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Abstract: Oleanolic acid is a triterpene widely distributed throughout the plant kingdom and present in virgin olive oil at a concentration of 57 mg/kg. To test the hypotheses that its long-term administration could modify hepatic gene expression in several animal models and that this could be influenced by the presence of APOA1-containing high-density lipoproteins (HDL), diets including 0.01% oleanolic acid were provided to Apoe- and Apoa1-deficient mice and F344 rats. Hepatic transcriptome was analyzed in Apoe-deficient mice fed long-term semipurified Western diets differing in the oleanolic acid content. Gene expression changes, confirmed by RT-qPCR, were sought for their implication in hepatic steatosis. To establish the effect of oleanolic acid independently of diet and animal model, male rats were fed chow diet with or without oleanolic acid, and to test the influence of HDL, Apoa1-deficient mice consuming the latter diet were used. In Apoe-deficient mice, oleanolic acid intake increased hepatic area occupied by lipid droplets with no change in oxidative stress. Bmal1 and the other core component of the circadian clock, Clock, together with Elovl3, Tubb2a and Cldn1 expressions were significantly increased, while Amy2a5, Usp2, Per3 and Thrsp were significantly decreased in mice receiving the compound. Bmal1 and Cldn1 expressions were positively associated with lipid droplets. Increased Clock and Bmal1 expressions were also observed in rats, but not in Apoa1-deficient mice. The core liver clock components Clock-Bmal1 are a target of oleanolic acid in two animal models independently of the diets provided, and this compound requires APOA1-HDL for its hepatic action

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Dietary oleanolic acid mediates circadian clock gene expression in liver independently of diet and animal model but requires apolipoprotein A1

Oleanolic acid and gene expression

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Abstract

Oleanolic acid is a triterpene widely distributed throughout the plant kingdom and present in virgin olive oil at a concentration of 57 mg/kg. To test the hypotheses that its long-term administration could modify hepatic gene expression in several animal models and that this could be influenced by the presence of APOA1-containing high-density lipoproteins (HDL), diets including 0.01% oleanolic acid were provided to *Apoe*- and *Apoa1*-deficient mice and F344 rats. Hepatic transcriptome was analyzed in *Apoe*-deficient mice fed long-term semipurified Western diets differing in the oleanolic acid content. Gene expression changes, confirmed by RT-qPCR, were sought for their implication in hepatic steatosis. To establish the effect of oleanolic acid independently of diet and animal model, male rats were fed chow diet with or without oleanolic acid, and to test the influence of HDL, *Apoa1*-deficient mice consuming the latter diet were used. In *Apoe*-deficient mice, oleanolic acid intake increased hepatic area occupied by lipid droplets with no change in oxidative stress. *Bmal1* and the other core component of the circadian clock, *Clock*, together with *Elovl3, Tubb2a* and *Cldn1* expressions were significantly increased, while *Amy2a5, Usp2, Per3* and *Thrsp* were significantly decreased in mice receiving the compound. *Bmal1* and *Cldn1* expressions were positively associated with lipid droplets. Increased *Clock* and *Bmal1* expressions were also observed in rats, but not in *Apoa1*-deficient mice. The core liver clock components *Clock-Bmal1* are a target of oleanolic acid in two animal models independently of the diets provided, and this compound requires APOA1-HDL for its hepatic action.

Keywords: apolipoprotein E-deficient mice, olive oil, oleanolic acid, *Clock, Bmal1*

1. Introduction

Oleanolic acid (OA) is a triterpene that is widely distributed throughout the plant kingdom [1] and present in the olive fruit at a concentration of 420±20 mg/kg [2]. The amount in olive oil depends on oil quality and fruit variety, its concentration being approximately 57.2±7.4 mg/kg in extra virgin olive oil, and higher in olive pomace oil [2]. Due to its thermal stability, it has been proposed to be an important contributor to the biological action of virgin olive oil [3]. In fact, hepatic gene expression changes were observed in mice consuming an unsaponifiable fraction-enriched olive oil with a high triterpene content [4], suggesting that oleanolic acid could be responsible for the changes. Indeed, several biological properties have been attributed to OA in different experimental settings: as an *in vitro* anti-inflammatory [5], modulating inflammatory processes in vascular cells [6], inhibiting the production of pro-inflammatory cytokines by human peripheral blood mononuclear cells [7] and inducing prostaglandin I_2 release by human coronary smooth muscle cells in a cyclooxygenase-2 dependent manner [8]; as an antioxidant, protecting against lipid peroxidation [9] [10] [11], suppressing superoxide anion generation [12], and reducing hydrogen peroxide induced cell apoptotic death of vascular smooth muscle cells [13]; and as a vasorelaxant in rat aorta [14]; and has been found to have antitumor [15, 16] [17], anti-diabetogenic [18-21], anti-HIV [22, 23], and anti-hyperlipidemic [24] [25] activities. Moreover, previous studies have reported hepatoprotective effects of OA [26, 27] [28, 29] with short-term administration in mouse (23 mg/kg body weight of OA for 4 days) [30] and in rat (20 to 60 mg/kg body weight for 7 days) [24].

The long-term administration was assessed in rats consuming a high-fat diet containing 50 mg/kg body weight of OA for 4 weeks [31], and a microarray analysis in liver revealed reduced expression of lipogenic genes. The animals consuming this

amount of OA showed less body weight gain and a significant reduction of liver weight. Lower food consumption and body weight were also observed when higher doses (100 mg/kg body weight/day for 8 weeks) were used in *Apoe*-deficient mice [32]. Therefore, these differences in body weight could mask the OA effect on hepatic gene expression and an administration regimen controlling food intake and maintaining body weight is crucial to unambiguously establish the OA properties in liver. In addition, the steatosis-prone liver of *Apoe*-deficient mice offers an excellent model for testing the properties of this compound since, as mentioned above, an unsaponifiable fraction-enriched olive oil with high triterpene content alleviated this ailment [4]. Thus, the aim of this study was to explore the effects of OA, in the absence of body weight changes, on hepatic gene expression, determined by microarray analysis and then confirmed by RT-qPCR, and fat content in *Apoe*-deficient mice fed a semipurified Western diet containing 0.01% of OA. To investigate the effect independently of diet and animal model, observed transcriptional changes were also studied in rats consuming a semipurified chow diet with and without OA. Since OA is a lipophilic compound that could be carried in lipoproteins, the hepatic changes were also tested in *Apoa1*-deficient mice that lack APOA1-containing high-density lipoproteins. Incubation of HepG2 cells in OA was also performed to investigate the direct action of this compound on gene expression. The results show an important role for OA in liver clock-controlled gene expression under all the conditions tested.

2. Material and methods

2.1. Animals:

2.1.1. Apoe-deficient mice

The experimental animals were 17 two-month-old male homozygous *Apoe*-deficient mice with a C57BL/6J genetic background, obtained from Charles River (Charles River Laboratories, Barcelona, Spain) and later bred in the *Servicio de Biomedicina y Biomateriales*, University of Zaragoza.

2.1.2. Rats

In this case, the animals were 20 two-month-old male F344 rats, obtained from the University of Wisconsin-Madison and later bred in the *Servicio de Experimentación Animal*, University of Zaragoza.

2.1.3. Apoa1-deficient mice

The experimental animals were 13 two-month-old male homozygous *Apoa1*-deficient mice with a C57BL/6J genetic background, obtained from the University of North Carolina and later bred in the *Unidad Mixta de Investigación*, University of Zaragoza.

To establish groups of animals of each type with similar baseline plasma cholesterol, blood samples were taken (after a four-hour fast) from the facial vein in mice and from the tail vein after overnight fasting in rats. All animals were housed in sterile filter-top cages in rooms maintained under a 12-h light/12-h dark cycle and had *ad libitum* access to food and water. The study protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza.

129 *2.2. Diets*

130 *2.2.1. Apoe-deficient mice*

131 Two study groups were established: a) one group (n=8) received a semipurified
132 Western diet [33] containing 0.15% cholesterol and 20% refined palm oil (Gustav
133 Heess, S.L., Barcelona, Spain) [34]; and b) the other group (n=9) received the same diet
134 but supplemented with 0.01% oleanolic acid (Extrasynthese, Genay, France), equivalent
135 to a dose of 10 mg/ kg mouse assuming a daily intake of 3 g per mouse.

136

137 *2.2.2. Rats*

138 Two study groups were established: ten male rats receiving a semipurified chow
139 diet, and ten male rats receiving the same chow diet containing 0.01% oleanolic acid
140 (Oskar Tropitzsch, Marktredwitz, Germany) for 11 weeks.

141

142 *2.2.3. Apoa1-deficient mice*

143 Two study groups were established: a) one group (n=6) received a semipurified
144 chow diet; and b) the other group (n=7) received the same chow diet supplemented with
145 0.01% oleanolic acid (Oskar Tropitzsch).

146 All diets were prepared weekly and kept under N_2 atmosphere at -20ºC. Fresh
147 food was provided daily. The animals were fed the experimental diets for 11 weeks.
148 Diets were well tolerated.

149

150 *2.3. HepG2 cell culture*

151 The human hepatocyte cell line HepG2 from passage 5 was grown in a
152 humidified atmosphere of 5% CO_2 at 37ºC in Dulbecco's modified Eagle's minimum
153 essential medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 10%

foetal bovine serum (FBS), 1% non essential amino acids, 1% penicillin (1000 U/ml), 1% streptomycin (1000 mg/ml), 4 mM L-glutamine and 1 mM sodium pyruvate in a 24 multiwell plate (in triplicate). Medium was changed every two days, and after one week of growth, this medium was removed, and cells were washed with phosphate buffered saline (PBS) prior to the addition of the serum-free media supplemented with 0.1% DMSO or 20 µM OA dissolved in DMSO. After a 12-h incubation, media were removed and cells were collected with Tri-reagent solution (Ambion, Austin, TX, USA). RNA isolation was performed and cDNA synthesis achieved as described in 2.4 and 2.5, respectively.

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

At sacrifice, the livers were immediately removed and frozen in liquid nitrogen. RNA from each liver was isolated using Tri-reagent (Ambion, Austin, TX, USA). 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 integrity of the 28S and 18S ribosomal RNAs was verified by agarose gel electrophoresis [35].

Five-µg aliquots of total RNA from each *Apoe*-deficient mouse of each group (n=8 for the control group and n=9 for the OA group) were pooled and purified using the *RNeasy Micro kit* (Qiagen, Barcelona, Spain). 200 ng of each RNA pool were used for biotin labeling with the Affymetrix *GeneChip 3' IVT Express* kit. Hybridization, washing, scanning, and analysis with the Affymetrix GeneChip Murine Genome MOE430A array (Affymetrix, Santa Clara, CA) were performed according to the standard Affymetrix protocols at Progenika Biopharma (Derio, Spain). Fluorometric

177 data were generated by Affymetrix software, and the fluorometric signal adjusted so
178 that all the probe sets provided intensities within a manageable range. Transcripts with
179 signal intensities that were lower than the limit of detection, estimated as background
180 matrix ±3 standard deviations, were not taken into account. The data obtained in the
181 microarray hybridizations were processed with Microarray Suite 5.0 (Affymetrix)
182 software. The identification of genes that were up- or down-regulated by OA was
183 performed by comparing gene expressions in the livers of animals from the two groups
184 (significance set at $P<0.01$). Of these, we selected only those whose signal $\log_2$ ratio
185 was higher than 1.5 (up-regulated genes) or lower than –1.5 (down-regulated genes).
186 The signal $\log_2$ ratio is recommended by Affymetrix software and several authors [36]
187 because of the linear response observed in contrast to fold change. The complete
188 datasets were deposited in the GEO database (accession number GSE43589).

189 *2.5. Quantification of mRNA*

190 The differences in mRNA expression observed with the microarrays were
191 confirmed by quantitative real-time RT-qPCR analysis of individual samples. Equal
192 amounts of DNA-free RNA from each sample of each animal were used in these
193 analyses. First-strand cDNA synthesis was achieved using the SuperScript™ II RT kit
194 (Invitrogen, Madrid, Spain) for *Apoe*-deficient mouse and First Strand synthesis kit
195 (Fermentas) for rat and *Apoa1*-deficient mouse cDNA synthesis. RT-qPCR reactions
196 were performed using the Sybr Green PCR Master Mix (Applied Biosystems). The
197 primers were designed using Primer Express® (Applied Biosystems, Foster City, CA)
198 and checked by BLAST analysis (NCBI) to verify gene specificity as well as to achieve
199 amplification of the cDNA but not of genomic DNA. The sequences are shown in
200 supplementary Table 1. Real time RT-qPCR reactions were performed in an ABI

PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and normalized to the reference cyclophilin B (*Ppib*) mRNA expression. The core OA-induced hepatic transcriptomic change network was constructed using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com, Ingenuity Systems, USA) based on the nine hepatic genes confirmed to be OA-responsive genes by RT-qPCR. This analysis allowed the identification of a network and of pathway interactions between genes based on an extensive manually curated database of published gene interactions.

2.6. Histological analysis of livers

Aliquots of liver were stored in neutral formaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and observed with 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 [37].

2.7. Liver homogenate preparation

Liver was homogenized in homogenization buffer (PBS with protease inhibitor cocktail), and used to estimate protein concentration and reactive oxygen species (ROS). Protein concentration was determined by the BioRad dye binding assay (BioRad, Madrid, Spain).

2.8. Determination of reactive oxygen species

The presence of ROS was estimated by the 2', 7'-dichlorofluorescein diacetate

(DCF) assay where liver homogenates (7 µg of protein) were incubated, at 37 ºC, with 50 µM DCF in PBS in a total volume of 50 µl and in presence of 8.3 µl of 0.12% sodium azide. Fluorescence was measured at 485 nm excitation and 535 nm emission after 3-hour incubation for *Apoe*-deficient mice or 24 h for rats.

2.9. Hepatic lipid extraction

Lipids were extracted according to the Folch method [38], and dissolved in 100 µl of isopropanol. Cholesterol and triglycerides were measured by colorimetric assay with Infinity kits (Thermo Scientific).

2.10. Extraction and analysis of oleanolic acid in rat liver

A piece of liver (200 mg) was homogenized in 200 µl of distilled water and 2 ml of diethyl ether, and centrifuged. The pellet was extracted four times, and the supernatants were combined and evaporated [39]. The dry residue was silanized using N,O-bis-(trimethylsilyl)trifluoroacetamide for 30 min at 90ºC. Derivatized samples were injected into a FinniganTrace-GC2000 gas chromatograph (GC) coupled to a Polaris-Q Ion-Trap mass spectrometer (ThermoFinnigan, Austin, TX, USA) equipped with an AS2000 autosampler, operating in full scan mode from *m/z* 50 to 800 Da at 1 scan/sec. The column used was a Zebron ZB-5 MS (Phenomenex, Torrance, CA, USA) fused silica capillary column (30 m length x 0.25 mm i.d x 0.25 µm film thickness). The GC conditions included helium as carrier gas at 1.0 mL min^{-1} in constant flow mode; the initial temperature of 105ºC was increased to 300ºC at a rate of 8 ºC/min^{-1} and maintained at the final temperature for 40 min. The split injection mode was used with an injection volume of 2 µL. Injector temperature was 300ºC. The mass spectrometry operating conditions were as follows: ion source and transfer line temperatures were

200 and 300ºC, respectively. The electron energy was 70 eV and the emission current 250 µA. Xcalibur version 1.4 software was used for data acquisition and processing of the results and Mass Frontier^{TM} version 4.0 software was used to assist in the interpretation of mass spectra. The standard used for oleanolic acid had an elution time of 37.9 min. The identification was carried out by mass spectrometry observing the fragmentation pattern of oleanolic acid as a trimethylsilyl-O (TMSIO) derivative and the registered mass spectra (Supplementary Fig 1 and Fig 2).

2.11. Statistical Analysis

Microarray results were analyzed using a Microarray Suite 5.0 algorithm (Affymetrix) in Affymetrix GeneChip Operating Software (GCOS) version 1.4. Significance was set at $P<0.01$. For non-microarray results, differences between pairs were analyzed by Mann-Whitney U-test using Instat 3.02 software for Windows (GraphPad, S. Diego, CA, USA). Results are expressed as mean ± SD. Differences were considered significant when $P<0.05$. Correlations between variables were tested by calculating the Pearson or Spearman correlation coefficient.

3. Results

3.1. Somatometric analyses

Long-term administration of OA-supplemented diet had no effect on body weight in *Apoe*-deficient mice (26.7 ± 1.4 and 26.6 ± 3.3 g for control and OA groups, respectively). In these animals, liver weight did not experience any change (1 ± 0.1 and 1 ± 0.2 g for control and OA groups). Similar results were obtained in rats and *Apoa1*-deficient mice (data not shown).

3.2. Effect of OA on gene expression in livers of Apoe-deficient mice

To determine the changes in hepatic gene expression induced by OA, the expression of 22,626 transcripts represented on the Affymetrix MOE430A GeneChip Murine Genome array was quantified in pooled liver samples from 8 animals that received the Western control diet and another 9 that received the same OA-enriched diet. The livers of control animals expressed 13,634 transcripts, while those of the OA animals expressed 13,786 (identified as "present" by Affymetrix software). Using the Mann-Whitney ranking feature of the Affymetrix software to determine significant differences in gene expression ($P<0.01$), and considering as compromised a limit of detection of variations in expressions lower than background matrix + 3 SD, we identified an increased expression of 513 sequences and a reduced expression of 774 sequences in samples from the animals on the control diet compared to those on the OA-supplemented diet. To select the most relevant examples, only differentially regulated genes with a signal $\log_2$ ratio higher than 1.5 (for those that are up-regulated) or lower than -1.5 (for those that are down-regulated) were taken into account. Table 1 lists the genes whose mRNAs reflected these expressions. Seven genes fulfilled the criterion for an increased expression in response to the OA content in the treatment diet.

One of these genes coded for circadian rhythm (*Bmal1*), two for lipid metabolism (*Elovl3, Chk*a), one for electron transport (*Cyp2b9*) and 3 for proteins with miscellaneous functions: the first was involved in the cell cycle (*Gadd45*), the second was involved in tight junctions (*Cldn1*) and the third was a cytoskeleton component (*Tubb2a*). Eight genes met the criterion for a reduced expression in response to the presence of OA in the diet (Table 1). Of these, 3 coded for circadian rhythm (*Per2, Per3, Nocturnin*), 1 for lipid metabolism (*Thrsp*) and 4 for proteins with miscellaneous functions (*Amy2a5, Usp2, Nocturnin* and *Igk-V28*).

To validate the results obtained with the microarray, the expressions of these fifteen genes *Bmal1, Cyp2b9, Elovl3, Tubb2a, Cldn1, Chka, Gadd45, Amy2a5, Usp2, Per3, Nrg4, Thrsp, Per2, Nocturnin* and *Igk-V28* that were up- or down-regulated (signal $\log_2$ ratio > 1.5 or < -1.5) were individually studied by specific RT-qPCR assays using *cyclophilin B* to normalize the results. The expressions of four out of the seven up-regulated genes selected –*Bmal1, Elovl3, Tubb2a* and *Cldn1*– were significantly increased by the presence of OA in the diet (Fig 1). The expressions of four of the eight down-regulated genes selected -*Amy2a5, Usp2, Per3* and *Thrsp*- were significantly decreased in mice receiving the OA-enriched diet. Considering that *Bmal1* and *Per3* are core circadian controllers in liver, and *Usp2, Thrsp,* and *Elovl3* expressions are known to be under circadian control, OA may modify the expression of circadian genes in liver. To reinforce this, the expression of another member of the circadian clock, *Clock,* was assayed and found to be significantly increased (Fig 1). Overall, OA is an important modifier of the expression of circadian genes in *Apoe*-deficient mice.

Figure 2A shows the correlation between the values of signal $\log_2$ ratio for the above-mentioned 15 genes according to the microarray assay performed with pooled *Apoe*-deficient mouse samples (Table 1), and the mean for each group obtained after the

analysis of samples from each animal by RT-qPCR in both experimental groups (Fig 1 and supplementary Table 2). The core gene of the liver circadian rhythm, *Clock,* was also included in the analysis. Good agreement between these procedures was obtained ($r= 0.877$, $P<0.0001$) and all samples were correctly classified, although the two methods differed in terms of the magnitude of the response. To evaluate the accuracy of setting a cut-off point at a signal $\log_2$ ratio 1.5 in microarray analysis, the value of the signal $\log_2$ ratio obtained in RT-qPCR analysis using individual samples was plotted against the probability value obtained for comparison of individual expressions for each gene (Fig 2B). A significant inverse relationship was observed that fitted a logarithmic equation where a signal $\log_2$ ratio of 1.51 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 despite the limitation of no information on biological variability. In addition, the high biological variation of mRNA indicates that a certain threshold of change to identify significant differences produced by dietary components is required, and the established $\log_2$ ratio of 1.5 is adequate.

3.3. Effect of OA on gene expression in rat livers

To verify whether the mRNA changes in response to OA were independent of the presence of apolipoprotein E and intake of a Western diet, rats were fed semipurified chow diets enriched in this compound and hepatic transcripts were assayed. Of the nine genes confirmed to be significantly modified in *Apoe*-deficient mice (Fig 1), only *Bmal1* and *Clock* mRNA expressions were significantly increased in rats consuming OA, and that of *Amy2a5* was significantly decreased (Table 2).

3.4. Gene expression in livers of Apoa1-deficient mice

The effect of OA on the mRNA changes was also tested in mice lacking *Apoa1* as a genetic model of absence of HDL and little possibility of delivering the hydrophobic molecule to the liver (Table 3). Interestingly, these mice showed no significant change for the nine selected genes. Collectively, these results suggest that the previous changes in expressions were a response to OA administration.

3.5. Hepatic parameters

The *Apoe*-deficient mouse is a model of spontaneous development of hepatic steatosis. To test the influence of OA on this ailment, histological analysis of liver was carried out. As shown in Fig 3, animals receiving the OA-supplemented diet had a significantly higher percentage of hepatic fat, assayed as surface of tissue occupied by lipid droplets. Chemical analysis of hepatic triglycerides showed no significant change (27 ± 8 and 29 ± 16 mg/g of liver tissue for control and OA group, respectively). A similar finding was observed for hepatic cholesterol content (7 ± 1 and 6 ± 1 mg/g of liver tissue for control and OA group, respectively). To verify whether the increase in lipid droplets on the liver surface was modifying oxidative stress, hepatic levels of ROS were determined. No variation in hepatic ROS levels was observed in *Apoe*-deficient mice that received OA (651 ± 134 and 742 ± 284 arbitrary fluorescence units for the two groups)

3.6. Association between mRNA expression changes and hepatic fat accumulation in Apoe-deficient mice

To establish a possible relationship between lipid droplet changes and gene expression, a correlation analysis was carried out. As shown in Fig 4A and 4B, *Bmal1*

and *Cldn1* gene expressions were positively correlated with hepatic fat content. *Usp2* and *Per3* gene expression changes were inversely associated (Fig 4, panels C and D). These data suggest a potential involvement of these genes in control of lipid droplets.

In addition, when changes in mRNA expression of different genes were considered, an interesting network of association emerged in *Apoe*-deficient mice (Fig 5A). In this regard, *Bmal1* appears to be an expression hub, as its expression correlates with that of *Cldn1, Tubb2a, Usp2, Per3 and Thrsp.*

3.7. Ingenuity Pathway Analysis (IPA).

Performing an IPA, we found an association with the network functions of behavior, nervous system development and function, and nutritional disease. According to this observation, the molecular function and canonical network with the highest significance and involving the greatest number of genes is represented in Fig 5B, showing that dietary OA modifies the expression of genes implicated in circadian rhythm and weight loss. Furthermore, IPA analysis gives *Bmal1* a central role as the top upstream regulator of the transcriptomic changes that take place after OA intake.

3.8. Oleanolic acid is delivered to the liver and BMAL1 mRNA expression is a direct target of oleanolic acid.

Chemical analysis of liver from control and OA-treated rats showed that OA was unambiguously present in liver of animals receiving this compound and was absent in control rats (Fig 6A).

To investigate whether OA alone was able to induce *BMAL1* expression, HepG2 cells were incubated in presence of 20 µM OA. As shown in Fig 6B, a significant expression was observed following 12-hour incubation. This reinforces the notion of a

387 key role of *BMAL1* in the hepatic effect of OA.

4. Discussion

This nutrigenomic approach was aimed to determine the hepatic transcriptomic changes taking place after eleven weeks of OA supplementation in the diet. We found that in *Apoe*-deficient mouse liver, OA mainly influenced two groups of potentially interconnected gene clusters, circadian clock genes and genes for fatty acid metabolism and bioenergetics, a circumstance that may lead to a significant increase in total liver fat deposition, but is not accompanied by an increase in oxidative stress. The gene changes were also assessed in rats receiving a chow diet supplemented with OA. Under these conditions, OA supplementation also induced an important increase in *Clock* and *Bmal1* expression, a finding that suggests that core circadian liver genes are a target of OA under different experimental conditions. To test our hypothesis that OA could be transported in HDL particles, *Apoa1*-deficient mice, as a model of absence of HDL, also received the diet supplemented with OA. Indeed, no significant changes were observed for hepatic *Clock* and *Bmal1*, an observation that is consistent with the fact that APOA1-containing HDL may participate in delivering OA to the liver. Furthermore, OA was detected in liver of OA-treated rats and OA incubation also elicited the expression of *BMAL1* in HepG2 cells.

In the present study, the microarray data derived from *Apoe*-deficient mice was subjected to a restrictive procedure, selecting only the genes with the most marked changes in expression, according to our previous experience [4] [40] [41] [42], setting the cut point at a signal $\log_2$ ratio of ± 1.5. In fact, as shown in Fig 2B, the biological variation of mRNA when individually studied is such that only genes with a certain threshold for change (signal $\log_2$ ratio ± 1.5) would be candidates to experience a significant biological response to a dietary intervention. With this criterion, only 7 genes were found to be remarkably up-regulated and 8 notably down-regulated in *Apoe*-

deficient mice. In our experience, this number is quite common when using such a highly restrictive criterion and a single dietary component [40, 41]. With a highly restrictive criterion and a complex herbal mixture, Klein et al. reported 24 important changes in gene expression [43], and we found 54 using olive oil enriched with the unsaponifiable fraction of this oil [4]. Thus, the more the dietary components added, the higher the number genes involved.

These microarray-detected changes were individually confirmed by RT-qPCR, and good agreement was observed between the Affymetrix chip and RT-qPCR data (Fig. 1A). The nature and extent of transcript variation differs across tissues in a given individual or among individuals in part due to circadian rhythms, growth hormone signaling, immune response, androgen regulation, lipid metabolism, social stress, extracellular matrix or epigenetic programming. In particular, this variation observed between genetically identical mice can influence the experimental design and the interpretation of data [44], particularly in studies addressing immune response, stress, amine metabolism, cell growth, ubiquitination or hormonally regulated genes in liver [45, 46]. For these reasons, and despite many concerns raised, mRNA samples are often pooled in microarray experiments to reduce the cost and complexity of analysis of transcript profiling. Pooling RNA samples from different subjects onto a single microarray chip was found to be statistically valid and efficient for microarray experiments. Appropriate RNA pooling can provide equivalent power and improve efficiency and cost-effectiveness for microarray experiments, with a modest increase in total number of subjects, and correct for the technical difficulty in getting sufficient RNA from a single subject [47]. Pooling hepatic RNA samples reflected the expression pattern of individual samples, and properly constructed pools provided measures of transcription response nearly identical to those of the individual RNA sample [48].

Accordingly, of the 15 genes whose expression was found to be strongly modified in the microarray, only four out of the seven up-regulated genes included in the validation analysis –*Bmal1, Elovl3, Tubb2a, Cldn1*– appeared to be significantly increased and four of the eight down-regulated genes selected –*Amy2a5, Usp2, Per3, Thrsp*– were significantly decreased in *Apoe*-deficient mice receiving the OA-enriched diet when studied individually. The core circadian liver gene, *Clock*, that drives the expression of circadian-controlled genes together with *Bmal1*, was included in the analysis and found to be increased by OA in *Apoe*-deficient mice.

Circadian rhythms are 24h oscillations in behavior and physiology that have been found to exist not only in the suprachiasmatic nucleus, but also in peripheral tissues [49] [50] [51]. Enhancement of rhythmic transcription in peripheral tissues provides the basic drive to the system through two transcription factors, circadian locomotor output cycles kaput (CLOCK) protein [52] and brain and muscle Arnt-like protein-1 (BMAL1) [50] [53]. The CLOCK-BMAL1 heterodimer directly or indirectly activates the transcription of various clock-controlled genes [50] [54] [55] [56], including Period (*Per*) 1, *Per2* and Cryptochrome (*Cry*) 1, *Cry2*. PER and CRY proteins then translocate back into the nucleus, and inhibit the activity of *Clock-Bmal1*, forming a negative feedback loop. Circadian regulatory genes and first-order transcription factors play a role in governing lipid metabolism-related genes and transcription factors, in the case of *Bmal1/Clock* through regulation of circadian activation of potential PPAR response element controlled target genes [50], with CLOCK being directly associated with the circadian expression of *PPARα* in the mouse liver [57]. The importance of tissue-specific regulation of *Bmal1* is supported by the growing evidence of the role of this gene in obesity and its related symptoms, as it is highly expressed during adipogenesis [58]. *Bmal1*-deficient mice show a loss of circadian rhythms, decreased

body weight, infertility, progressive arthropathy, shortened life span [59], and impaired adipogenesis, adipocyte differentiation, and hepatic carbohydrate metabolism [60]. Our results indicate that increasing BMAL1 may regulate lipid droplet amount or enlargement, an aspect that requires further experimental support.

Circadian clocks, especially peripheral clocks, can be strongly entrained by daily feedings, but few papers have reported the effects of food components on circadian rhythm. Clock components and biochemical processes are species-specific. However, previous findings showing that caffeine lengthens circadian rhythms in species ranging from bacteria to insects and in mice, suggest that the target of caffeine is a universal mechanism that determines 24-h clocks in all life forms [61]. Caffeine lengthened circadian rhythm in cultured cell lines as well as in mouse liver explants [61]. In liver, caffeine supplementation led mainly to phase advance and change in expression level of clock genes [62]. Resveratrol was found to regulate circadian clock genes in Rat-1 fibroblasts [63]. Folate depletion was found to change circadian cycle gene expression in male mice [64]. Furthermore, R-α-lipoic acid up-regulates genes in the positive arm (*Bmal1* and *Npas2*, a functional homologue of the *Clock* gene) and down-regulates genes in the negative arm (*Per2, Per3, Nr1d2*) of the circadian core oscillators; thus, it may alter the rhythmicity of the central hepatic clock genes and attenuate expression of first-order clock transcription factors [65]. Contrary to the anti-hyperlipidemic effect of OA described in previous studies [24] [25] with similar or even higher OA content in the diet, the dose of OA used in our study is clearly a modulator of the clock system in liver and induced an increase in the percentage of the surface occupied by lipid droplets in *Apoe*-deficient mice, without changes in triglyceride or cholesterol contents or oxidative stress. Therefore, OA is an agent that modifies lipid distribution among cellular stores. This is the first study using a long-term administration, three months,

while previous studies lasted ~~for~~ no more than a week, and OA has been proved to be delivered to the liver. Interestingly, OA was found to act as a transcriptional modulator of circadian expression independently of diet and animal model and directly in cell culture.

In conclusion, through transcriptomic profiling, we have assessed the influence of OA on hepatic gene expression. Using a selection procedure previously validated by our group, those genes undergoing extreme changes have been confirmed and tested in two additional animal models. Independently of animal model and diet, OA also induced important increases in the expression of *Clock* and *Bmal1*, core circadian liver genes. Changes in *Bmal1* were associated with liver surface occupied by lipid droplets. These changes in gene expression were not observed in mice lacking APOA1, the main HDL apolipoprotein, a fact that suggests that these particles may be involved in delivering OA to the liver.

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No competing financial interests exist.

Abbreviations used: IPA, Ingenuity Pathway Analysis; OA, oleanolic acid; PCR, polymerase chain reaction; RT, reverse transcriptase; BSTFA, N,O-bis-(trimethylsilyl)trifluoroacetamide

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Fig 1. Effect of oleanolic acid on hepatic gene expression in male *Apoe*-deficient mice. Individual data represent arbitrary units normalized to the *cyclophilin B* expression in control and treated mice according to the RT-qPCR assay for the genes significantly up- or down-regulated in *Apoe*-deficient liver by OA administration, plus *Clock* gene. Statistical analysis was carried out by Mann-Whitney U-test. **P<0.01 and *P<0.05.

Fig 2. **Quality and biological meaning of microarray data in *Apoe*-deficient mice.** (A) Correlation analysis between microarray and RT-qPCR data. The expression of 15 genes –*Bmal1, Cyp2b9, Elovl3, Tubb2a, Cldn1, Chka, Gadd45, Amy2a5, Usp2, Per3, Nrg4, Thrsp, Per2, Nocturnin and Igk-V28*– was individually studied by RT-qPCR and normalized to the invariant *cyclophilin B* gene. The mean values obtained for signal $\log_2$ ratio from individual analyses (Fig 1) were plotted against the microarray values based on pooled samples (Table 1). The agreement between the procedures was good (r= 0.877, P<0.0001). (B) Correlation analysis between signal $\log_2$ ratio of RT-qPCR data and *P* values obtained in individual comparisons using nonparametric Mann-Whitney U test. Signal $\log_2$ ratios are taken as absolute values. SL_2R, signal $\log_2$ ratio; RT-qPCR, real-time quantitative polymerase chain reaction.

Fig 3. Liver histology and hepatic fat content. Representative liver micrographs at x400 magnification from control (A) and oleanolic (B) *Apoe*-deficient male mice. Bar denotes 20 μm. Morphometric changes in hepatic fat content (C) in *Apoe*-deficient mice consuming the different diets, quantified with Adobe Photoshop 7.0 and expressed as percentage of area of total liver section. Data are expressed as mean ± SD for each group. Statistical analyses were done with the Mann-Whitney test. *P<0.003.

Fig 4. Relationship between hepatic gene expression and hepatic fat content in *Apoe*-deficent mice. A, B: direct correlations between hepatic fat content and *Bmal1* and *Cldn1* gene expressions. C, D: inverse correlations between hepatic fat content and *Usp2* and *Per3* gene expressions. Statistical analysis was carried out using the Pearson test for parametric distributions (A) and the Spearman test for nonparametric distributions (B, C, D).

Fig 5. Relationship among hepatic gene expressions in *Apoe*-deficient mice. A. Significant direct correlations (*Bmal1* versus *Tubb2a* and *Cldn1*) and significant inverse correlations (*Bmal1* versus *Usp2, Per3* and *Thrsp*) according to the Spearman test. B. Network of genes showing statistical significance in *Apoe*-deficient mice according to Ingenuity Pathway Analysis. Fx represents the main molecular and canonical pathways involved.

Fig 6. Detection and direct action of oleanolic acid (OA) in liver. A. Gas chromatograms of OA standard and liver extracts from control and OA-treated rats. B. Expression of *BMAL1* in a human hepatocyte cell line. HepG2 cells were incubated in presence of 0.1% DMSO (control) and 20 µM oleanolic acid dissolved in 0.1% DMSO for 12 hours. The experiment was performed in triplicate, with n=6 in each experiment for control and treated animals. *BMAL1* expression was quantified relative to *cyclophilin B* according to $2^{-\Delta\Delta Ct}$ method by RT-qPCR. Results are expressed as mean and standard deviation, and statistical analysis was done according to the Mann-Whitney test. **P<0.001. TMSIO, N,O-bis-(trimethylsilyl)-O-derivative.

TABLE 1. Hepatic genes differentially regulated by the administration of oleanolic acid at the level of signal log_2 ratio >1.5 or <-1.5 in male *Apoe*-deficient mice

Biological process	GenBank	Affymetrix ID	Name	Gene symbol	Control	Oleanolic acid	Signal log_2 ratio
Up-regulated genes							
Circadian rhythm	BC011080	1449479_at	Aryl hc-receptor nuclear translocator-like	*Bmal1*	25	92	2.2
Electron transport	NM_010000	1419590_at	cytochrome P450, 2b9	*Cyp2b9*	57	200	2.3
Fatty acid metabolism	BC016468	1432466_a_at	Elongase of very long chain fatty acids-like	*Elovl3*	59	3126	1.8
Cytoskeleton component	BC003475	1422257_s_at	Tubulin, beta 2A	*Tubb2a*	377	1355	1.8
Tight junction	NM_016674	1450014_at	Claudin 1	*Cldn1*	33	97	1.5
Lipid biosynthesis	NM_013490	1450264_a_a	Choline kinase alpha	*Chka*	106	259	1.5
Cell cycle	AK010420	1450971_at	Growth arrest, DNA-damage-inducible 45 b	*Gadd45*	187	614	1.5
Down-regulated genes							
Glucidic metabolism	NM_009669	1417168_a_at	Amylase 2a5, pancreatic	*Amy2a5*	680	111	-2.4
Ubiquitin catabolic process	NM_016808	1421087_at	Ubiquitin specific peptidase 2	*Usp2*	880	239	-1.9
Circadian rhythm	BB757992	1421681_at	Period homolog 3	*Per3*	293	82	-1.9
Growth factor	NM_032002	1422973_a_at	Neuregulin 4	*Nrg4*	132	33	-1.9
Fatty acid metabolism	NM_009381	1417602_at	Thyroid hormone responsive (Spot14)	*Thrsp*	1653	476	-1.9
Circadian rhythm	NM_011066	1425837_a_at	Period homolog 2	*Per2*	202	75	-1.7
Circadian rhythm	AF199491	1427455_x_at	Nocturnin/carbon catabolite repression 4l	*Nocturnin*	815	266	-1.6
Immune response	BI107286	1416055_at	Similar to Chain L, Structural Basis	*Igk-V28*	578	213	-1.6

Data represent intensity of signal for control and treated mice with the Affymetrix chip.

TABLE 2. Effect of oleanolic acid on hepatic gene expression in male rats

	Control (n=10)	Oleanolic acid (n=10)	Fold change	Signal log_2 ratio
Up-regulated genes				
Bmal1	2.3 ± 2.3	10.3 ± 12[a]	4.5	2.6
Elovl3	4.8 ± 8.5	10.0 ± 11.5	2.1	1.8
Tubb2a	4.8 ± 10.8	1.2 ± 1.0	0.3	-0.2
Cldn1	3.1 ± 6.0	1.0 ± 1.2	0.3	-0.6
Clock	1.1 ± 0.5	2.4 ± 1.6[b]	2.2	1
Down-regulated genes				
Amy2a5	1.9 ± 1.9	0.2 ± 0.2[a]	0.1	-3.3
Usp2	3.1 ± 4.3	3.1 ± 2.9	1	0.9
Per3	4.1 ± 6.9	3.1 ± 5.0	0.7	-1.8
Thrsp	1.1 ± 0.4	2.0 ± 1.8	1.8	0.6

Data (mean ± SD) represent arbitrary units normalized to the *cyclophilin B* expression for control and treated rats according to ~~the~~ RT-qPCR. Statistical analysis was carried out by Mann-Whitney-U test. a, $P<0.01$ and b, $P<0.05$.

TABLE 3. Effect of oleanolic acid on hepatic gene expression in male *Apoa1*-deficient mice

	Control (n= 6)	Oleanolic acid (n= 7)	Fold change	Signal log_2 ratio
Genes				
Bmal1	1.8 ± 1.6	0.9 ± 1.0	0.5	-1.1
Elovl3	1.9 ± 2.0	2.4 ± 1.5	1.3	0.8
Tubb2a	1.2 ± 0.7	0.9 ± 1.1	0.7	-1.7
Cldn1	1 ± 0.3	0.9 ± 0.4	0.9	-0.2
Clock	1.5 ± 1.0	1.0 ± 0.6	0.7	-0.4
Amy2a5	1.7 ± 1.8	5.3 ± 5.0	3.1	1.5
Usp2	1.5 ± 1.3	1.4 ± 1.1	0.9	-0.3
Per3	1 ± 1.9	1.2 ± 0.4	1.2	0.3
Thrsp	1 ± 3.4	1.3 ± 0.5	1.3	0.4

Data (mean ± SD) represent arbitrary units normalized to the *cyclophilin B* expression for control and treated mice according to ~~the~~ RT-qPCR. Statistical analysis was carried out by Mann-Whitney-U test.

Figure 1

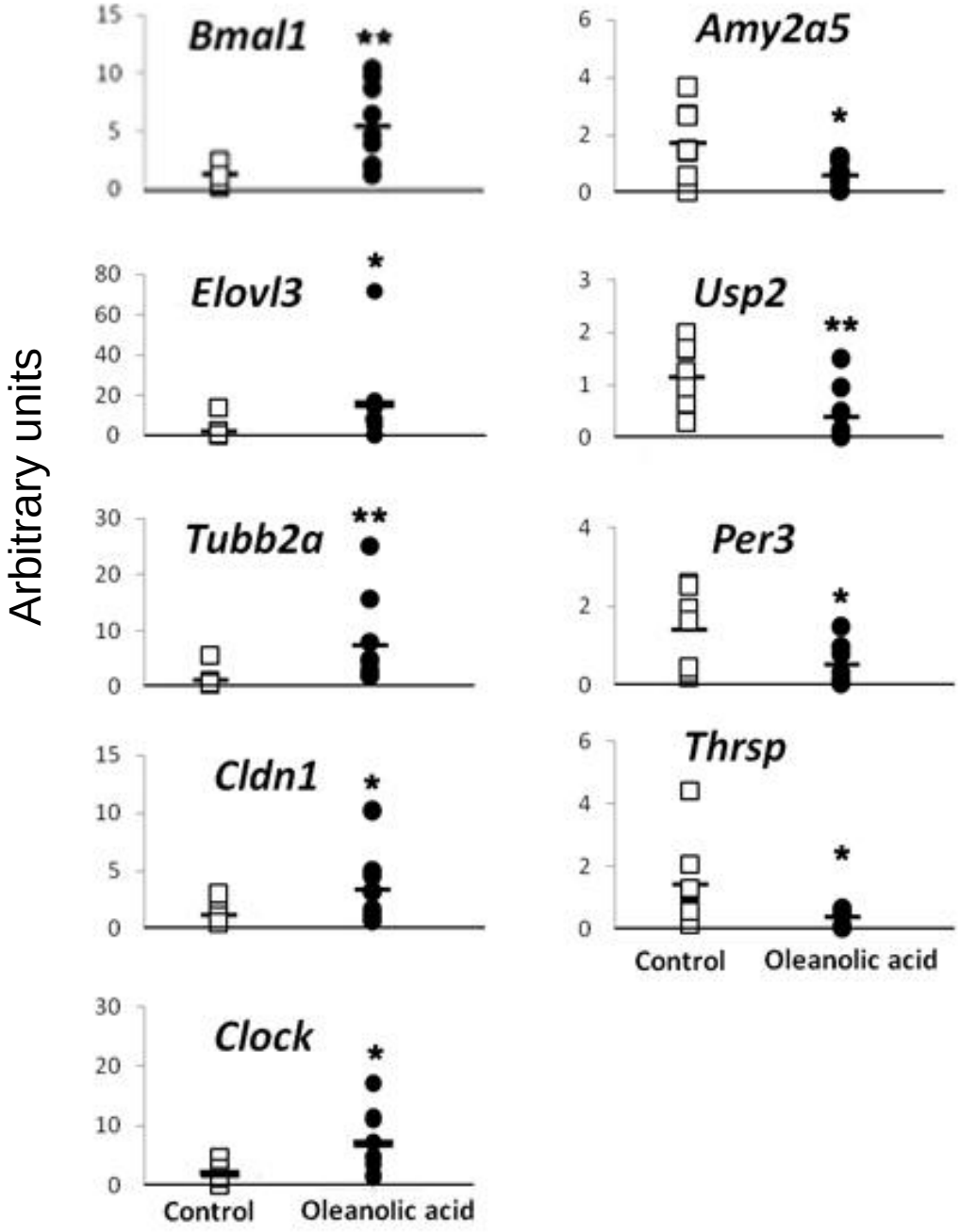
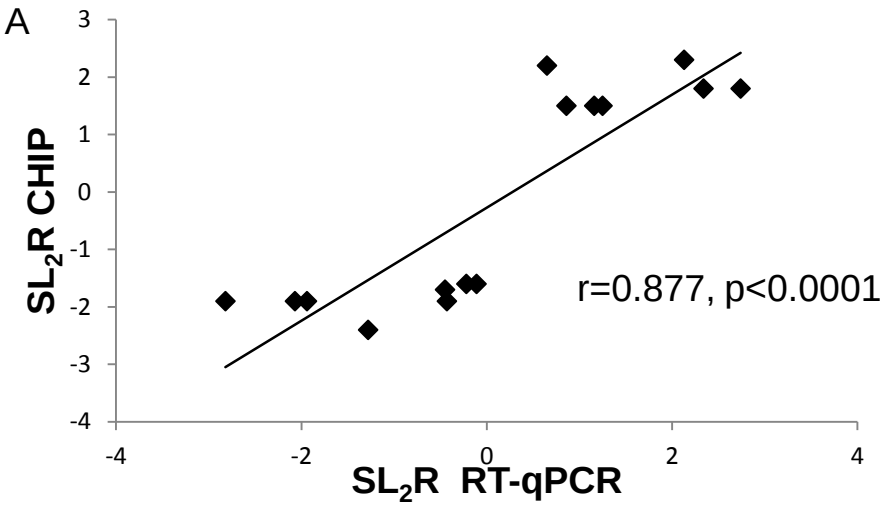

Arbitrary units
Bmal1
**
15
10
5
0
Amy2a5
*
6
4
2
0
Elovl3
*
80
60
40
20
0
Usp2
**
3
2
1
0
Tubb2a
**
30
20
10
0
Per3
*
4
2
0
Cldn1
*
15
10
5
0
Thrsp
*
6
4
2
0
Clock
*
30
20
10
0
Control
Oleanolic acid
Control
Oleanolic acid

Figure 2


A
SL2R CHIP
SL2R RT-qPCR
r=0.877, p<0.0001

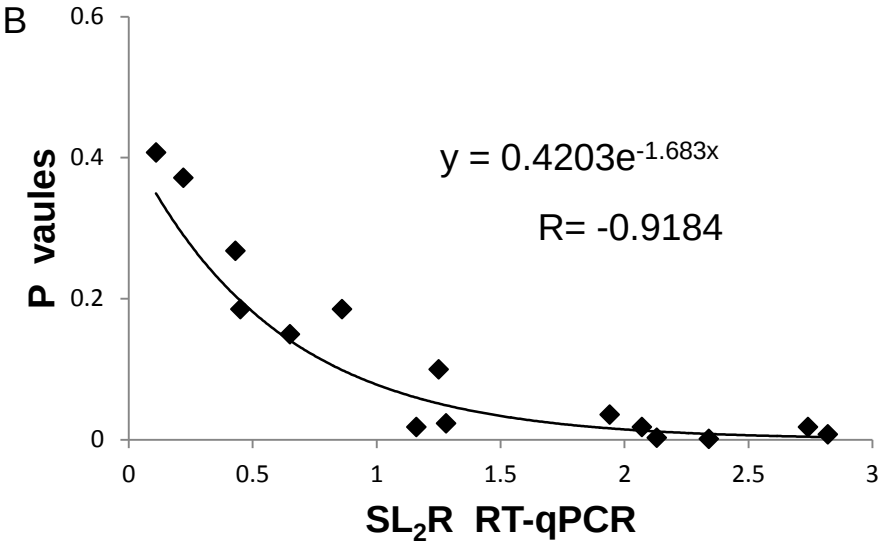

B
P vaules
SL2R RT-qPCR
y = 0.4203e-1.683x
R= -0.9184

Figure 3

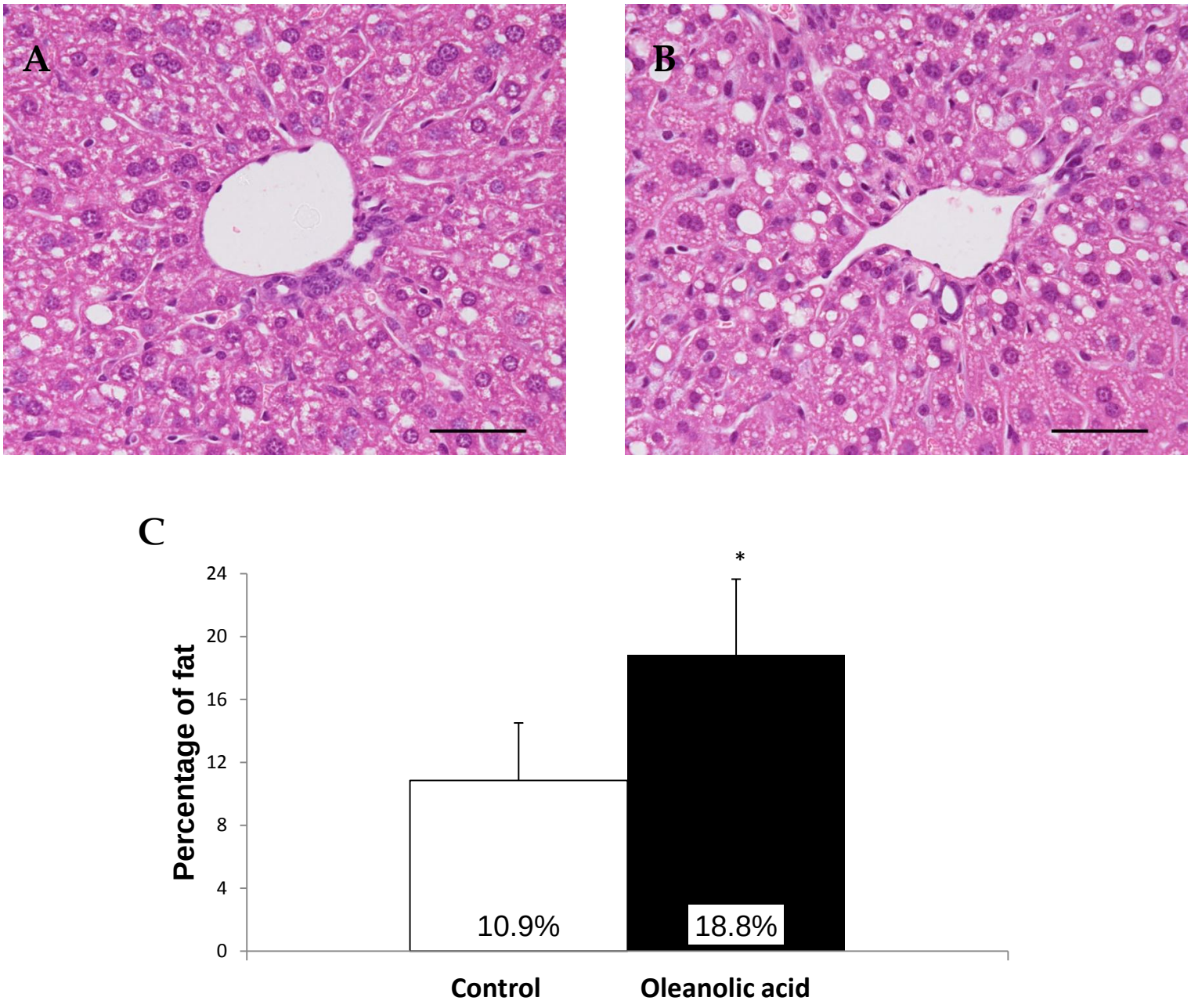

A
B
C
Percentage of fat
24
20
16
12
8
4
0
*
10.9%
18.8%
Control
Oleanolic acid

Figure 4

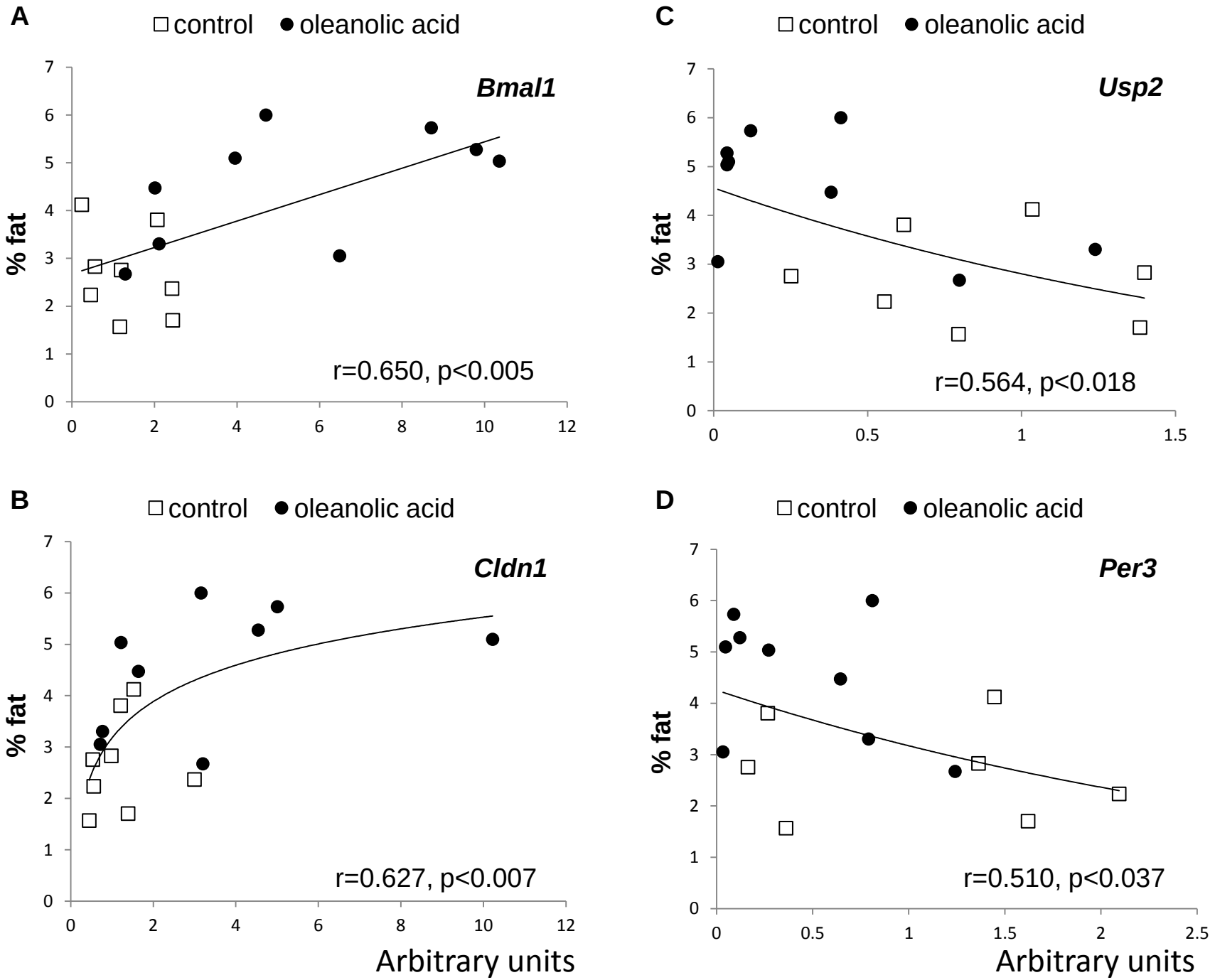

A
control
oleanolic acid
Bmal1
% fat
r=0.650, p<0.005
C
control
oleanolic acid
Usp2
% fat
r=0.564, p<0.018
B
control
oleanolic acid
Cldn1
% fat
r=0.627, p<0.007
Arbitrary units
D
control
oleanolic acid
Per3
% fat
r=0.510, p<0.037
Arbitrary units

Figure 5

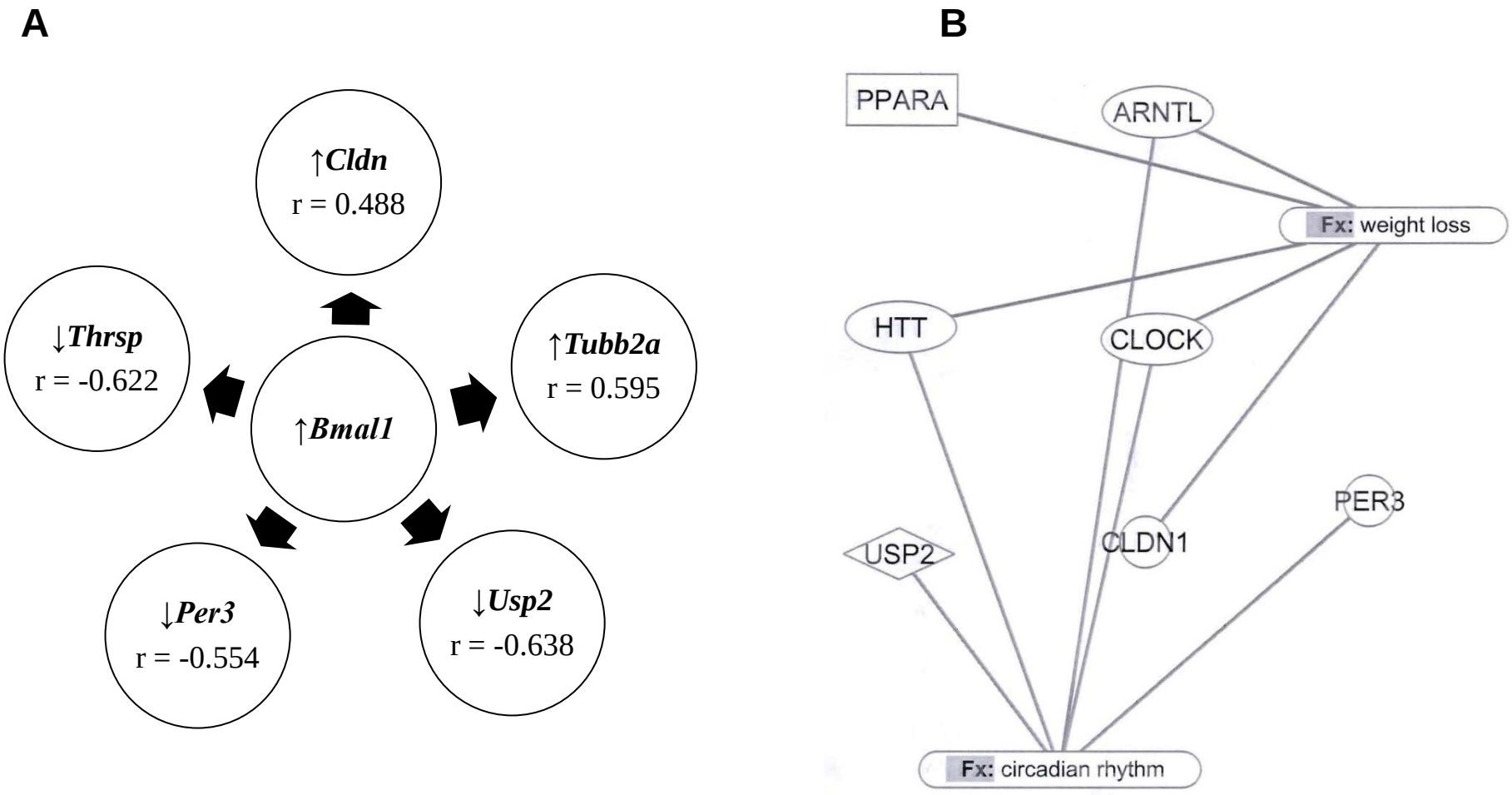
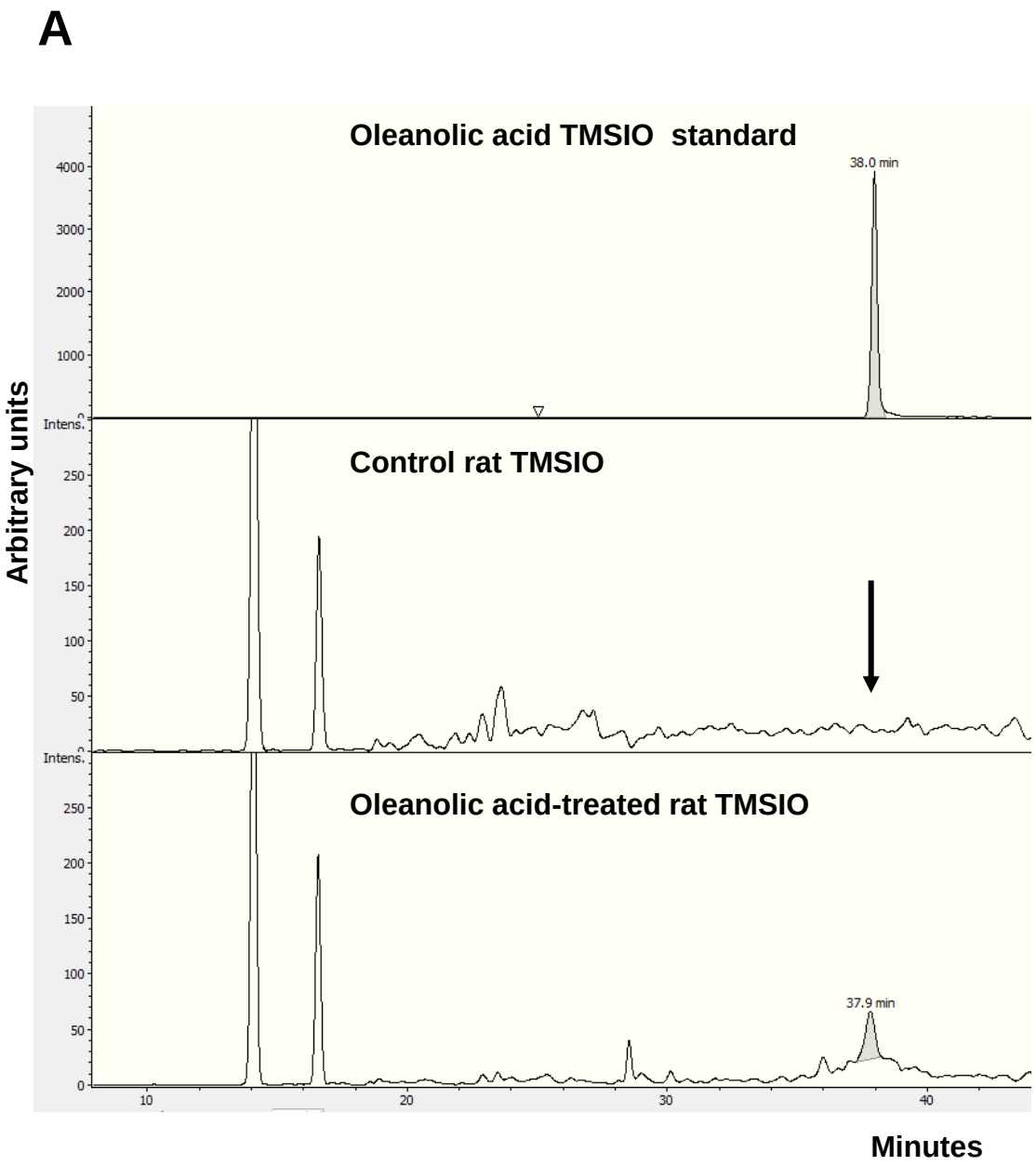
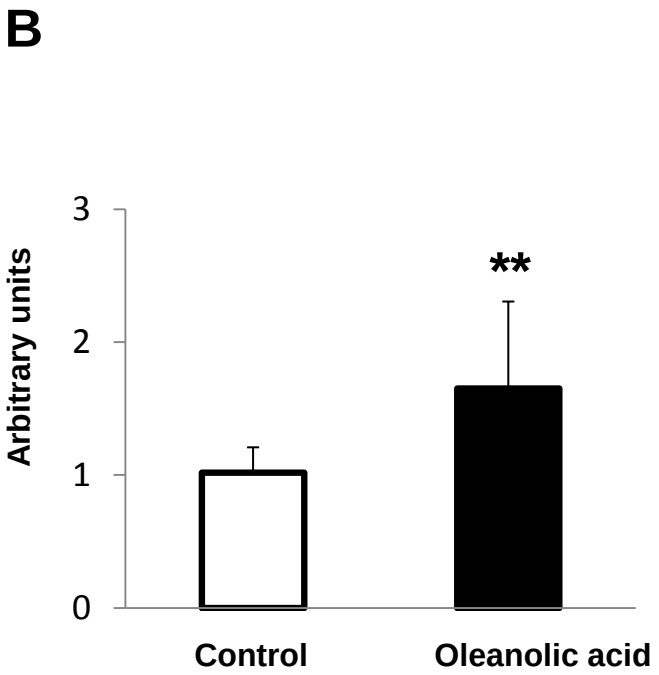

A
↑Cldn
r = 0.488
↓Thrsp
r = -0.622
↑Tubb2a
r = 0.595
↑Bmal1
↓Per3
r = -0.554
↓Usp2
r = -0.638
B
PPARA
ARNTL
Fx: weight loss
HTT
CLOCK
PER3
CLDN1
USP2
Fx: circadian rhythm

Figure 6


A
Oleanolic acid TMSIO standard
38.0 min
Control rat TMSIO
Oleanolic acid-treated rat TMSIO
37.9 min
Intens.
Arbitrary units
Minutes
4000
3000
2000
1000
250
200
150
100
50
0
10
20
30
40


B
Arbitrary units
3
2
1
0
**
Control
Oleanolic acid

Supplemental Table 1

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Supplemental Table 2

Click here to download Supplemental file for online publication: Supplementary Table 2.doc

Supplemental Figure 1

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Supplemental Figure 2

Click here to download Supplemental file for online publication: Supplementary Fig 2.pptx