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# Microarray analysis of hepatic gene expression identifies new genes involved in steatotic liver

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[1]*Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Instituto Aragonés de Ciencias de la Salud (Universidad de Zaragoza-Salud del Gobierno de Aragón),* [2]*Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain;* [3]*Nutrigenomics Research Group, UCD Conway Institute, University College-Dublin, Belfield, Dublin, Ireland;* [4]*Departamento de Anatomía y Embriología y Genética Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza; and* [5]*CIBER de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain*

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**Guillén N, Navarro MA, Arnal C, Noone E, Arbonés-Mainar JM, Acín S, Surra JC, Muniesa P, Roche HM, Osada J.** Microarray analysis of hepatic gene expression identifies new genes involved in steatotic liver. *Physiol Genomics* 37: 187–198, 2009. First published March 3, 2009; doi:10.1152/physiolgenomics.90339.2008.— *Trans*-10, *cis*-12-conjugated linoleic acid (CLA)-enriched diets promote fatty liver in mice, while *cis*-9, *trans*-11-CLA ameliorates this effect, suggesting regulation of multiple genes. To test this hypothesis, apoE-deficient mice were fed a Western-type diet enriched with linoleic acid isomers, and their hepatic gene expression was analyzed with DNA microarrays. To provide an initial screening of candidate genes, only 12 with remarkably modified expression between both CLA isomers were considered and confirmed by quantitative RT-PCR. Additionally mRNA expression of 15 genes involved in lipid metabolism was also studied. Ten genes *(Fsp27, Aqp4*, *Cd36, Ly6d, Scd1, Hsd3b5, Syt1, Cyp7b1,* and *Tff3)* showed significant associations among their expressions and the degree of hepatic steatosis. Their involvement was also analyzed in other models of steatosis. In hyperhomocysteinemic mice lacking *Cbs* gene, only *Fsp27, Cd36, Scd1, Syt1,* and *Hsd3b5* hepatic expressions were associated with steatosis. In apoE-deficient mice consuming olive-enriched diet displaying reduction of the fatty liver, only *Fsp27* and *Syt1* expressions were found associated. Using this strategy, we have shown that expression of these genes is highly associated with hepatic steatosis in a genetic disease such as *Cbs* deficiency and in two common situations such as Western diets containing CLA isomers or a Mediterranean-type diet. Conclusion: The results highlight new processes involved in lipid handling in liver and will help to understand the complex human pathology providing new proteins and new strategies to cope with hepatic steatosis.

conjugated linoleic acid; apolipoprotein e-deficient mice; olive oil; hepatic steatosis; DNA microarrays; *Syt1*

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is the most common form of liver disease in Westernized societies, reaching a prevalence as high as 25% in some ethnic groups in the United States (17). The etiology and progression of NAFLD to steatohepatitis and cryptogenic cirrhosis are a matter of intense scrutiny due to the high variability among individuals. Commonly associated with obesity, it may be also found in lean people, in lipodystrophic patients lacking adipose tissue (52), in insulin resistance or hyperinsulinemia (9), in hyperhomocysteinemia (55), or after antiretroviral therapy (40). A hallmark of NAFLD is the accumulation of hepatic triglycerides (TG), which are the result of the availability of fatty acids (FA) from the circulation, de novo lipogenesis of FA from carbohydrates, oxidation of FA, and the secretion of TG in very low-density lipoproteins (VLDL) (22). Despite the accurate description of involved metabolic pathways, the precise regulation and coordination are becoming quite complex in mammals. Thus, endocrine, metabolic, and transcriptional pathways are involved (16). Regarding the first aspect, not only insulin influences the processes (21), but also other hormones such leptin or growth factors (28). In consequence, the hepatic intracellular cascades involved may be also complex with sterol regulatory element binding proteins (SREBP-1c or SREBP-2) (25), carbohydrate response element binding protein (ChREBP) (9), peroxisome proliferator-activated receptors (PPARα, PPARγ2) (20), liver X receptors (LXR), AMP-activated protein kinase (16), Foxo1 (2), or hepatocyte nuclear factor 4α (HNF-4α) (32) participating at different stages. Likewise, the secretion of TG in VLDL has been complicated with the discovery of proteins involved in the dynamics of lipid droplets such as adipophilin (11). Since NAFLD is considered the origin of other hepatic pathologies and a frequent finding in the metabolic syndrome (51), much attention should be focused on its understanding and prevention.

The term conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid. The *cis*-9, *trans*-11-CLA (c9,t11-CLA) isomer is the most abundant in meat and dairy products (37), although the *trans*-10, *cis*-12-CLA (t10,c12-CLA) isomer appears in almost equal concentrations in most processed CLA-containing foods and supplements (19). Multiple biological effects have been ascribed to CLA, including the control of body composition and energy metabolism, the modulation of the immune response, lipid metabolism, insulin resistance, and atherosclerosis (4, 6, 30, 34, 36, 42, 48, 53). Several authors report that the different CLA isomers have different effects on lipid metabolism in animals and humans (14, 47, 49). We, as well as others, have

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previously reported that t10,c12-CLA is the isomer responsible for the presence of fatty liver in mice associated with the loss of adipose tissue (4, 12, 15, 27, 35). This steatotic effect has been attributed to increased hepatic TG, cholesterol, cholesterol esters, and free fatty acids (27), reflecting increased hepatic fatty acid synthesis (45). However, the mechanisms by which the liver becomes steatotic in response to t10,c12-CLA appear to be more complex since t10,c12-CLA also induces β-oxidation (15) and reduces microsomal oxidation of fatty acids (38). Furthermore, using the steatotic prone model such as the apoE-deficient mice (43), we have also proved that c9,t11-CLA administration in Western diets ameliorated the fatty liver (3). Likewise, administration of a diet enriched in 10% (wt/wt) olive oil has been also observed to reduce hepatic fat (1). Thus, these dietary interventions represent valuable tools to explore new mechanisms involved in fatty liver by using microarray technology to systematically examine the expression of a large number of hepatic genes. Therefore, our aim was to address the finding of new genes involved in fatty liver. Here, with use of nutritional (feeding Western diets containing either c9,t11-CLA or t10,c12-CLA isomers or 10% olive oil-enriched diets) and genetic (*Cbs*-deficient and hyperhomocysteinemic mice) models of hepatic steatosis, and gene expression determined by microarray analysis and confirmed by real-time RT-PCR, several new genes were associated with this pathological finding.

## MATERIAL AND METHODS

### *Mice and Diets*

Three experimental designs using different genetic models as well as dietary conditions were carried out. All mice were housed in sterile filter-top cages (three or four per cage), had ad libitum access to chow diet and water, and were kept under light cycle conditions of 12 h light/12 h dark. Fresh food was provided daily. The study protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza.

In the first experiment, 29 three-mo-old homozygous apoE knockout (KO) male mice, bred at the Unidad Mixta de Investigación, Zaragoza, and having a C57BL/6JxOla129 genetic background and similar baseline plasma cholesterol concentrations, were randomly assigned to three different Western diets: linoleic (c9,c12-LA) acid ($n$ = 10), c9,t11-CLA-enriched ($n$ = 10) or t10,c12-CLA-enriched ($n$ = 9) diet. All three diets were isocaloric and isonitrogenous; fat provided 30% of the energy intake. The linoleic acid diet contained 1.0% (wt/wt) of this fatty acid, while the others contained an equivalent amount of either c9,t11-CLA or t10,c12-CLA, as previously described (4, 14). All diets were prepared by Unilever (Vlaardingen, The Netherlands) and stored in an $N_2$ atmosphere at −20°C. Fresh food was provided daily. The mice were fed the different experimental semipurified diets for 12 wk.

In the second experiment, 17 2-mo-old, male, homozygous apoE KO mice with a C57BL/6JxOla129 genetic background underwent an overnight fast, and blood samples from the retroorbital plexus after isoflurane anaesthetization were taken. Two study groups of similar initial plasma cholesterol were established: *1*) control group ($n$ = 8) received a chow diet (Teklad Mouse/Rat Diet no. 2014, Harlan Teklad; Harlan Ibérica, Barcelona, Spain) and *2*) the olive group ($n$ = 9) received the same chow diet but supplemented with 10% (wt/wt) olive oil. The animals were fed the experimental diets for 11 wk. Both diets were isocaloric (10). The olive oil diet was prepared weekly and stored in an $N_2$ atmosphere at −20°C.

For the third experiment, 11 cystathione β-synthase KO and seven control male mice with a C57BL/6JxOla129 genetic background were characterized as previously described (24). Nonweaned nine-day-old pups were killed after 2 h fast.

### *Histological Analysis*

A sample of liver from each mouse 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

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

| Gene | Forward 5′-3′ | Reverse 5′-3′ |
|---|---|---|
| *Sept4* | AACAACACAGAGTGCTGGAAGC | TGGATGTTCTTGCGGTTCAG |
| *Ly6d* | TGCCCGTCCAACTTCTACTTCT | TAGTCGGAGGTGCATGAGTTTG |
| *Fsp27* | GACTTTATTGGCTGCCTGAACG | ATCTCCTTCACGATGCGCTT |
| *Pqlc3* | ACGTGAAGCAAGCCTTACCCT | GACTGTGCATAAGTTCATGGCC |
| *Aqp4* | TACATCATTGCACAGTGCCTGG | TTTCCATGAACCGTGGTGACTC |
| *Sdcbp* | TGTGACAGGTAACGATGCTGGA | GGAATTAGCCTGGACGAGTTGA |
| *Col1a1* | ATCCAACGAGATCGAGCTCAGA | ATGACTGTCTTGCCCCAAGTTC |
| *Uck1* | GTTGAAGGCAAAACTGTCGAGG | TCGAACAGAACCACATCAGCTG |
| *Vnn1* | CCTCAAGTAAACTGGATACCCTGTG | TACACGGCTTCTTGTCTCCCAT |
| *Cln6* | CCATCTTGACCTCTGGTTCTACTTC | TGGATCGGGAATACCAGCAT |
| *Hsd3b5* | GCTCTTGGAAACCACAAGGAAC | GACAATCCTCTGGCCAAGAAAC |
| *Syt1* | CAAAAGTCCACCGGAAAACC | TGCCACCTAATTCCGAGTATGG |
| *Tff3* | TCTGGGATAGCTGCAGATTACG | GTAGCCACAGTCCACTCTGACATTT |
| *Cyp7b1* | AATTGGACAGCTTGGTCTGCCT | TGTGTATGAGTGGAGGAAAGAGGG |
| *Actb* | CTGACTGACTACCTCATGAAGATCCT | CTTAATGTCACGCACGATTTCC |
| *Ppib* | GGAGATGGCACAGGAGGAA | GTAGTGCTTCAGCTTGAAGTTCTCAT |
| *Acaa* | ATGATGGTCTGAAGGCAGCTGA | CTTTTCTGATCCCTTTCCCTCC |
| *Acab* | AATGTGGCAGACGCGATGTT | CAACGTCCACAATGTTCAGCAG |
| *Srbp2* | CATTGAAAAGCGCTACCGGT | CAGACTTGTGCATCTTGGCATC |
| *Scd1* | TGTCTCGGTGTGTGTCGGAGT | TGTACCACTACCTGCCTGCATG |
| *Cd36* | GGTAGAGATGGCCTTACTTGGGAT | CGGCTTTACCAAAGATGTAGCC |
| *Cpt1a* | TTCAATACTTCCCGCATCCC | TGTGGTACACGACAATGTGCCT |
| *Ppargc1a* | GTGTTCCCGATCACCATATTCC | AGGGTGACCTTGAACGTGATCT |
| *Ppargc1b* | TTGTGTCAAGGTGGATGGCA | TTATGCAGTTCCGTACAAGGCC |
| *Apoa5* | CTCTGGGACTACTTCAGCCAAAAC | TCTTGCTCGAAGCTGCCTTT |

droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section (1).

*Hepatic Lipid Analysis*

Tissues were homogenized in 10 volumes of PBS per gram of tissue. An aliquot was saved to determine protein concentration by the Bio-Rad dye binding assay (Bio-Rad, Madrid, Spain). One volume of homogenate was extracted with two volumes of chloroform-methanol (2:1) twice. The separated organic phases were combined and evaporated under $N_2$ stream. Extracts were dissolved in 100 μl of isopropanol and used to estimate triglyceride concentrations using commercial kits from Sigma Chemical (Madrid, Spain).

*RNA Isolation, Affymetrix Oligonucleotide Array Hybridization, and Data Analysis*

At the end of the experimental period the mice were killed by suffocation with $CO_2$. Blood was drawn from the heart, and livers of all mice were immediately removed and frozen in liquid nitrogen. RNA from each liver was isolated using Trigent reagent (MRC, Cincinnati, OH). DNA contaminants were removed by TURBO DNase treatment using the DNA removal kit from AMBION (Austin, TX). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was >1.75). The integrity of the 28S and 18S ribosomal RNAs was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining and the 28S/18S ratio was greater than 2. We pooled 20 μg aliquots of total liver RNA from each mouse of each group ($n$ = 10 for the control, $n$ = 10 for the c9,t11-CLA-enriched and $n$ = 9 for t10,c12-CLA-enriched diets) to avoid problems of individual variation and purified them using the RNeasy system (Qiagen, Barcelona, Spain). Eight micrograms of total liver RNA were then used for biotin labeling. Hybridization, washing, scanning, and analysis with the Affymetrix GeneChip Murine Genome U74A version 2 array (Affymetrix, Santa Clara, CA) were performed according to the standard Affymetrix protocols. Fluorometric data were generated by Affymetrix software, and the fluorometric signal was adjusted so that all the probe sets provided intensities within a manageable range. Transcripts whose signal intensities were lower than the noise of the matrix ± 3 SD were not taken into account. The data obtained in the microarray hybridizations were processed with Microarray Suite 5.0 (Affymetrix) software. Annotation was confirmed by checking the MGI Gene Ontology Browser at the Jackson website http://www.informatics.jax.org/searches/GO.cgi?id=GO:0042416. The identification of genes that were up- or downregulated by the presence of steatosis was carried out by first selecting only those whose signal $\log_2$ ratio between CLA isomers were higher than 1.5 (upregulated genes) or lower than −1.5 (downregulated genes) and those whose signal was significantly different from control group. To evaluate this global situation, a steatotic index was generated also considering signal $\log_2$ ratios ($SL_2R$) between t10,c12 CLA and control and between c9,t11-CLA and control, and according to the following equation: ($SL_2R$ t/cis) + ($SL_2R$ t/la) − ($SL_2R$ cis/la). Those with a score higher than 6 in this index were selected for further analysis. The complete datasets were deposited in the GEO database (accession number GSE7076).

*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 (3). Primers and probes for *Acox1, Fas1*, *Lxrα, Mtp1, Ppara,* and *Srbp1* were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). The remaining primers were designed by Primer Express (Applied Biosystems) and checked by BLAST analysis (National Center for Biotechnology Information) to verify gene specificity and selective amplification of cDNA vs. genomic DNA. The sequences are shown in Table 1. The specificity of the PCR reaction was confirmed by sequencing the products after their electrophoretic separation in agarose gels. Real-time RT-PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosys-

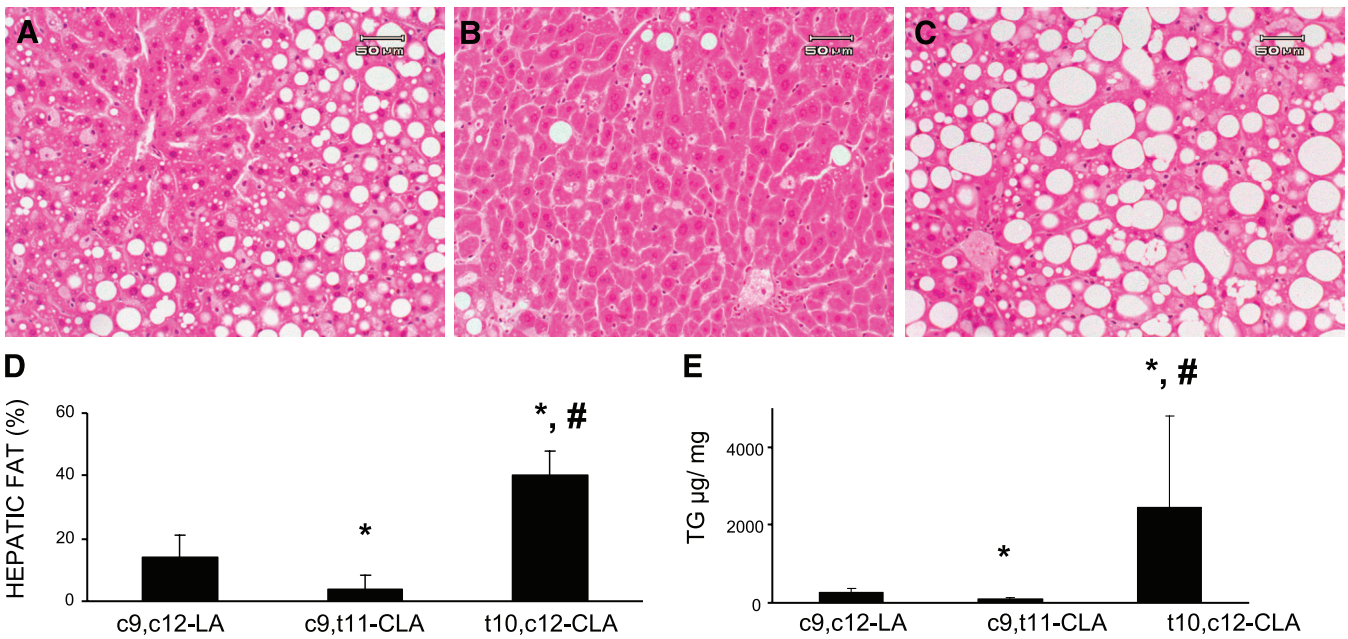


Fig. 1. Effects of Western diets with different linoleic acid isomers on hepatic steatosis in apoE-deficient mice. Representative liver micrographs at ×200 magnification from apoE-deficient mice consuming the c9,c12 linoleic (*A*), c9,t11-conjugated linoleic acid (CLA; *B*), and t10,c12-CLA-enriched (*C*) diets. Liver sections (4 μm) from all 29 mice were stained with hematoxylin and eosin and evaluated blind. Morphometric changes of hepatic fat content (*D*) and analysis of triglyceride (TG) content (*E*) in mice consuming the different diets where data are means ± SD for each group. Statistical analyses were done according to Mann-Whitney *U*-test. *$P < 0.01$ vs. c9,c12-LA; #$P < 0.01$ vs. c9,t11-CLA.

tems) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. *β-Actin* (*Actb*) and *cyclophilin B* (*Ppib*) mRNA were used as the invariant control.

*Tissue Homogenates and Western Blot Analysis*

We homogenized 1 g of tissue in 10 ml of PBS (136 mM NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4$, and 0.24 g/l $KH_2PO_4$, pH 7.4) homogenization buffer with protease inhibitor cocktail (Roche, Barcelona, Spain) at 0–4°C and centrifuged for 10 min at 10,000 *g* to obtain the postmitochondrial supernatant. Protein concentration was determined by the Bio-Rad dye binding assay (Bio-Rad).

We loaded 10 μg of protein onto a 12% SDS-polyacrylamide gel and electrophoresed it for 150 min at 90 V in a Bio-Rad Miniprotean cell (Hercules, CA). Proteins were transferred electrophoretically to PVDF membranes (GE Healthcare, Madrid, Spain) using a Bio-Rad Trans-Blot SD (semidry transfer cell) apparatus with 1.5 mA $cm^2$ membrane for 30 min. Membranes were blocked with PBS buffer containing 5% BSA and 2% Tween 20 for 1 h at room temperature and thereafter kept in blocking solution overnight (at 6°C). The primary antibody, diluted in PBS buffer containing 5% BSA and 1% Tween 20, was added, and the membranes were incubated overnight at 6 °C and then 2 h at room temperature. FSP27 protein expression was evidenced by using a rabbit polyclonal antibody against mouse protein (diluted 1/5,000, NB100–430; Novus Biologicals, Littleton, CO). Equal loadings were confirmed by using a goat polyclonal anti-HSC70 (diluted 1/1,000, sc-1059; Santa Cruz Biotechnology, Madrid, Spain). Membranes were washed with PBS containing 0.1% Tween 20 and then incubated with secondary antibodies linked to horseradish peroxidase (anti-rabbit, diluted 1/100,000, NA934GE, Healthcare; and anti-goat, diluted 1/70,000, Sigma Chemical) in PBS buffer containing 5% BSA and 1% Tween 20 for 90 min at room temperature. Detection was carried out using ECL detection kit (GE Healthcare). Membranes were exposed to Hyperfilm-ECL (GE Healthcare) for several time periods to achieve signal intensity within the dynamic range of quantitative detection, and films scanned at a 600 dpi resolution. Intensity of bands for each condition, taken as volume of pixels per $mm^2$, was calculated using Quantity One software version 4.5.0 (Bio-Rad) and normalized to that corresponding to HSC70 signal.

*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, Chicago, IL). Significance was set at $P \leq 0.05$.

## RESULTS

### *Histological and Histomorphometric Analysis in ApoE-Deficient Mice Receiving Western LA isomer Diets*

The apoE-deficient mice receiving the linoleic (c9,c12-LA) diet showed macrovesicular steatosis restricted to the hepatocytes of liver zone 2 and microvesicular steatosis in hepatocytes in and outside this region (Fig. 1*A*). The accumulation of lipids was almost absent in livers of mice receiving the c9,t11-CLA diet (Fig. 1*B*) and were dramatically worsened in mice fed the t10,c12-CLA diet (Fig. 1*C*). Quantitative morphological evaluation of the percentage of hepatic fat areas of all animals is shown in Fig. 1*D* and corroborates the above

Table 2. *Hepatic genes differentially expressed by the administration of LA isomers at the level of signal $log_2$ ratio ($SL_2R$) >3.0 between both CLA isomers*

| Biological Process | GeneBank | Affymetrix ID | Name | Gene Symbol | LA | c9,t11 CLA | t10,c12 CLA | $SL_2R$ *t/cis* | $SL_2R$ *t*/la | $SL_2R$ *cis*/la | Steatotic Index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | | | | | |
| Cytokinesis | X61452 | 94079_at | Septin 4 | *Sept4* | 297 | 23 | 573 | 4.6 | 0.9 | −3.7 | 9.3 |
| Immunity | X63782 | 160553_at | Lymphocyte antigen 6D | *Ly6d* | 681 | 163 | 4002 | 4.6 | 2.6 | −2.1 | 9.2 |
| Adipocyte differentiation | M61737 | 102016_at | Adipocyte-specific gene | *Fsp27* | 19 | 21 | 388 | 4.2 | 4.4 | 0.1 | 8.4 |
| Membrane protein | AI553536 | 99366_at | PQ loop repeat containing | *Pqlc3* | 30 | 23 | 282 | 3.6 | 3.2 | −0.4 | 7.2 |
| Small molecule transport | U88623 | 102704_at | Aquaporin 4 | *Aqp4* | 123 | 15 | 167 | 3.5 | 0.4 | −3.0 | 7.0 |
| Cytoskeleton | AF077527 | 93017_at | Syntenin | *Sdcbp* | 273 | 27 | 267 | 3.3 | 0.0 | −3.3 | 6.6 |
| Extracellular matrix | U03419 | 94305_at | alpha-1 type I procollagen | *Col1a1* | 403 | 176 | 1606 | 3.2 | 2.0 | −1.2 | 6.4 |
| Nucleotide biosynthesis | L31783 | 94381_at | Uridine kinase | *Uck1* | 813 | 221 | 1883 | 3.1 | 1.2 | −1.9 | 6.2 |
| *Downregulated* | | | | | | | | | | | |
| C21-steroid hormone biosynthesis | L41519 | 94795_at | 3-Ketosteroid reductase | *Hsd3b5* | 3103 | 4618 | 73 | −6.0 | −5.4 | 0.6 | −12.0 |
| Exocytosis | D37792 | 93005_at | Synaptotagmin 1 | *Syt1* | 42 | 158 | 5 | −5.0 | −3.1 | 1.9 | −10.0 |
| Tissue differentiation | D38410 | 100106_at | Trefoil factor 3 intestinal | *Tff3* | 352 | 1923 | 104 | −4.2 | −1.8 | 2.4 | −8.4 |
| Bile acid biosynthesis | AV141027 | 161345_f_at | Cytochrome P450, 7b1 | *Cyp7b1* | 6375 | 10426 | 1300 | −3.0 | −2.3 | 0.7 | −6.0 |

Data represent intensity of signal with the Affymetrix chip. Steatotic index was calculated as ($SL_2R$ *t/cis*) + ($SL_2R$ *t*/la) − ($SL_2R$ *cis*/la). LA, linoleic acid; CLA, conjugated linoleic acid.

pattern. Analysis of hepatic TG content (Fig. 1*E*) was also in agreement with the previous data. Therefore, this dietary approach constitutes a perfect paradigm where to study presence and absence of steatotic liver and the involved mechanisms.

### *Gene Expression in Livers of ApoE-deficient Mice Fed the Different CLA Diets*

To determine the changes in hepatic gene expression induced by the presence of steatosis, the expression of 12,489 transcripts represented on the Affymetrix GeneChip Murine Genome U74A version 2 array was quantified in pooled liver samples of 11 mice that received the linoleic (c9,c12-LA) diet, 10 mice fed the c9,t11-CLA diet and 9 mice on the t10,c12-CLA diet. The livers of c9,c12-LA group expressed 4,603 transcripts (identified as "present" by Affymetrix software), while those of the animals receiving c9,t11- and t10,c12-CLA isomers expressed 3,563 and 4,424, respectively. Using the Mann-Whitney ranking feature of the Affymetrix software to determine significant differences in gene expression ($P < 0.01$), we identified the increased expression of 369 sequences plus the reduced expression of 122 transcripts in samples from the animals on the t10,c12-CLA diet compared with those on the c9,t11-CLA diet when no multiple-test correction was applied. When we took the latter into consideration by removing expressions with similar gene symbols, identical accession number, and identical UNIGENE number, the number of genes with increased and repressed expression was reduced to 225 and 76, respectively.

As significant differences in levels of fat (Fig. 1) were observed between t10,c12 CLA and c9,c12-LA and between c9,t11-CLA and c9,c12-LA, $\log_2$ ratios of the latter comparisons were also calculated and are shown in Table 2. An steatotic index according to the following equation: ($SL_2R$ t/cis) + ($SL_2R$ t/la) − ($SL_2R$ cis/la) was obtained. To select fatty liver associated genes, only those with a value higher than 6 were chosen for further studies and are listed in Table 2. Empirically, the relevance of the index was tested by analyzing biological variation and association with degree of steatosis. *Cln6* and *Vnn1* with scores of 5 and 5.6 were individually studied (Table 3) and tested their association of individual expression with the steatosis values (Data not shown). Since these gene expressions failed in all of the criteria, only genes (*Sept4, Ly6d, Fsp27, Pqlc3, Aqp4, Sdcbp, Col1a1, Uck1, Hsd3b5, Syt1, Tff3,* and *Cyp7b1*) with values higher or lower than 6 or −6, respectively, were considered potential candidates and their validated microarray results, by individual analysis of expression using qRT-PCR normalized to *cyclophilin* mRNA level, are shown in Table 3. Of 10 genes upregulated, three of these genes coded for intracellular organization proteins (*Sept4*, *Sdcbp,* and *Cln6*), one coded for a protein belonging to the plasma membrane (*Pqlc3*), one coded for a protein belonging to the extracellular space (*Col1a1*), one was involved in immunity (*Ly6d*), one for metabolite transport proteins (*Aqp4*). A further three for proteins with miscellaneous functions (e.g., one was involved in adipocyte differentiation [*Fsp27*], another was an enzyme involved in nucleotide metabolism [*Uck1*], and one was an enzyme of the pantothenic metabolism [*Vnn1*]). Of the 10 upregulated genes included in the validation analysis (Table 3), only nine (*Ly6d, Fsp27, Pqlc3, Aqp4, Sdcbp, Col1a1, Uck1, Vnn1,* and *Cln6*) appeared significantly increased in their expressions by the t10,c12 CLA diet compared with c9,t11-CLA. Four genes met the criterion of showing a reduced expression by the administration of the t10,c12-CLA diet compared with those on the c9,t11-CLA (Tables 2 and 3). Of these, one coded for an enzyme involved in bile acid metabolism (*Cyp7b1*), one was involved in steroid hormone metabolism (*Hsd3b5*) and two for genes with miscellaneous functions (one

Table 3. *Modification of hepatic gene expression in apoE-deficient mice following the consumption of Western diets enriched in LA isomers*

| | LA | c9, t11-CLA | t10, c12-CLA | $SL_2R$ t/*cis* |
|---|---|---|---|---|
| *n* | 10 | 10 | 9 | |
| | | *Genes Upregulated* | | |
| *Sept4* | 1±0.5[a] | 0.9±0.4[a] | 1.5±0.6[a] | 0.7 |
| *Ly6d* | 1.4±1.0[b] | 1.7±1.0[b] | 6.3±2.3[a] | 1.9 |
| *Fsp27* | 1.1±0.4[b] | 0.4±0.2[c] | 17±9[a] | 5.4 |
| *Pqlc3* | 1±0.4[a] | 0.8±0.6[a] | 1.3±0.3[b] | 0.7 |
| *Aqp4* | 1.1±0.6[b] | 0.2±0.2[c] | 1.8±1.1[a] | 3.2 |
| *Sdcbp* | 1.1±0.6[a] | 0.6±0.4[a] | 1.4±0.4[b] | 1.2 |
| *Col1a1* | 1.1±0.6[a] | 1.1±0.9[a] | 7.2±6[b] | 2.7 |
| *Uck1* | 1.1±0.5[a] | 0.7±0.5[a] | 2.3±1.2[b] | 1.7 |
| *Vnn1* | 1.0±0.4[a] | 0.2±0.1[b] | 0.9±0.4[a] | 2.1 |
| *Cln6* | 1±0.4[a] | 0.8±0.2[a] | 1.5±0.7[b] | 0.9 |
| | | *Genes Downregulated* | | |
| *Hsd3b5* | 1.1±0.4[b] | 1.8±1.3[b] | 0.01±0.01[a] | −7.5 |
| *Syt1* | 1.2±0.7[a] | 1.1±0.4[a] | 0.5±0.2[b] | −1.1 |
| *Tff3* | 3.4±3[b] | 12.5±4[a] | 0.9±0.5[c] | −3.8 |
| *Cyp7b1* | 0.5±0.2[b] | 2.3±1.2[a] | 0.3±0.1[c] | −2.9 |

Values are the means ± SD in arbitrary units normalized to the cyclophilin B (*Ppib*) expression for each condition with the qRT-PCR. Means in the same row without a common letter differ significantly ($P \leq 0.05$; Mann-Whitney *U*-test).

Table 4. *Hepatic expression of genes related to fatty acid metabolism following consumption by apoE-deficient mice of Western diets enriched in LA isomers*

| | LA | c9, t11-CLA | t10, c12- CLA | $SL_2R$ t/ *cis* |
|---|---|---|---|---|
| *n* | 10 | 10 | 9 | |
| | | *Fatty Acid Transport and Biosynthesis* | | |
| *Acaa* | 1.1±0.5[a] | 1.1±0.5[a] | 2.1±1.0[b] | 0.9 |
| *Acab* | 1.2±0.8[a] | 1.7±1.7[a] | 6.0±3.7[b] | 1.8 |
| *Fas* | 1.0±0.4[a] | 0.9±0.3[a] | 0.9±0.4[a] | 0.0 |
| *Srbp1* | 1±0.4[a] | 0.7±0.6[a] | 0.7±0.4[a] | 0.0 |
| *Srbp2* | 0.5±0.2[a] | 1.0±0.6[a] | 1.4±0.7[b] | 0.5 |
| *Scd1* | 1.3±1.0[a] | 0.7±0.7[a] | 9.8±3.0[b] | 3.8 |
| *Cd36* | 1.1±0.6[b] | 0.7±0.4[b] | 4.1±1.9[a] | 2.6 |
| | | *Fatty Acid Catabolism* | | |
| *Acox* | 1.0±0.3[a] | 0.5±0.2[b] | 0.6±0.1[b] | 0.3 |
| *Cpt1a* | 1.1±0.5[a] | 0.6±0.3[b] | 1.4±0.3[c] | 1.2 |
| *Pparα* | 1±0.9[a] | 0.7±0.7[a] | 0.5±0.4[a] | −0.5 |
| *Ppargc1a* | 1±0.4[a] | 2.1±1.4[a] | 5.4±4.2[b] | 1.4 |
| *Ppargc1b* | 0.5±0.2[a] | 1.3±1.0[a] | 1.3±0.7[a] | 0.0 |
| | | *TG Transport* | | |
| *Apoa5* | 0.6±0.1[a] | 0.7±0.1[a] | 0.3±0.1[b] | −1.2 |
| *Lxrα* | 1±0.5[a] | 0.6±0.4[a] | 0.5±0.3[b] | −0.3 |
| *Mtp1* | 1±0.3[a] | 1.0±0.2[a] | 0.6±0.2[a] | −0.7 |

Values are the means ± SD. in arbitrary units normalized to the cyclophilin B (*Ppib*) expression for each condition with the qRT-PCR. Means in the same row without a common letter differ significantly ($P \leq 0.05$; Mann-Whitney *U*-test).

exocytosis protein [*Syt1*] and one differentiation protein [*Tff3*]).The four downregulated genes selected, *Hsd3b5, Syt1, Tff3,* and *Cyp7b1*, were significantly decreased in mice receiving the diet enriched in the t10,c12 CLA isomer compared with c9,t11-CLA.

Previous literature has shown that CLAs affect hepatic TG (27), hepatic synthesis (45), and oxidation of fatty acids (15, 38). For this reason, mRNA expression of 15 genes involved in fatty acid synthesis and transport (*Acaa, Acab, Fas, Srbp1, Srbp2, Scd1, and Cd36*), oxidation (*Acox, Cpt1a, Pparα,*

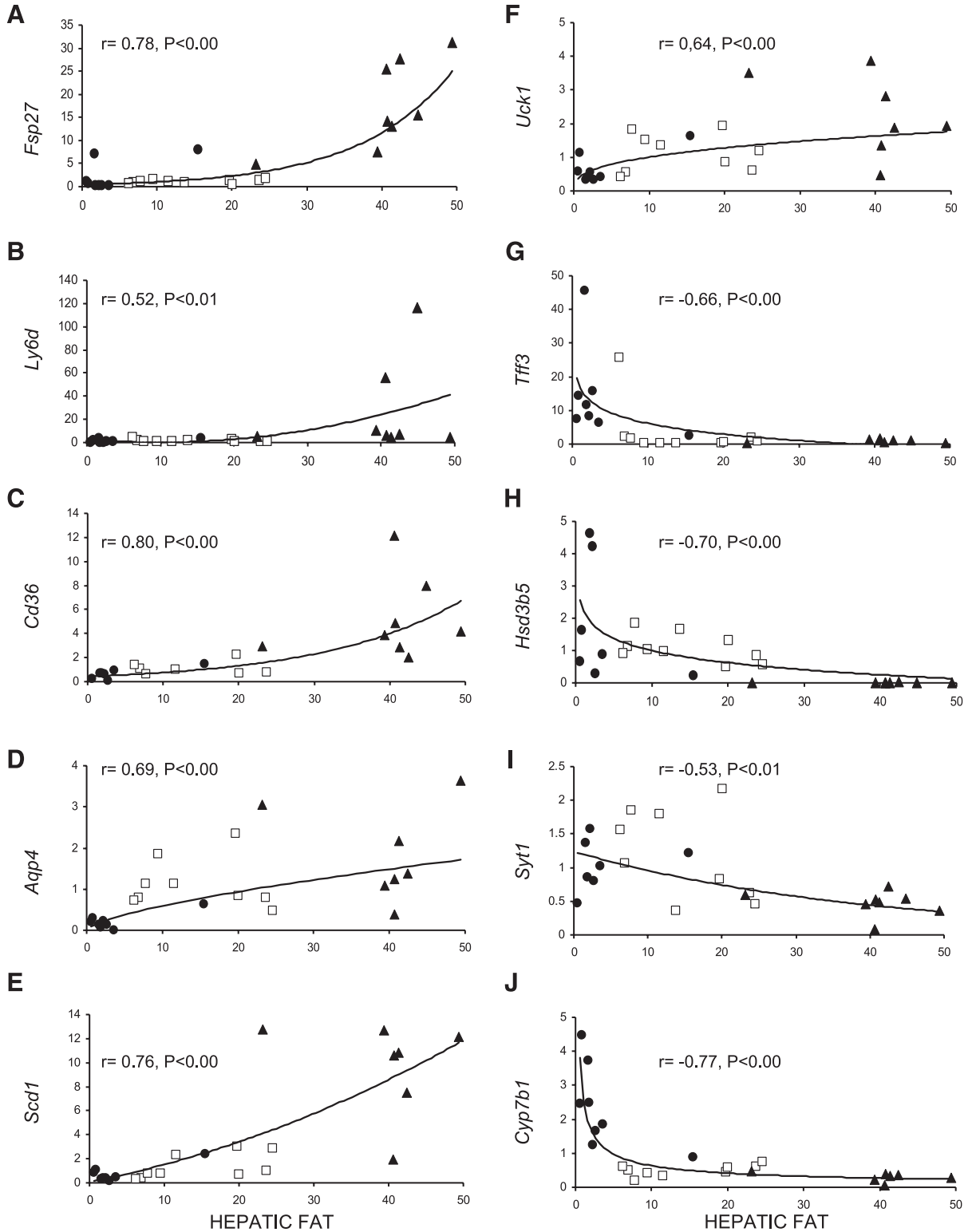


Fig. 2. Relationship between hepatic fat area and hepatic gene expression in apoE-deficient mice consuming linoleic acid (LA)-containing diets. □, LA; ● c9,t11-CLA; ▲, t10,c12-CLA-fed animals. Correlations were calculated according to Spearman's test.

*Ppargc1a, Ppargc1b*), and hepatic TG dynamics (*Lxra, Mtp1, Apoa5*) were tested in individual samples by RT-PCR (Table 4). Seven genes (*Acaa, Acab, Srbp2, Scd1, Cd36, Cpt1a,* and *Ppargc1a*) were significantly increased in their expressions in mice receiving the t10,c12 CLA diet compared with those on the c9,t11-CLA one. Only *Apoa5* expression was significantly decreased in mice receiving the diet enriched in the t10,c12 CLA isomer compared with c9,t11-CLA. In the supplementary file and Supplementary Fig S1 the degree of concordance between the microarray and RT-PCR are shown.[1]

To verify the degree of association between gene expression (Tables 3 and 4) and the presence of hepatic fat (Fig. 1), analyses of association of individual values obtained by qRT-PCR were carried out. Figure 2 shows those that appeared statistical significantly associated *Fsp27, Ly6d, Cd36, Aqp4, Scd1*, *Uck1*, *Tff3*, *Hsd3b5, Syt1,* and *Cyp7b1*. Interestingly, *Fsp27* and *Cd36* mRNA expressions showed the highest positive associations with hepatic fat content ($r_s = 0.78$, $P < 0.00$ and $r_s = 0.8$, $P < 0.00$, respectively) and *Cyp7b1* the lowest negative ($r_s = -0.77$, $P < 0.00$). In contrast, other parameters associated with lipid metabolism and presented in Table 4 such as *Ppara*, *Srbp1, Srbp2, Ppargc1a, Ppargc1b,* or *Lxra* mRNA expressions were not found associated [$r = -0.02$, not significant (ns); $r = -0.17$, ns; $r = -0.21$, ns; $r = 0.16$, ns; $r = 0.02$, ns and $r = -0.2$, ns; respectively]. These results indicate that these new selected genes are potential good candidates to be involved in fat accumulation in the liver.

[1] The online version of this article contains supplemental material.

### *Histological Analysis and Gene Expression in Livers of Cbs-Deficient Mice Receiving Maternal Milk*

The ten genes significantly associated with fatty liver (*Fsp27, Ly6d, Cd36, Aqp4*, *Scd1*, *Uck1*, *Tff3*, *Hsd3b5, Syt1,* and *Cyp7b1*) were tested in homozygous *Cbs*-deficient mice as a model of genetic fatty liver (54). As shown in Fig. 3*A*, livers of wild-type mice did not show any lipid accumulation. However, *Cbs*-deficient mice showed a liver with a macrovesicular and microvesicular steatosis (Fig. 3*B*). Quantitative evaluation of the percentage of hepatic lipid areas of all animals is shown in Fig. 3*C* and corroborates the above pattern. This genetic deficiency provides a field in which to test the relevance of selected genes in the steatotic process. As shown in Fig. 3*D*, *Cd36, Fsp27, Ly6d,* and *Scd1* expressions were significantly increased in *Cbs*-deficient mice. On the other hand, *Hsd3b5* and *Scd1* were significantly decreased, as expected, but *Aqp4*

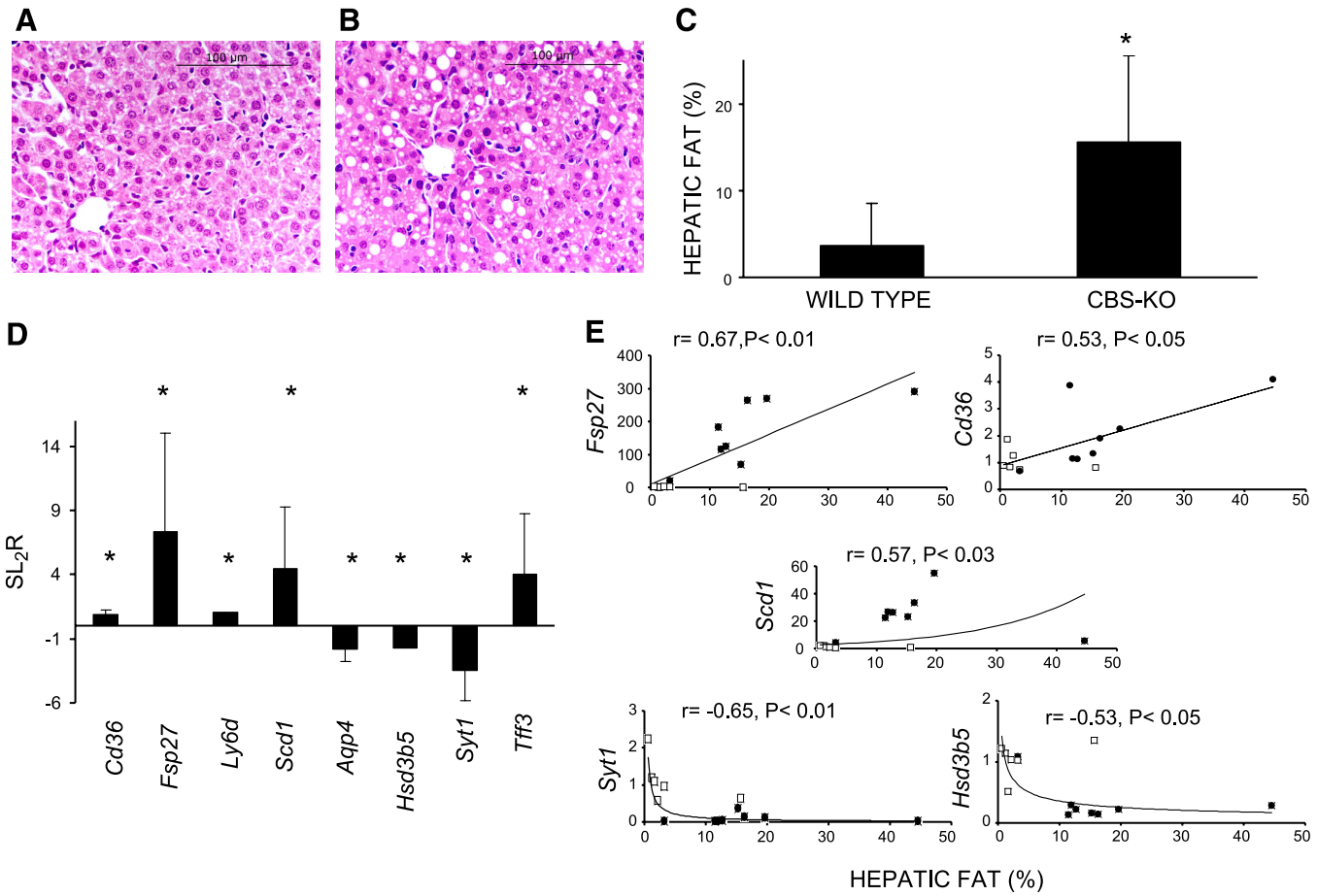


Fig. 3. Hepatic steatosis and gene expression in *Cbs*-deficient mice. Representative liver micrographs at ×400 magnification from wild-type (*A*) and *Cbs*-deficient mice (KO; *B*). Morphometric changes of hepatic fat content in mice (*C*). Hepatic gene expressions (*D*) in which data are expressed as signal $\log_2$ ratios ($SL_2R$) of hepatic mRNA expression changes (as determined by qRT-PCR) for each gene in Cbs-deficient vs. wild-type mice used as the reference and normalized to *Ppib*. Data represent means and SD for each group. $^*P < 0.01$ between groups according to Mann-Whitney's *U*-test. *E*: relationship between hepatic fat area and hepatic gene expression. □, Wild-type animals; ●, *Cbs*-deficient mice. Correlations were calculated according to Spearman's test.

*and Tff3* messages were unexpectedly found significantly decreased and increased, respectively in these mice.

To verify the degree of association between gene expression (Fig. 3*D*) and the presence of hepatic fat (Fig. 3*C*), analyses of association were carried out. Figure 3*E* shows those that appeared significantly associated. Interestingly, *Fsp27*, *Cd36* and *Scd1* mRNA expressions showed positive associations ($r_s = 0.67$, $P < 0.01$; $r_s = 0.53$, $P < 0.05$ and $r_s = 0.57$, $P < 0.03$, respectively) while *Hsd3b5* and *Syt1* showed negative ones ($r_s = -0.53$, $P < 0.05$ and $r_s = -0.65$, $P < 0.01$, respectively). These results indicate that, in this genetic model of hepatic steatosis, *Fsp27, Cd36, Syt1, Scd1,* and *Hsd3b5* expressions are also good candidates to control fat accumulation in the liver.

### *Histological Analysis and Gene Expression in Livers of ApoE-Deficient Mice Receiving Olive Oil-Enriched Diets*

To investigate whether or not the selected genes (*Fsp27, Cd36, Ly6d, Scd1, Hsd3b5, Syt1, and Tff3*) underwent expression changes in the correction of steatotic features from apoE-deficient mice, the livers of animals consuming chow or 10% (wt/wt) olive oil-enriched diet were examined histologically and their mRNA expressions assayed by qRT-PCR. Figure 4 shows representative images of livers from apoE-deficient mice receiving the two diets. The liver of those receiving the chow diet (Fig. 4*A*) showed steatosis, in agreement with the described accumulation of lipids in this model (1, 44). Interestingly, the olive oil-enriched diet alleviated these steatotic features (Fig. 4*B*). Quantitative confirmation of those effects is shown in Fig. 4*C*. Of the seven genes studied, only hepatic expressions of *Fsp27, Scd1,* and *Syt1* were significantly modified along with the decrease of steatosis by consuming the olive oil-enriched diet (Fig. 4*D*). Interestingly, for *Hsd3b5* and *Tff3* gene expression the existence of responders and nonresponders to the olive oil diet was observed. These *Fsp27* and *Syt1* gene expressions were also significantly associated with the presence of hepatic fat ($r_s = 0.51$ $P < 0.04$ for *Fsp27* and $r_s = -0.59$, $P = 0.03$ for *Syt1*) (Fig. 4*E*). These results indicate

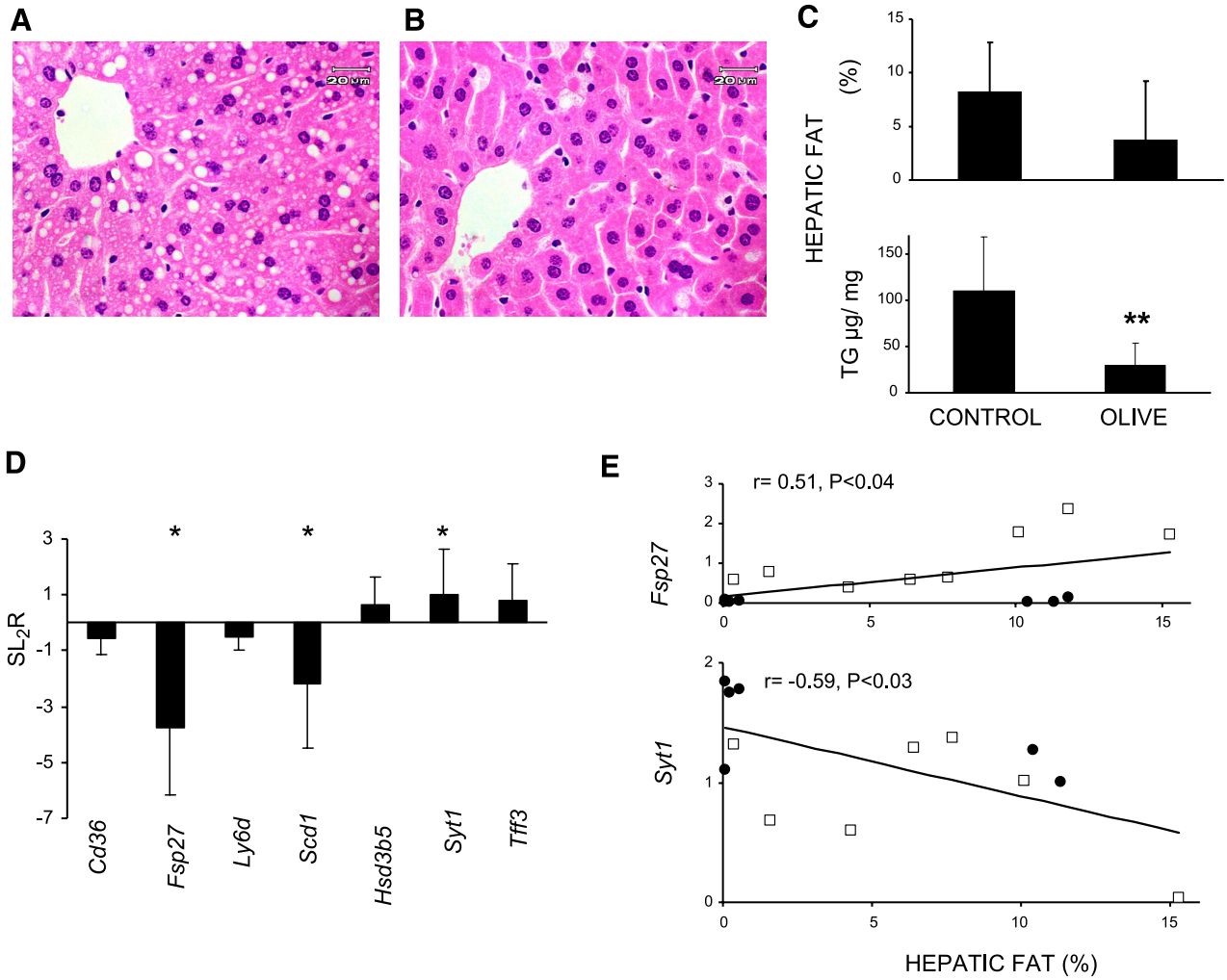


Fig. 4. Hepatic steatosis and gene expression in apoE-deficient mice receiving olive oil-enriched diets. Representative liver micrographs at ×400 magnification from apoE-deficient mice consuming the control (*A*) and olive oil-enriched diets (*B*). *C*: hepatic fat content in mice consuming the different diets as morphometric analysis (*top*) and as TG content (*bottom*). *D*: hepatic gene expressions where data are expressed as signal $\log_2$ ratios of hepatic mRNA expression changes (as determined by qRT-PCR) for each gene in mice consuming olive oil-enriched diet vs. control mice used as the reference and normalized to *Ppib* expression. Data represent means ± SD for each group. $^{**}P < 0.001$; $^{*}P < 0.01$ between groups according to Mann-Whitney's *U*-test. Relationship between hepatic fat area and hepatic gene expression (*E*). □, Chow-fed mice; ●, olive-fed mice. Correlations were calculated according to Spearman's test.

that in moderate dietary control of hepatic steatosis, *Fsp27* and *Syt1* expressions are also good candidates associated with fat accumulation in the liver.

*Hepatic Protein Levels of FSP27 in ApoE-deficient Mice Fed the Different CLA Diets*

To verify whether the changes in *Fsp27* mRNA were reflected into protein, Western blot analysis was carried out and results are shown in Fig. 5. It is noticeable that the single protein band recognized by the antibody showed the proper molecular weight in adipose tissue (Fig. 5*A*, *lane 2*), while the hepatic protein displayed a molecular mass >50 kDa (Fig. 5*A*, *lane 3*). Likewise, the abundance of the cytosolic protein is much more abundant in liver than in adipose tissue. As shown in Fig. 5, *B* and *C*, FSP27 levels were modulated by the diets containing linoleic isomers. Thus, animals receiving the c9,t11-CLA showed significant lower values than those receiving the c9,c12-LA diet, and mice receiving t10,c12-CLA diet had the significant highest values compared with the other groups. These results demonstrate that mRNA changes are reflected in protein expression.

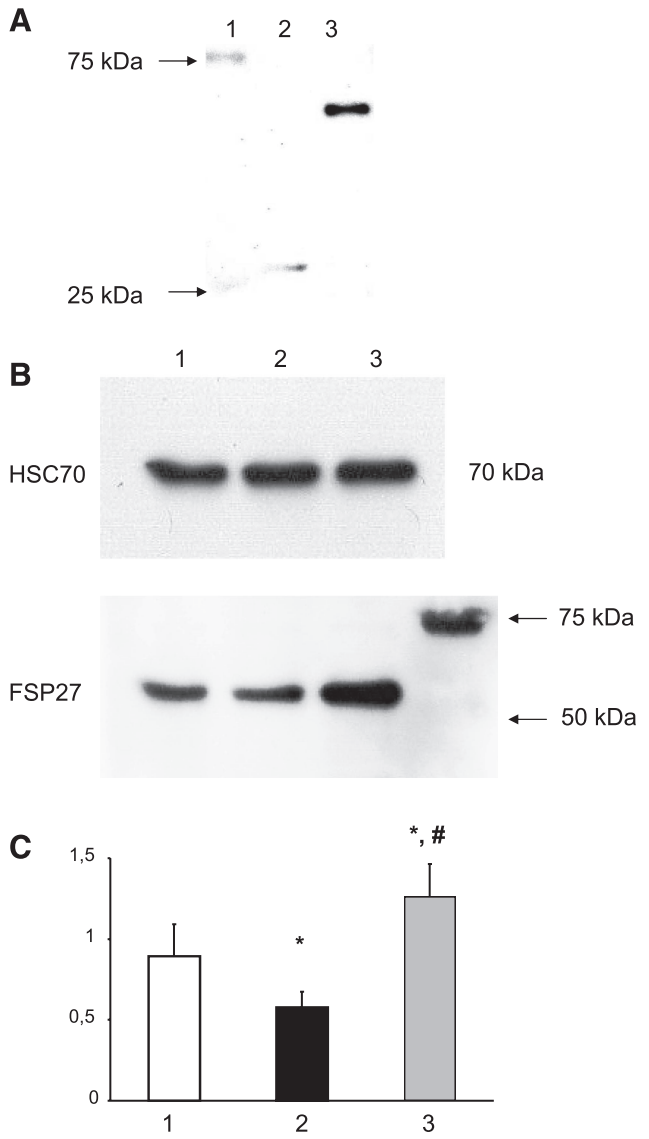


Fig. 5. FSP27 protein expressions in mice fed the LA-containing diets. *A*: Western blot showing the molecular size in adipose tissue and liver. *Lane 1*, molecular weight markers; *lanes 2* and *3*, adipose tissue and hepatic homogenates, respectively. *B*: representative blots showing the hepatic FSP27 and HSC70 expressions of mice consuming diets containing c9,c12-LA (*lane 1*), c9,t11-CLA (*lane 2*), and t10,c12-CLA (*lane 3*). *C*: quantification of Western results. Data (means ± SD) expressed as arbitrary absorbance units normalized to the HSC70 expression are given. Western analysis was carried out as described in MATERIAL AND METHODS. Statistical analysis was done according to Mann-Whitney *U*-test. *$P < 0.05$ vs c9,c12-LA; #$P < 0.01$ vs c9,t11-CLA.

## DISCUSSION

Using nutritional and genetic models of hepatic steatosis in mice, together with microarray analysis and its confirmation by RT-PCR, we have provided a new panoply of genes whose expression is associated with hepatic steatosis as well as potential nutritional strategies able to cope with the disease through regulating the expression of those genes.

Our work shows that the CLA dietary intervention in a Western diet markedly influenced the lipid content of the liver on both directions providing an interesting experimental approach to test the involvement of different genes. So far, the effects of CLA have been explained through interactions with a number of transcription factors, particularly the PPAR. Indeed, both c9,t11-CLA and t10,c12-CLA are PPARα and PPARγ ligands (12, 31). PPARα activates lipolytic pathways and apolipoprotein production, ultimately reducing the fat content of the liver (18). Thus, c9,t11-CLA could promote PPARα activation, leading to reduced liver fat. However, our present results prove that there is no association between hepatic fat and expression levels of this transcription factor. Furthermore, no such changes were observed in genes controlled by this transcription factor (4), suggesting that PPARα activation does not explain satisfactorily the model. These CLA isomers have been found to promote downregulation of LXRα (41). As in the case of PPARα, no association was found between LXRα expression and hepatic fat. The profound hepatic steatosis showed by animals consuming the t10,c12-CLA isomer, an effect reported in other studies (7, 12, 50), has been attributed to increased hepatic TG, cholesterol, cholesterol esters, and free fatty acids (27), reflecting increased hepatic fatty acid synthesis (45). However, the mechanisms by which the liver becomes steatotic in response to t10,c12-CLA appear to be complex since t10,c12-CLA induces β-oxidation (15), which would not be associated with lipid accumulation, and reduces microsomal oxidation of fatty acids (38), which could be associated to a certain level. This scenario suggests that not every participating actor has been conveniently identified. Eventually, studying the expression levels of the differentially regulated genes in microarray experiments under CLA isomer-containing diets may reveal interesting candidate genes and unravel the metabolic pathways involved. By this approach and the expression analysis of known genes involved in lipid metabolism (Tables 3 and 4), only six appeared significantly increased in their expressions by the t10,c12 CLA diet compared with c9,t11-CLA (*Ly6d, Fsp27, Aqp4*, *Uck1*, *Scd1,* and *Cd36*) and were positively associated with hepatic fat content (Fig. 2). In the same experimental setting, four gene expressions (*Hsd3b5, Syt1*, *Tff3,* and *Cyp7b1*) were significantly decreased and negatively associated with hepatic steatosis.

Hyperhomocysteinemia is a cause of fatty liver (55), and consequently, homozygous *Cbs*-deficient mice as a model of genetic hyperhomocysteinemia present such findings (54). When tested, of the 10 candidate genes in this model, only *Fsp27, Cd36, Scd1, Syt1,* and *Hsd3b5* expressions resulted as good candidates to control fat accumulation in the liver. Our study also shows that in moderate dietary control of hepatic steatosis with a Mediterranean diet with low cholesterol content and enriched in 10% (wt/wt) olive oil, *Hsd3b5* and *Tff3* gene expression interestingly revealed the existence of responders and nonresponders to the olive oil diet, and only hepatic *Fsp27* and *Syt1* expressions were good candidate genes. Using this biological procedure of selection, we have shown that expression of these genes is highly associated with hepatic steatosis in two common dietary situations, such as Western diets containing CLA isomers or a Mediterranean-type diet and in a genetic disease such as *Cbs* deficiency. In addition, using this scheme of selection in different experimental approaches, we have identified several genes with variable involvement in the same phenotypic outcome, hepatic steatosis, which raises the notion of several pathways conveying into a final result. This fact may have potential clinical implications: not all fat accumulated in liver might have the same origin and consequences. In this sense, we have recently demonstrated, using a proteomic approach, that animals with fatty liver consuming Western-type diets with extra virgin olive oil develop less aortic atherosclerosis than those consuming diets containing palm oil (5), and likewise, fatty liver of *Cbs* deficiency is not translated into atherosclerosis (54). However, the opposite situation of hepatic fat liver content in close association with atherosclerosis has also been found by our group and others (3, 4, 8, 23). Our work provides a new arena in which to address future work, both in mice liver as well as in other animal models, and may provide clues to solve the urgent dilemma of transplanting or not a fatty liver in light of the scientific evidence, particularly these days when liver donors are lacking and, to complicate more things, fatty liver prevalence in Western societies is soaring.

The changes of expression of *Fsp27* (13), traditionally considered a transcription factor, and *Syt1,* a protein involved in vesicular transport (46), indicate that new processes seem to be involved in lipid handling in hepatic cells. Recently, Fsp27 has been found as a positive factor responsible for hepatic steatosis in leptin-deficient mice (29), and a role for it in storage of triglycerides by binding lipid droplet (26) and regulating its size has been proposed (33). These results confirm the value of our strategy in delimiting genes involved in hepatic steatosis. Furthermore, our results provide the first evaluation of protein levels of FSP27, suggesting that its expression is highly abundant in liver compared with adipose tissue. Besides, its molecular mass in liver suggests a posttranslational modification. In the Western blot displaying the overexpression of FSP27 fusion protein by Matsusue et al. (29), a faint band was observed at the molecular size >50 kDa. This fact and our results suggest that the posttranslational modification may be a saturable process and that in physiological conditions the protein form >50 kDa is only present in liver. In addition, protein changes paralleled those observed at the mRNA level in the experiment using LA isomers. Overall, these results provide an important role and new avenues to explore the regulation of this protein in hepatic steatosis.

The precise molecular mechanism by which *Syt1* acts is not known. It could be hypothesized that the decrease of a vesicular protein *Syt1* may hamper the release of VLDL particles. A general involvement of this protein in secretion processes besides neurons (46) may be drawn from the present work and the finding of expression of this molecule in kidney podocytes (39) and lactating mammary gland (GEO deposit GDS1805). Considering the robustness of the association of its changes in all studied models of hepatic steatosis, it is tempting to suggest that its hepatic levels are necessary to facilitate the adequate transport and exocytose of VLDL-containing vesicles. In addition, this challenging view has been confirmed with realistic nutritional interventions such as those presented.

As a whole, our results provide new evidences to advance in the knowledge of hepatic steatosis. Through microarray analysis, genes not previously associated with this entity have been found differentially expressed and significantly associated in three different experimental conditions. In this respect, our findings for the mouse hepatic steatosis will help in the understanding of complex human pathology and provide new proteins, like *Fsp27,* with new basic molecular mechanisms that have emerged during revision of this manuscript. At the same time, dietary prevention strategies such as the inclusion of c9,t11-CLA in Western diets or the use of Mediterranean diets with low cholesterol content are presented as good alternatives to control hepatic steatosis.

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