



## Minor components of pomace olive oil enhance VLDL-receptor expression in macrophages when treated with postprandial triglyceride-rich lipoproteins

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**SUMMARY:** Pomace olive oil (POO) is rich in minor components, which can modulate the composition of postprandial triglyceride-rich lipoproteins (TRL) and their uptake by macrophages. The aim of the present study was to investigate the effects of postprandial TRL obtained after the ingestion of meals containing POO or refined olive oil (ROO) on foam cell formation, one of the initial steps of atherogenesis. Meals were administered to 9 healthy men and TRL were isolated from serum 4h after the intake. POO intake led to TRL with lower triglyceride/apo B48 and triglyceride/apo B100 ratios compared to ROO. Upon incubation of THP-1 macrophages with the TRL, an increase in the intracellular triglyceride content and foam cell formation was observed. Compared to ROO-TRL, the only receptor involved in lipoprotein uptake that showed changes in the mRNA expression after treatment with POO-TRL was the VLDL-receptor (VLDLr). In conclusion, the intake of POO modified the composition of human TRL, which increased the VLDLr gene expression in macrophages. However, the changes were not sufficient to enhance foam cell formation.

**KEYWORDS:** *Macrophages; Pomace olive oil; Postprandial; Triglycerides; VLDL-receptor*

**RESUMEN:** *Los componentes menores del aceite de oliva de orujo aumentan la expresión del receptor VLDL en macrófagos tratados con lipoproteínas ricas en triglicéridos postprandiales.* El aceite de orujo de oliva (AOO) es rico en componentes menores que pueden modular la composición de lipoproteínas ricas en triglicéridos postprandiales (TRL) y su captación por macrófagos. El objetivo del presente estudio fue investigar los efectos de TRL obtenidas después de la ingesta de comidas que contenían AOO o aceite de oliva refinado (AOR) sobre la formación de células espumosas, uno de los pasos iniciales de la aterogénesis. Las comidas fueron administradas a 9 hombres sanos y las TRL fueron aisladas del suero sanguíneo 4h después de la ingesta. La ingesta de AOO dio lugar a TRL con menor ratio triglicéridos/apo B48 y triglicéridos/apo B100 en comparación con AOR. Tras la incubación de macrófagos THP-1 con las TRL, se observó un aumento en el contenido de triglicéridos intracelular y la formación de células espumosas. En comparación con TRL-AOR, el único receptor implicado en la captación de lipoproteínas que mostró cambios en la expresión génica después del tratamiento con TRL-AOO fue el receptor VLDL (VLDLr). En conclusión, la ingesta de AOR modificó la composición de TRL humanas, lo que aumentó la expresión de VLDLr en macrófagos, aunque los cambios no fueron suficientes para mejorar la formación de células espumosas.

**PALABRAS CLAVE:** *Aceite de orujo de oliva; Macrófagos; Postprandial; Receptor VLDL; Triglicéridos*

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## 1. INTRODUCTION

It has become clear in the last years that postprandial lipemia, which is caused by raised levels of triglyceride-rich lipoproteins (TRL) present in the blood after a meal containing fat, may be a risk factor for atherosclerosis (Jackson *et al.*, 2012). According to the European Atherosclerosis Society Consensus Panel the association between triglycerides (TG) and cardiovascular disease (CVD) is potentially driven by the number of TRL (Chapman *et al.*, 2011). Circulating TRL include chylomicrons (CM), VLDL and their remnants. During the postprandial phase, TG are carried in the circulation by CM, which are synthesized in the intestine and transport fat of dietary origin (exogenous fat), and VLDL, which are synthesized and secreted by the liver (endogenous fat) (Jackson *et al.*, 2012). TRL can cross the endothelial barrier and enter into the vascular wall (Mamo and Wheeler, 1994), where they can interact with lipoprotein receptors on macrophages, enhancing lipid accumulation and leading to foam cell formation (Napolitano *et al.*, 2003).

The magnitude and duration of the postprandial TG response is influenced by a number of metabolic processes, including the rate of TG secretion from the intestine and the liver, the activity of enzymes involved in the TRL processing and the rate of clearance of TRL remnants by receptor-mediated pathways (Jackson *et al.*, 2012). The type of dietary lipids carried in TRL is crucial in these processes. Diets enriched in MUFA are associated with a low rate of cardiovascular mortality and it has been demonstrated that different MUFA-rich oils produce different effects on the magnitude and duration of postprandial triglyceridemia (Abia *et al.*, 2001). Despite the multiple advantages attributed to MUFA, an increasing number of studies point out that the content of beneficial fatty acids alone cannot fully explain the impact of MUFA-rich oils on health and that minor components can exert an important role (Perona *et al.*, 2006). Minor lipid components from dietary fats are absorbed in the intestine similarly to other lipids and they are incorporated into postprandial TRL for their transport in plasma. Once delivered to the tissues as part of TRL they may have important physiological effects (Perona *et al.*, 2008).

In previous studies, CM remnants have been shown to induce extensive lipid accumulation in a variety of macrophage types, including those derived from the human monocyte cell line THP-1 (Moore *et al.*, 2005). The exact mechanisms by which TRL are taken up by macrophages are not yet clearly defined. However, there are several membrane receptors identified as possible candidates that appear to be involved in this process (Elsegood *et al.*, 2001). In the present study, we investigated the effects of postprandial TRL obtained after the ingestion of pomace olive oil (POO) and refined

olive oil (ROO) on lipid accumulation and foam cell formation in THP-1 macrophages. The composition of both oils is similar with regard to fatty acids but they differ in minor components of the unsaponifiable fraction. POO is obtained from the solid phase resulting after mechanical extraction of virgin olive oil (VOO), and is rich in unsaponifiable compounds but lacks phenolics. TRL obtained after the intake of POO and ROO present differences in their content of minor components (Cabello-Moruno *et al.*, 2014) and it is possible, therefore, that this may affect their uptake by macrophages and their promotion of foam cell formation. We also aimed to determine the contribution of apolipoprotein (apo) E-dependent (LDLr, LRP, VLDLr) and scavenger (SR-A2, SR-B1 and CD36) receptors to the removal of apoE-containing native TRL by macrophages. We have previously shown that TRL particles isolated at 4 h after the oil intake is the time point at which the maximum cellular incorporation of TG is found (Cabello-Moruno *et al.*, 2014). Therefore, we investigated the effects of postprandial TRL obtained 4 h after the ingestion of meals containing POO or ROO on foam cell formation by analyzing the intracellular lipid accumulation and gene expression of membrane receptors involved in the process.

## 2. MATERIALS AND METHODS

### 2.1. Subjects and Study Design

Nine healthy men aged  $26.2 \pm 4.3$  years with a BMI  $23.7 \pm 2.0 \text{ kg} \cdot \text{m}^{-2}$  participated in the study. Subjects were excluded if they suffered from any digestive or metabolic disorder, were taking dietary supplements, or were under medication of any kind. A fasting blood sample was collected to ensure that recruited subjects had plasma TG and glucose concentrations within normal limits (Table 1).

TABLE 1. Baseline data of normo-lipidemic men participating in the study<sup>a</sup>

	Concentration (mg·dL <sup>-1</sup> )	Reference range (mg·dL <sup>-1</sup> ) <sup>b</sup>
Total Cholesterol	151.5±7.3	<200
LDL-Cholesterol	97.6±4.0	<100
HDL-Cholesterol	60.7±6.0	>40
Triglyceride	67.5±5.7	<200
Apo A	147.8±7.7	>120 <sup>c</sup>
Apo B	59.3±2.3	<90 <sup>c</sup>
LDL/HDL	1.6±0.1	–
Apo B/apo A	0.4±0.1	–

<sup>a</sup>Data are expressed as means±SEM, n=9.

<sup>b</sup>Reference values for men according to the American Heart Association.

<sup>c</sup>Data for patients with a high cardiovascular risk.

The experiment was carried out over three weeks, being the second week a wash-out period in which volunteers returned to their normal diet. Participants were given 1 L of ROO in the week before the beginning of the study in order to minimize the effect of the habitual dietary oil. On the last day of that week, the subjects consumed a meal that consisted of 1 slice of brown bread (28 g), 1 skim yoghurt (125 g) and plain pasta (100 g, cooked with 200 mL of water) with tomato sauce (130 g) previously mixed with POO or ROO (70 g). The oils contributed with 2587 kJ of energy while the whole meal provided 4523 kJ, distributed as follows: 32.5% carbohydrate, 7.6% protein, and 59.9 % fat. POO and ROO were kindly supplied by Oleicola El Tejar, S.A. (El Tejar, Cordoba, Spain). The fatty acid composition of the two oils was similar (Table 2), but there were marked differences in the content of their unsaponifiable fractions, with POO having considerably higher levels of sterols, tocopherols, erythrodiol + uvaol and waxes (Table 3).

TABLE 2. Fatty acid composition of refined (ROO) and pomace (POO) olive oil employed in the study

Fatty acid	ROO (g·100 g <sup>-1</sup> )	POO (g·100 g <sup>-1</sup> )
16:0	10.6	10.4
18:0	3.3	2.8
20:0	0.8	0.9
16:1 (n-7)	0.8	0.9
18:1 (n-9)	78.7	76.2
18:2 (n-6)	5.2	7.6
Others	0.8	1.0

TABLE 3. Unsaponifiable fraction composition of refined (ROO) and pomace (POO) olive oils employed in the study

	ROO (mg·kg <sup>-1</sup> )	POO (mg·kg <sup>-1</sup> )
Unsaponifiable fraction (%)	1.3	1.6
Sterols	1120.0	2245.0
Cholesterol (%)	0.3	0.3
Brassicasterol (%)	0.1	0.1
Campesterol (%)	3.2	2.4
Stigmasterol (%)	0.9	0.9
β-sitosterol (%)	95.5	95.2
Δ7-stigmasterol (%)	0.2	0.3
Tocopherols	220.5	981.2
α-tocopherol (%)	94.4	80.7
β-tocopherol (%)	4.1	8.9
γ-tocopherol (%)	1.5	10.4
Squalene	2642.0	2543.0
Erythrodiol+uvaol	17.0	507.0
Waxes	123.0	3484.0

Participants were asked to have a low-fat dinner the evening prior to the postprandial study and to abstain from alcohol drinking and smoking for 24 h. On arrival, after an overnight fast (12 h), a cubital vein was catheterized and a baseline blood sample was taken immediately before consumption of the test meal. Four hours after the intake blood samples were collected. The postprandial time point for TRL isolation was chosen according to the hour at which the maximum TRL incorporation into cells had been observed previously (Cabello-Moruno *et al.*, 2014). During the course of the experiment, subjects were allowed to drink water and to undertake only light activities. Serum was recovered by centrifugation (1620×g, 30 min, 4 °C) and sodium azide, phenylmethylsulfonyl fluoride and aprotinin (Sigma-Aldrich, Poole, UK) were added to a final concentration of 1 mmol·L<sup>-1</sup>, 10 μmol·L<sup>-1</sup>, and 0.5 mg·L<sup>-1</sup>, respectively.

## 2.2. Postprandial TRL isolation

Postprandial TRL were isolated from 4.5 mL of serum collected at 4 h after the intake of the test meal. Serum was layered under 6 mL of NaCl solution (d=1.006 kg·L<sup>-1</sup>) and TRL were obtained by a single ultracentrifugation spin (39,000 rpm, 16 h, 12 °C). Ultracentrifugation was performed using a SW41Ti swinging bucket rotor in a Beckman L8-70M preparative ultracentrifuge (Beckman Instruments, Palo Alto, USA). TRL were rapidly frozen at -80 °C and thawed just before use. Since severe changes in temperature might cause an alteration in TRL structure, particles were checked for aggregations before use in culture.

## 2.3. TRL lipid and apolipoprotein B composition

TRL obtained from 4.5 mL of serum (aprox. 1 mL) were adjusted to a final volume of 1.5 mL with a KCl solution (0.1 M). Total lipids in TRL were extracted following a modification to the method of Folch *et al.* (1957). The extracted lipids were dissolved in 1 mL of chloroform:methanol (2:1, v/v). Aliquots were made and kept at -20 °C until use.

Lipid class composition was determined by HPLC as described by Perona and Ruiz-Gutierrez (2004). In brief, 10 mL of lipids were dissolved in chloroform-methanol (2:1, v/v) and injected by an automatic sampler into a 2690 Alliance liquid chromatograph (Waters, Milford, MA) controlled by the Empower System (Waters, Milford, MA). Detection was accomplished with a model 2420 light-scattering detector (Waters, Milford, MA). The separation of lipid classes was carried out on a Lichrosphere diol column (250 mm, 4.6 mm, 5 mm particle size; Merck, Darmstadt, Germany) using a gradient-elution system containing hexane, 2-propanol and methanol. Stock solutions of cholesteryl oleate, triolein, cholesterol and phosphatidylcholine were prepared in chloroform-methanol (2:1, v/v) and used as standard

for the identification and quantification of cholesteryl esters (CE), TG, free cholesterol (FC) and phospholipids (PL), respectively. The precision of the method ranged from 2.4–4.0% for lipid standards and 6.8–11.7% for TRL.

Apo B48 and apo B100 were quantified after separation by SDS-PAGE. Electrophoresis was carried out using the buffer system described by Laemmli (1970). The gels contained a 4–15% acrylamide gradient, 0.1% SDS, and 0.375 M Tris. A stacking gel (5% acrylamide, 0.1% SDS, 2 mM EDTA, 0.11 M Tris-HCl, pH 6.8) was added with a 10-slot well-forming comb. Non-delipidated lipoprotein samples (3 mg of protein) were reduced in an SDS sample buffer (3% SDS, EDTA 0.8 mM, 5% mercaptoethanol, 0.004% bromophenol blue, 0.05 M Tris-HCl, 10% glycerol, pH 6.8) for 3 min at 100 °C. Gels were run at 60 V for 3 hr. Gels were fixed with a 10% methanol: 7% acetic acid solution for 30 min and stained overnight with SYPRO Ruby Protein Gel Stain (Molecular Probes). De-staining was achieved with a 1 h wash with the 10% methanol: 7% acetic acid solution. The two molecular weight forms of apo B were clearly separated in this gel system and were identified by comparing the distance they migrated into the gels with known molecular weight standards (Sigma Marker, Wide Range (M.W. 6.5–250.0 kDa). The gel was scanned using the Gel Doc 1000 system (Bio-Rad, Richmond, CA) and analyzed with the software Molecular Analyst v.1.6 (Bio-Rad, Richmond, CA). All samples from the same subject were run on the same gel to enable comparison after separation and staining in the same conditions. Considering that apo B-100 and B-48 show the same chromogenicity (Karpe and Hamsten, 1994), the relative amounts of the two isoforms were calculated from the staining intensity, and their absolute masses were calculated from the total apo B mass determined by immunoturbidimetry (Sigma Diagnostics, St. Louis, MO).

#### 2.4. Culture of THP-1 macrophages

THP-1 monocytes ( $3\text{--}9 \times 10^5$  cells/ml) in RPMI 1640 supplemented with fetal bovine serum (10% v/v), penicillin ( $100 \text{ U} \cdot \text{mL}^{-1}$ ), streptomycin ( $100 \text{ mg} \cdot \text{mL}^{-1}$ ) and 2-mercaptoethanol ( $20 \mu\text{M}$ ) were differentiated into macrophages by incubation with phorbol 12-myristate 13-acetate (PMA,  $200 \text{ ng} \cdot \text{mL}^{-1}$ ) for 72 h at 37 °C in 5% CO<sub>2</sub>:95% air. Confluency of adherent cells was 70–80% in all experiments and cell viability was >95% and was unaffected by incubation with TRL.

#### 2.5. Oil red O staining

Cells ( $3 \times 10^5$  cells·mL<sup>-1</sup>) were incubated with TRL ( $15 \mu\text{g}$  cholesterol·mL<sup>-1</sup>) for 24 h, washed with PBS and 60% propan-2-ol, then Oil red O (0.5% (w/v) in

40% propan-2-ol/H<sub>2</sub>O (v/v)) was added. After 15 min, the stain was removed and the cells were washed twice with PBS.

#### 2.6. Intracellular lipid composition

The cells ( $8 \times 10^5$  cells/ml) were incubated with TRL ( $15 \mu\text{g}$  cholesterol·mL<sup>-1</sup>) for 48 h, after which they were washed with PBS and scraped off the plates with 700 mL of PBS. The cells were lysed by sonication for 5 seconds (2 cycles) at 50W using a Bandelin Sonoplus HD2070 apparatus (Bandelin electronic, Berlin, Germany). An aliquot of 50 ml was used to determine the protein content using the method of Bradford. Lipids were extracted using the method of Folch *et al.* (1957). TG, FC and CE content were determined by enzymatic analysis using commercial enzymatic reagent kits (Alpha Laboratories, Eastleigh, UK).

#### 2.7. mRNA analysis

THP-1 macrophages ( $6 \times 10^6$  cells·mL<sup>-1</sup>) were incubated with TRL ( $15 \mu\text{g}$  cholesterol·mL<sup>-1</sup>) for 16 h and total RNA was then extracted (GenElute Mammalian Total RNA kit, Sigma-Aldrich, St. Louis, USA) with DNAase I treatment according to the manufacturer's instructions. RT and oligo-(dT) were from Promega (Promega, Southampton, UK) and were used for reverse transcription. The mRNA abundance for LDLR, LRP, VLDLR, CD36, SRA2, SRB1 and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by RT-PCR using SYBR green quantitative fluorescence (SYBR Green JumpStart Taq ReadyMix, sigma-Aldrich, Poole, UK) and an Opticon 2 LightCycler system (MJ Research, Waltham, Massachusetts, USA). Ct values were determined by automated threshold analysis. Data were normalized using the values obtained for GAPDH and the fold change in mRNA expression in TRL-treated as compared to control macrophages was determined by the method of Pfaffl (2001).

#### 2.8. Statistical analysis

The results were expressed as means±SEM (n=9) unless otherwise stated. Data analyses were performed with the GraphPad Prism® 5 statistical package (GraphPad Software Inc., San Diego, CA). Statistical significance of differences were assessed using the following tests: for differences in TRL composition paired Student's t-test, for differences in mRNA expression unpaired Student's t-test and for intracellular lipid composition one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered statistically significant at p<0.05.

TABLE 4. Lipid class composition of triglyceride-rich lipoproteins (TRL) obtained from healthy volunteers 4 hours after the intake of meals rich in refined (ROO) or pomace (POO) olive oils

	ROO (mg·mg <sup>-1</sup> TRL protein)	POO (mg·mg <sup>-1</sup> TRL protein)
TG	3.28±0.60	1.51±0.17*
TC	0.73±0.11	0.43±0.08
CE	0.23±0.04	0.18±0.04
FC	0.49±0.82	0.25±0.04*
PL	0.34±0.06	0.15±0.02*
Total lipids	5.07±1.60	2.09±0.26*
Apo B48 (µg·mg <sup>-1</sup> TRL protein)	0.004±0.001	0.003±0.001
Apo B100 (µg·mg <sup>-1</sup> TRL protein)	0.070±0.010	0.066±0.004
TG/apo B48	929.43±108.82	360.83±54.91*
TG/apo B100	41.72±7.64	17.77±0.34*

\*TG, triglycerides; TC, total cholesterol; CE, cholesterol esters; FC, free cholesterol; PL, phospholipids; Apo B: apolipoprotein B; TG/apo B: triglyceride/apo B ratio. Data are expressed as means±SEM, n=9. \*p<0.05, vs ROO.

### 3. RESULTS

#### 3.1. Lipid class composition of postprandial TRL

Table 4 shows the lipid class composition of postprandial TRL 4h after the intake of the experimental meals. TG, FC and PL and total lipid concentrations were significantly lower in TRL after consumption of POO. No significant differences were observed in apo B48 and apo B100 concentrations in TRL after the intake of both dietary oils, but the lower TG content in TRL formed after POO ingestion led to particles with significantly lower TG/apo B48 and TG/apo B100 ratios.

#### 3.2. Intracellular lipid composition in THP-1 macrophages

THP-1 macrophages were incubated with postprandial TRL in order to evaluate the influence of the modifications in their composition on the cellular uptake and intracellular lipid accumulation. Microphotographs of THP-1 cells showing

intracellular lipids can be found in Figure 1 and the results in Figure 2. The intracellular concentration of both FC and CE tended to be higher when macrophages were incubated with TRL obtained after the intake of ROO but differences were not significant. In macrophages incubated with POO-TRL intracellular accumulation of CE and FC was similar to that seen in control cells (absence of TRL). However, a 5 fold increase in TG intracellular accumulation was found when THP-1 macrophages were treated with TRL obtained after the intake of either ROO- or POO-TRL compared to the control cells (ROO-TRL, p<0.01; POO-TRL, p<0.05), and there was no significant difference between the two oils.

#### 3.3. Macrophage receptors gene expression

The influence of postprandial TRL obtained at 4h after the intake of the experimental meals containing ROO or POO on the mRNA expression of membrane receptors known to be involved in lipoprotein uptake by macrophages is shown in Figure 3.

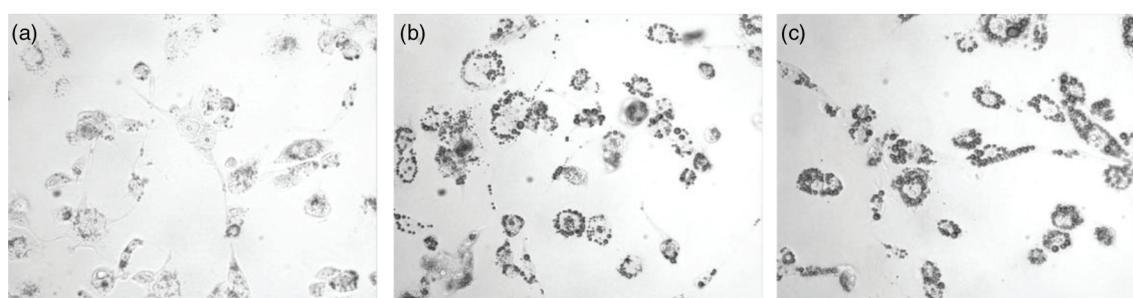


FIGURE 1. Microphotographs of THP-1 macrophages incubated for 24 h in the presence or absence (control, a) of TRL (15 µg total cholesterol·mL<sup>-1</sup>) isolated 4 h after the consumption of meals containing ROO (b) or POO (c) and stained with Oil Red O for visualizing lipid accumulation. Typical images from nine experiments are shown.

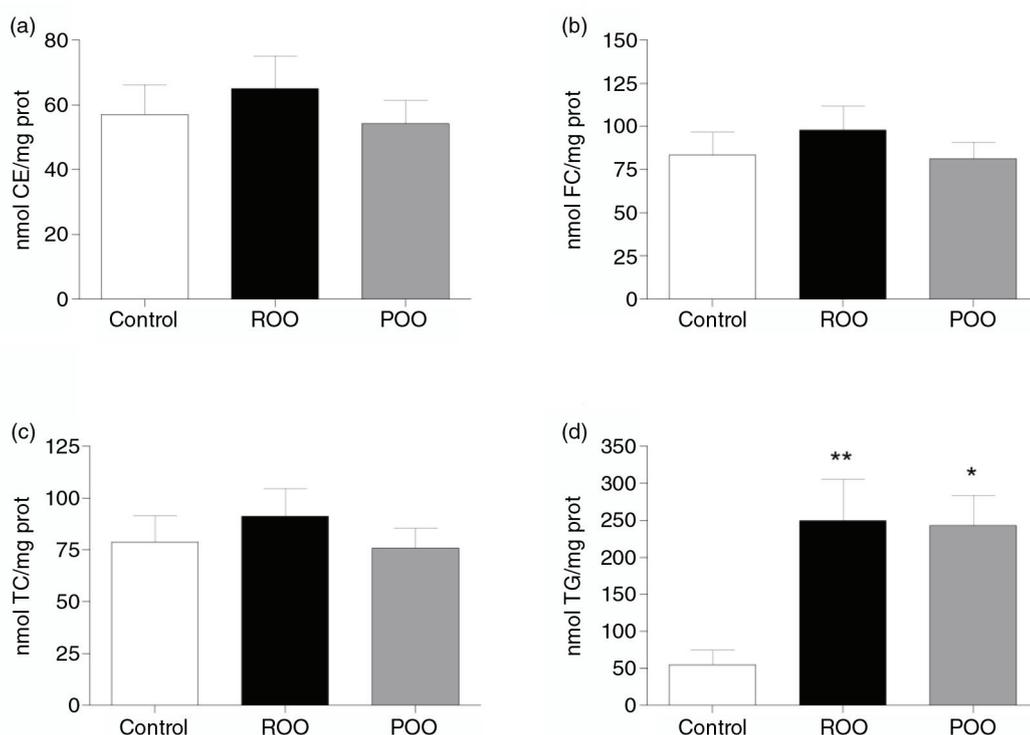


FIGURE 2. Intracellular lipid accumulation in macrophages incubated 24 hours in the absence of (control, white bar) or with TRL (15  $\mu\text{g}$  total cholesterol· $\text{mL}^{-1}$ ) obtained at 4 h after the consumption of meals containing ROO (black bar) or POO (grey bar). a: Cholesteryl esters CE; b: Free cholesterol, FC; c: Total Cholesterol, TC; d: Triglycerides, TG. Statistical significance of differences between the effects of both experimental oils compared with the Control was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. \* $p < 0.05$ , \*\*  $p < 0.01$ , vs. Control. Data are expressed as means  $\pm$  SD,  $n = 9$ .

The relative abundance of mRNA transcripts for the LDLR was reduced by about 50% after incubation with TRL obtained after the ingestion of either oil. mRNA levels for LRP were less affected after incubation with TRL, although there was a slight tendency towards inhibition of the expression with TRL after the intake of POO. In contrast to LDLR and LRP, for which differences between TRL from the two oils were not significant, the increase in mRNA levels for the VLDLR caused by TRL after the intake of POO-TRL particles was significantly higher than that observed with ROO-TRL.

Unlike apo E-dependent receptors, the amounts of mRNA for scavenger receptors in the macrophages were increased in almost all cases by TRL particles obtained after the ingestion of ROO and POO. In the case of CD36, the relative abundance of mRNA transcripts was increased by up to 80% after incubation with ROO-TRL, and the effect induced by TRL obtained after POO intake was similar. The particles isolated after the ROO meal induced an increase in the mRNA expression for SR-A2 and SR-B1 genes, while those obtained after POO also showed raised levels of mRNA for SR-A2 to a lesser extent, but had little effect on the expression of mRNA for SR-B1. However, in both cases, the mRNA expression levels

for SR-A2 and SR-B1 were not significantly different between ROO- and POO-TRL.

#### 4. DISCUSSION

The unsaponifiable content of POO can affect the size and composition of human postprandial TRL, which might have an influence on lipoprotein atherogenicity (Cabello-Moruno *et al.*, 2007; Cabello-Moruno *et al.*, 2014). The present study was designed to test the hypothesis that TRL particles obtained after the intake of POO, with a high unsaponifiable content, may influence foam cell formation in macrophages.

Human TRL were collected from healthy donors 4h after the intake of meals rich in POO or ROO because we had observed previously that at that time point the effect on intracellular lipid accumulation after TRL uptake was at its maximum in THP-1 cells (Cabello-Moruno *et al.*, 2014). We now show that POO as compared to ROO, significantly decreased the content of TG, FC and PL in TRL. In earlier studies, we had reported that the unsaponifiable fraction of olive oil does not exert changes in postprandial serum TG concentrations but can affect the TRL lipid composition, with

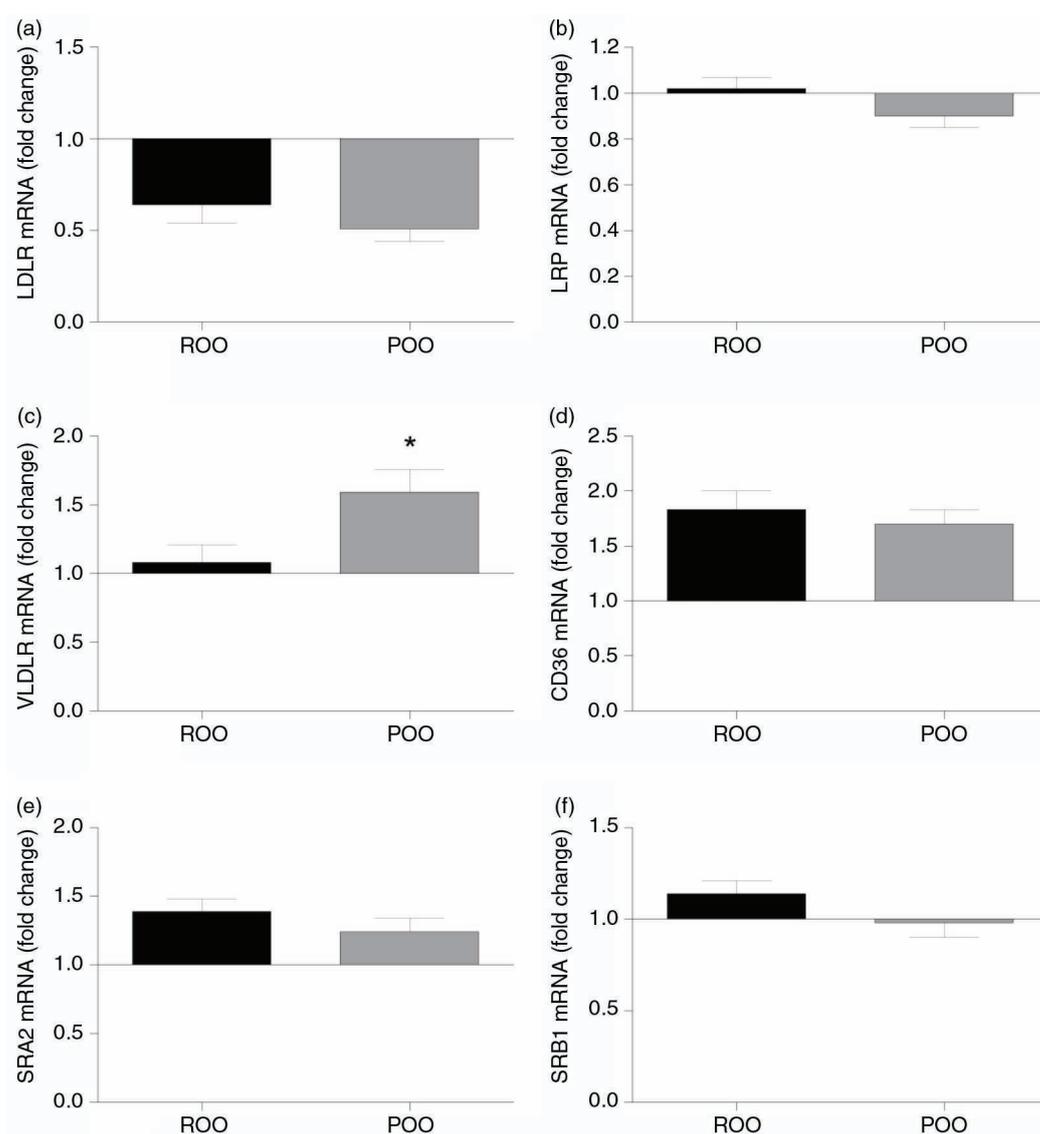


FIGURE 3. Relative abundance of mRNA expression of membrane receptors in macrophages incubated with TRL ( $15 \mu\text{g total cholesterol}\cdot\text{mL}^{-1}$ ) obtained at 4 h after the intake of a meal containing refined olive oil (ROO, black bar) or pomace olive oil (POO, grey bar). a: LDLr (low-density lipoprotein receptor); b: LRP (LDLr-related protein); c: VLDLR (VLDL receptor); d: CD36 (Scavenger receptor CD36); e: SRA2 (Scavenger receptor class A type 2); f: SRB1 (Scavenger receptor class B type 1). Data are expressed as means $\pm$ SEM, n=9. \*p<0.05, \*\* p<0.01, vs ROO.

important consequences for lipid uptake in different cell types (Cabello-Moruno *et al.*, 2007; Perona *et al.*, 2008). In the present work, the intake of either ROO or POO did not lead to significant differences in TRL apo B48 and apoB100 concentrations, suggesting that the number of both apo B48-containing particles (CM) and apo B100-containing particles (VLDL) was similar after the intake of both dietary oils. As a consequence of the modifications in the TG content but not in apo B48 and apo B100, the TG/apo B48 and TG/apo B100 ratios were significantly lower in TRL particles obtained after the POO meal. This ratio is believed to be an indicator

of particle size, and it has been previously reported that TRL particles with differences in size and composition can modulate TRL clearance postprandially, which may affect TG incorporation into the tissues (Elsegood *et al.*, 2001). The current findings suggest, therefore, that POO intake leads to smaller TRL, containing less TG molecules per particle, which could interfere with lipid incorporation into macrophages both via LPL hydrolysis and the receptor-mediated pathways (Palmer *et al.*, 2005).

Postprandial TRL particle size may be modified by the fatty acid composition of dietary oils, with TRL obtained from MUFA-rich oils tending to be

larger, compared to those derived from those rich in SFA, n-6 PUFA and n-3 PUFA (Jackson *et al.*, 2002; Perez-Martinez *et al.*, 2011). In a previous study, we showed that the unsaponifiable fraction of POO induced the release of larger TRL particles that were more rapidly hydrolyzed to their corresponding remnant particles (Cabello-Moruno *et al.*, 2007), which was subsequently associated with a preference for the most apolar TG molecular species by LPL (Cabello-Moruno *et al.*, 2014).

Palmer *et al.* (2005), demonstrated that the highest TG uptake by macrophages takes place when cells were incubated with TRL obtained at the time point at which the particle size is intermediate (Sf 60–400), ie, not as large as nascent particles (Sf >400) and not as small as small-VLDL particles (Sf 20–60). The current study indicates that although POO-TRL were estimated from the TG/apoB ratio to be smaller than ROO-TRL, the two types of particles caused a similar level of intracellular lipid accumulation in macrophages, although both types of particles caused a strikingly similar increase in TG levels.

Modifications of the fatty acid composition of chylomicrons have been shown to affect their induction of lipid accumulation in macrophages (Napolitano *et al.*, 2003). However, very little is known about the effect of olive oil minor components. Actually, to the best of our knowledge, and despite their lipophilic nature, the presence of unsaponifiable components of olive oil in postprandial TRL has not been demonstrated. Therefore, most of the biological effects of the components of the unsaponifiable fraction of olive oil have been tested *in vitro* and not considering their presence within postprandial TRL. Although we did not measure the content of each component of this fraction in TRL, the effects observed should be ascribed to their presence since that was the main difference between ROO and POO, as no difference was observed in the fatty acid composition of the oils.

Nevertheless, a number of authors have explored the effects of these minor components in *in vitro* studies. In agreement with the current results, we had found that the unsaponifiable fraction of VOO did not affect lipid accumulation in rat hepatocytes after incubation with human TRL (Perona *et al.*, 2008). Squalene, a main component of the unsaponifiable fraction of olive oil, does not prevent monocyte migration and activation into macrophages, but it has been shown to inhibit oxidated-LDL uptake by macrophages, probably by reducing the CD36 expression (Granados-Principal *et al.*, 2012). In combination with oleic acid, the incubation of  $\beta$ -sitosterol induced TG accumulation in 3T3-L1 adipocytes (Awad *et al.*, 2000). However, Vejux *et al.* (2012) did not find accumulation of polar lipids in human monocytic U937 cells after incubation with phytosterols. There are very scarce data on tocopherols, but Wallert *et al.* (2014) demonstrated that long-chain metabolites of  $\alpha$ -tocopherol occurring in

human serum can inhibit macrophage foam cell formation from THP-1 macrophages when treated with oxidated-LDL. These authors suggested that these compounds might be considered as new class of regulatory metabolites. The greatest difference between POO composition and ROO is the concentration of the aliphatic alcohol fraction (waxes) (Table 3). Policosanol, a mixture of aliphatic alcohols containing mainly octacosanol, has been demonstrated to reduce lipofundin-induced foam cell formation in rabbits and rats and to prevent the development of foam cells in carrageenan-induced granulomas in rats (Noa *et al.*, 1996). Unfortunately, the mechanisms of these effects remain obscure and it is not presently known whether or not they are related to lower lipid accumulation in cells involved in atherogenesis, such as macrophages or smooth muscle cells.

The ingestion of POO or ROO led to TRL that were capable of reducing the expression of LDLr and inducing that of CD36 and SR-A2 to similar extents, with little effect on LRP and SR-B1. The inhibition of the LDLr expression can be explained by massive intracellular lipid accumulation, and the induction of SR-A2 and CD36 was associated with a greater TG uptake in a previous (Cabello-Moruno *et al.*, 2014). Nonetheless, significant differences between POO-TRL and ROO-TRL were observed for the VLDLr, a member of the LDLr family that mediates the uptake of TRL by peripheral tissues (Takahashi *et al.*, 2004). Re-introduction of VLDLr in VLDLr-KO mice significantly increases atherosclerotic lesion development, indicating that the VLDLr expressed in macrophages is a pro-atherogenic factor (Eck *et al.*, 2005). Since VLDLr expression is down-regulated by the activation of the peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), it has been suggested that PPAR $\alpha$  agonists might help to reduce TRL uptake in macrophages (Dushkin *et al.*, 2007).

To the best of our knowledge, there is no evidence for a direct effect of the unsaponifiable fraction of olive oil on PPAR $\alpha$  or VLDLr expression. Nevertheless, an association cannot be discarded, since Cardeno *et al.* (2013) reported that the unsaponifiable fraction of olive oil induced apoptosis in HT-29 human colon adenocarcinoma cells through PPAR $\gamma$  and NF $\kappa$ B signaling pathways. There have also been no previous studies concerning the effect of isolated components of this unsaponifiable fraction on VLDLr expression, and very little regarding PPARs. However, Plat *et al.* (2009) evaluated the effects of plant stanol ester consumption on PPAR $\alpha$  mRNA expression in isolated peripheral mononuclear blood cells and found no changes.

## 5. CONCLUSIONS

To summarize, the results presented here show that POO intake leads to smaller postprandial TRL compared to ROO, which is likely be related to the

unsaponifiable content in the oil. Upon the incubation of macrophages with TRL particles, an increase in the TG content of the cells was observed but there was no difference between TRL obtained after POO or ROO intake. The two types of particles also had similar effects on the expression of mRNA for most of the receptors studied, however, the expression of the VLDLr was significantly higher in POO- than in ROO-TRL treated cells. Notwithstanding these results suggest that the VLDLr pathway might not be a main mechanism for TRL uptake in macrophages, we cannot exclude an indirect role of VLDLr via enhancement of TG hydrolysis by LPL. Indeed, Goudriaan *et al.* (2004) suggested that apart from its chaperone function in increasing LPL mRNA expression, VLDLr might participate in CM catabolism through the bridging of particles to the endothelial surface, thereby facilitating the LPL-particle interaction. Whether the unsaponifiable fraction of POO affects LPL expression or the interaction of VLDLr and LPL will need to be further studied.

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