad y Nutricion as an initiative of ISCIII, CICYT-FEDER (SAF2007-60173), Gobierno de Aragon (PI025/08) and Redes DGA (B-69). M.A.N., S.A. and MAG were recipients of Miguel Servet contracts and PROMEP fellowships. No competing financial interests exist.

7. REFERENCES

1. Fujioka, Y. & Y. Ishikawa: Remnant lipoproteins as strong key particles to atherogenesis. *J Atheroscler Thromb*, 16, 145-54 (2009)

2. Redgrave, T. G.: Chylomicrons in disease-future challenges Invited keynote address. *Atheroscler Suppl*, 9, 3-6 (2008)

3. Nordestgaard, B. G., A. Langsted & J. J. Freiberg: Nonfasting hyperlipidemia and cardiovascular disease. *Curr Drug Targets*, 10, 328-35 (2009)

4. Kannel, W. B. & R. S. Vasan: Triglycerides as vascular risk factors: new epidemiologic insights. *Curr Opin Cardiol*, 24, 345-50 (2009)

5. Lopez-Miranda, J., C. Williams & D. Lairon: Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. *Br J Nutr*, 98, 458-73 (2007)

6. Perez-Martinez, P., J. Lopez-Miranda, F. Perez-Jimenez & J. M. Ordovas: Influence of genetic factors in the modulation of postprandial lipemia. *Atheroscler Suppl*, 9, 49-55 (2008)

7. Cianflone, K., S. Paglialunga & C. Roy: Intestinally derived lipids: metabolic regulation and consequences--an overview. *Atheroscler Suppl*, 9, 63-8 (2008)

8. Lairon, D.: Macronutrient intake and modulation on chylomicron production and clearance. *Atheroscler Suppl*, 9, 45-8 (2008)

9. Xu, T., X. Li, X. Ma, Z. Zhang, T. Zhang & D. Li: Effect of diacylglycerol on postprandial serum triacylglycerol concentration: a meta-analysis. *Lipids*, 44, 161-8 (2009)

10. Perona, J. S., M. Avella, K. M. Botham & V. Ruiz-Gutierrez: Uptake of triacylglycerol-rich lipoproteins of differing triacylglycerol molecular species and unsaponifiable content by liver cells. *Br J Nutr*, 95, 889-97 (2006)

11. Overturf, M. L. & D. S. Loose-Mitchell: *In vivo* model systems: the choice of the experimental animal model for analysis of lipoproteins and atherosclerosis. *Curr Opin Lipidol*, 3, 179-185 (1992)

12. Fielding, C. J., R. J. Havel, K. M. Todd, K. E. Yeo, M. C. Schloeter, V. Weinberg & P. H. Frost: Effects of dietary

cholesterol and fat saturation on plasma lipoproteins in an ethnically diverse population of healthly young men. *J Clin Invest*, 95, 611-618 (1995)

13. Cozzi, J., I. Anegon, V. Braun, A. C. Gross, C. Merrouche & Y. Cherifi: Pronuclear DNA injection for the production of transgenic rats. *Methods Mol Biol*, 561, 73-88 (2009)

14. Geurts, A. M., G. J. Cost, Y. Freyvert, B. Zeitler, J. C. Miller, V. M. Choi, S. S. Jenkins, A. Wood, X. Cui, X. Meng, A. Vincent, S. Lam, M. Michalkiewicz, R. Schilling, J. Foeckler, S. Kalloway, H. Weiler, S. Menoret, I. Anegon, G. D. Davis, L. Zhang, E. J. Rebar, P. D. Gregory, F. D. Urnov, H. J. Jacob & R. Buelow: Knockout rats via embryo microinjection of zinc-finger nucleases. *Science*, 325, 433 (2009)

15. Calleja, L., M. C. Trallero, C. Carrizosa, M. T. Mendez, E. Palacios-Alaiz & J. Osada: Effects of dietary fat amount and saturation on the regulation of hepatic mRNA and plasma apolipoprotein A-I in rats. *Atherosclerosis*, 152, 69-78 (2000)

16. Acin, S., M. A. Navarro, J. S. Perona, J. M. Arbones-Mainar, J. C. Surra, M. A. Guzman, R. Carnicer, C. Arnal, I. Orman, J. C. Segovia, J. Osada & V. Ruiz-Gutierrez: Olive oil preparation determines the atherosclerotic protection in apolipoprotein E knockout mice. *J Nutr Biochem*, 18, 418-24 (2007)

17. Maeda, N., H. Li, D. Lee, P. Oliver, S. H. Quarfordt & J. Osada: Targeted disruption of the apolipoprotein C-III gene in mice results in hypertriglyceridemia and protection from postprandial hypertriglyceridemia. *J Biol Chem*, 269, 23610-23616 (1994)

18. Guillen, N., S. Acin, M. A. Navarro, J. S. Perona, J. M. Arbones-Mainar, C. Arnal, A. J. Sarria, J. C. Surra, R. Carnicer, I. Orman, J. C. Segovia, V. Ruiz-Gutierrez & J. Osada: Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice. *Atherosclerosis*, 196, 558-564 (2008)

19. Arbonés-Mainar, J. M., M. A. Navarro, S. Acín, M. A. Guzmán, C. Arnal, J. C. Surra, R. Carnicer, H. M. Roche & J. Osada: trans-10, cis-12- and cis-9, trans-11-Conjugated Linoleic Acid Isomers Selectively Modify HDL-Apolipoprotein Composition in Apolipoprotein E Knockout Mice. *J Nutr*, 136, 353-359 (2006)

20. Guillen, N., S. Acín, J. C. Surra, C. Arnal, J. Godino, A. García-Granados, P. Muniesa, V. Ruiz-Gutiérrez & J. Osada: Apolipoprotein E determines the hepatic transcriptional profile of dietary maslinic acid in mice. *J Nutr Biochem*, 20, 882-893 (2009)

21. Guillen, N., M. A. Navarro, C. Arnal, E. Noone, J. M. Arbones-Mainar, S. Acin, J. C. Surra, P. Muniesa, H. M. Roche & J. Osada: Microarray Analysis of Hepatic Gene Expression Identifies New Genes Involved in Steatotic Liver. *Physiol Genomics*, 37, 187-198 (2009)

22. Acin, S., M. A. Navarro, J. S. Perona, J. C. Surra, N. Guillen, C. Arnal, A. J. Sarria, J. M. Arbones-Mainar, R. Carnicer, V. Ruiz-Gutierrez & J. Osada: Microarray analysis of hepatic genes differentially expressed in the presence of the unsaponifiable fraction of olive oil in apolipoprotein E-deficient mice. *Br J Nutr*, 97, 628-38 (2007)

23. Munteanu, M., D. Messous, D. Thabut, F. Imbert-Bismut, M. Jouys, J. Massard, A. Piton, L. Bonyhay, V. Ratziu, B. Hainque & T. Poynard: Intra-individual fasting versus postprandial variation of biochemical markers of liver fibrosis (FibroTest) and activity (ActiTest). *Comp Hepatol*, 3, 3 (2004)

24. Hussain, M. M., D. K. Strickland & A. Bakillah: The mammalian low-density lipoprotein receptor family. *Annu Rev Nutr*, 19, 141-72 (1999)

25. Liu, C. J.: The role of ADAMTS-7 and ADAMTS-12 in the pathogenesis of arthritis. *Nat Clin Pract Rheumatol*, 5, 38-45 (2009)

26. Baker, A. H., D. R. Edwards & G. Murphy: Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci*, 115, 3719-27 (2002)

27. Markovich, D. & H. Murer: The SLC13 gene family of sodium sulphate/carboxylate cotransporters. *Pflugers Arch*, 447, 594-602 (2004)

28. Inoue, K., L. Zhuang & V. Ganapathy: Human Na+ - coupled citrate transporter: primary structure, genomic organization, and transport function. *Biochem Biophys Res Commun*, 299, 465-71 (2002)

29. Gopal, E., S. Miyauchi, P. M. Martin, S. Ananth, S. R. Srinivas, S. B. Smith, P. D. Prasad & V. Ganapathy: Expression and functional features of NaCT, a sodium-coupled citrate transporter, in human and rat livers and cell lines. *Am J Physiol Gastrointest Liver Physiol*, 292, G402-8 (2007)

30. Metges, C. C. & G. Wolfram: Different (13)CO(2) recovery of orally administered [1-(13)C]- and [8-(13)C] triolein in postprandial humans: an effect of phosphoenolpyruvate-carboxykinase (EC 4.1.1.32) in peripheral tissues? *Clin Nutr*, 12, 337-43 (1993)

31. Taylor, G. A., E. Hudson, J. H. Resau & G. F. Vande Woude: Regulation of P311 expression by Met-hepatocyte growth factor/scatter factor and the ubiquitin/proteasome system. *J Biol Chem*, 275, 4215-9 (2000)

32. da Costa, N., C. McGillivray, Q. Bai, J. D. Wood, G. Evans & K. C. Chang: Restriction of dietary energy and protein induces molecular changes in young porcine skeletal muscles. *J Nutr*, 134, 2191-9 (2004)

33. Leung, J. K., S. Cases & T. H. Vu: P311 functions in an alternative pathway of lipid accumulation that is induced by retinoic acid. *J Cell Sci*, 121, 2751-8 (2008)

34. Lecker, S. H., R. T. Jagoe, A. Gilbert, M. Gomes, V. Baracos, J. Bailey, S. R. Price, W. E. Mitch & A. L. Goldberg: Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb J*, 18, 39-51 (2004)

Abbreviations: A2m, alpha2- macroglobulin; LRP, low density receptor related protein; NaCT, *Slc13a5* or Na^+-coupled citrate transporter; OO olive oil; PCR, polymerase chain reaction; RT, reverse transcriptase; qRT, quantitative real time; TG, triglycerides; TRL, triglyceride rich lipoproteins

Key Words: Postprandial triglyceridemia, olive oil, hepatic gene expression, DNA microarrays

Send correspondence to: Jesus Osada, Department of Biochemistry and Molecular Biology, Veterinary School, University of Zaragoza, Miguel Servet, 177, E-50013 Zaragoza, Spain, Tel: 34-976-761-644, Fax: 34-976-761-612, E-mail: Josada@unizar.es

http://www.bioscience.org/current/volE3.htm