Minor components of olive oil facilitate the triglyceride clearance from
postprandial lipoproteins in a polarity-dependent manner in healthy men

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ABBREVIATIONS

Apo; apolipoprotein
CN; carbon number
DB; number of double bonds
ECN; equivalent carbon number
LRP; LDL receptor-related protein
LPL; lipoprotein lipase
MPP; myristoyl-dipalmitoyl-glycerol
MTP; microsomal triacylglycerol transfer protein
MUFA; monounsaturated fatty acids
NUFA; number of unsaturated fatty acids
OLIVE, refined olive oil
OLL; oleoyl-dilinoleoyl-glycerol
OOL; dioleoyl-linoleoyl-glycerol
OOO; trioleoyl-glycerol
PLL; palmitoyl-dilinoleoyl-glycerol
POMACE; pomace olive oil
POL; palmitoyl-oleoyl-linoleoyl-glycerol
POO; palmitoyl- dioleoyl-glycerol;
PN; partition number
SLL; stearoyl-dilinoleoyl-glycerol
SOL; stearoyl- oleoyl-linoleoyl-glycerol;
SOO; stearoyl-dioleoyl-glycerol
47 SPE; solid-phase extraction

48 TG; triglyceride
ABSTRACT

Postprandial triglyceride-rich lipoproteins (TRL) are recognized as atherogenic particles whose lipid composition and function can be modified by the composition of dietary oils. This study was designed to test the hypothesis that minor components of pomace olive oil (POMACE) can not only change the composition of postprandial TRL but also affect the clearance of triglyceride (TG) molecular species of postprandial TRL. Meals enriched in POMACE or refined olive oil (OLIVE) were administrated to 10 healthy young men. TRL were isolated from serum at 2, 4 and 6 hours postprandially and their fatty acid and TG molecular species compositions were analyzed by gas chromatography. The apolipoprotein B concentration was determined by immunoturbidimetry. POMACE and OLIVE, differing mainly in their unsaponifiable fraction, led to similar fatty acid and TG molecular species profiles in postprandial TRL. However, POMACE-TRL presented a higher particle size, estimated as TG to apolipoprotein B ratio, which was also found for the main TG molecular species (trioleoyl-glycerol, palmitoyl-dioleoyl-glycerol, palmitoyl-oeloyl-linoleoyl-glycerol, and dioleoyl-linoleoyl-glycerol). TG from POMACE-TRL also showed higher clearance rates. In this regard, apolar TG (with a higher equivalent carbon number) disappeared more rapidly from TRL particles obtained after the ingestion of either POMACE and OLIVE. In conclusion, minor components of POMACE facilitated TG clearance from TRL by modifying their particle size and the hydrolysis of the most apolar species.

KEY-WORDS: olive oil; human; triglyceride; lipoprotein; triolein; apolipoprotein B; fatty acid.
1. INTRODUCTION

There is growing agreement that postprandial hypertriglyceridemia is a potential independent cardiovascular risk factor [1]. Triglyceride-rich lipoproteins (TRL) can cross the endothelial barrier and enter into the vascular wall [2], where they can enhance lipid accumulation into macrophages, leading to foam cell formation [3]. TRL consist of chylomicrons, which are secreted by the small intestine and contain apolipoprotein (apo) B-48 as the structural protein, and VLDL, originated in the liver and containing apo B-100. In addition, TRL also include chylomicron and VLDL remnant particles, partially depleted of triglycerides (TG) and enriched with cholesteryl esters. The transformation of TRL into remnant particles is dependent upon TG hydrolysis by lipoprotein lipase (LPL), which is attached to the surface of the vascular endothelium [4]. The enzyme can differentiate between substrates and exhibits specificity with respect to fatty acid length chain and unsaturation [4,5]. Therefore, the composition of TRL-TG is decisive for the activity of LPL and the formation of TRL remnants.

The Mediterranean diet, characterized by a high consumption of monounsaturated fatty acids (MUFA), has been proposed as a healthy dietary standard because it is associated with a low rate of cardiovascular mortality [6]. However, we have demonstrated that not all MUFA-rich oils exert the same effects on the magnitude and duration of postprandial triglyceridemia [7]. Other factors, such as minor non-fatty acid constituents (unsaponifiable fraction), rather than the content of oleic acid, may be responsible for the postprandial responses to virgin olive oil, and for the effects of TRL and their remnants. In this regard, we have recently reported that
the unsaponifiable fraction of virgin olive oil, contained in circulating TRL, improves the balance between vasoprotective and pro-thrombotic factors released by endothelial cells [8].

Pomace olive oil (POMACE) is obtained by chemical processes from residues of the extraction of virgin olive oil. The new improved procedures for POMACE extraction allow the presence of a number of unsaponifiable components from the skin of the olive, including elevated amounts of sterols, tocopherols, waxes and triterpenic acids and alcohols, such as oleanolic acid and erythrodiol [9]. To our knowledge there is no study assessing the effects of POMACE on the composition and clearance of TG molecular species contained in postprandial lipoproteins. Therefore, the hypothesis of the present work was that, in addition to modification of postprandial TRL-TG composition, minor components of POMACE can affect the clearance of their TG molecular species in men. To test that hypothesis, we aimed to determine the TG composition of postprandial TRL after the intake of POMACE and to compare this effect with that of a refined olive oil (OLIVE), with a low unsaponifiable content, in order to evaluate its potential impact on TRL metabolism and their metabolic consequences. Since postprandial studies are only slightly invasive, they allow they use of human beings for experimentation, provided all ethical issues are considered.
2. METHODS AND MATERIALS

2.1. Subjects and Study Design

Ten healthy men aged 26.2 ± 4.3 years with body mass index 23.7 ± 2.0 kg/m$^2$ participated in the study. Subjects were excluded if they suffered from any digestive or metabolic disorder, were taking dietary supplements, or under medication of any kind. The number of participants was chosen in accordance to similar studies [7,8,10-12]. A fasting blood sample was collected to ensure that recruited subjects had plasma TG and glucose concentrations within normal limits (Table 1). These parameters were checked at the beginning of the two phases of the study, i.e. at baseline before administration of either experimental meal. Participants gave written, informed consent to a protocol approved by the Institutional Committee on Human Research (Hospital Universitario Virgen del Rocio, Seville, Spain). All procedures were in accordance with the Institutional and National ethical standards for human experimentation and the Helsinki Declaration of 1964 and its later amendments.

The study was designed as a randomized cross-over trial. On the day of the experiment, the subjects consumed two different meals enriched with either of the test oils, POMACE or OLIVE. Meals consisted of 1 slice of brown bread (28 g), 1 skimmed yogurt (125 g) and plain pasta (100 g, cooked with 200 mL of water) with fresh tomato (130 g) previously mixed with the corresponding oil (70 g). A washout period of two weeks was established between experiments. 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.
Participants were asked to have a low-fat dinner the prior evening and to abstain from alcohol drinking and smoking for 24 h before the postprandial study. 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. Following the intake, blood samples were collected at 2, 4 and 6 h postprandially. During the course of the experiment, subjects were allowed to drink water and undertake only light activities.

Serum was recovered by centrifugation (1620 x 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, 10 μmol/L, and 0.5 mg/L, respectively.

2.2. Olive oil composition

OLIVE and POMACE were kindly supplied by Oleicola El Tejar, S.A. (El Tejar, Cordoba, Spain). To determine the fatty acid composition of the oils, TG were transmethylated using a solution of KOH (2N) in methanol, following the procedure described on the EU Regulations (CE Nº2568/91). Resultant fatty acid methyl esters were analyzed by gas chromatography (GC), using a model 5890 series II gas chromatograph (Hewlett-Packard Co, Avondale, USA) equipped with a flame ionization detector and a capillary silica column Supelcowax 10 (Sulpeco Co, Bellefonte, USA) of 60 m length and 0.25 mm internal diameter with hydrogen as a carrier gas. The injector and detector were set at a constant temperature of 250 °C during the analysis. The oven temperature was programmed to start at 180 °C during 10 min and then to increase until 250 °C at 2 °C/min rate. External standards used for identification and
quantification of the resulting chromatographic peaks were purchased from Sigma-Aldrich (Poole, UK). Fatty acid methyl esters were quantified as weight percentages.

TG molecular species contained in the oils were also determined by GC after dissolving oil samples in hexane. The gas chromatograph (model 5890 series II, Hewlett-Packard Co, Avondale, USA) was equipped with a Quadrex Aluminium-Clad 400-65HT column (Quadrex, Woodbridge, USA) with 30 m length and 0.25 mm internal diameter, using a linear gas rate of 50 cm/s and a split ratio 1:80. The injector and detector temperatures were both 380 °C, the oven temperature was 345 °C and a head pressure gradient from 70 to 120 kPa was applied.

The composition of the unsaponifiable fraction of the oils, including total unsaponifiable matter, tocopherols, sterols, squalene, waxes and erythrodiol+uvaol, was provided by the manufacturer (Oleicola El Tejar, S.A., El Tejar, Cordoba, Spain).

2.3. Postprandial TRL isolation

Postprandial TRL were isolated from 4.5 mL of serum collected at 2, 4, and 6 h after the intake of the test meals enriched with OLIVE or POMACE. Serum was layered under 6 mL of NaCl solution (d = 1.006 kg/L) and TRL were obtained by a single ultracentrifugation spin (39,000 rpm, 18 h, 12°C). Ultracentrifugation was performed using a SW 41Ti swinging bucket rotor in a Beckman L8-70M preparative ultracentrifuge (Beckman Instruments, Palo Alto, USA). Different postprandial time points for TRL isolation were chosen according to the hours at which the maximum and minimum serum TG concentration values had been previously found [10].
2.4. Triglyceride-rich lipoprotein composition

Total lipids in TRL were extracted following the method of Folch et al. [13]. To determine the fatty acid composition of TG in TRL, this lipid class was separated from a 150 μL total lipid aliquot by solid-phase extraction, using diol bonded-phase columns (Supelclean LC-Diol, Supelco, Bellefonte, USA). TG were transmethylated using sodium methoxide in methanol (0.5 %, m/v) and the resulting fatty acid methyl esters were analyzed by GC, using the same equipment and conditions described above. The analysis of TG molecular species in TRL was carried out by GC using the equipment described above for the TG analysis of the oils, and following the same experimental conditions. The equivalent carbon number (ECN) of TRL-TG was calculated as described elsewhere [14] using the following equation: ECN=CN-2·DB-0.2·NUFA, where CN is the number of carbon atoms in the TG molecule corresponding to fatty acids, DB is the number of double bonds of the fatty acids and NUFA is the number of unsaturated fatty acids in the molecule.

The apo B composition was determined by immunoturbidimetry (Tina-quant; Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions.

2.5. Statistical analyses

Results were expressed as means ± SD (n=10). Data analyses and graphs were performed using the GraphPad Prism® 5 statistical package (GraphPad Software Inc., San Diego, USA). Statistical significance of differences between the effects of both experimental oils was evaluated by paired t-test analyses, while postprandial changes were analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison test. Correlations between variables
were assessed using Pearson’s correlation coefficients. Differences were considered statistically significant at P<0.05.
3. RESULTS

3.1. Fatty acid and molecular species composition of OLIVE and POMACE

The fatty acid composition of OLIVE and POMACE was similar (Table 2) in terms of MUFA (nearly 80%) and saturated fatty acids (SFA) concentrations, although the amount of stearic acid (18:0) was slightly higher in OLIVE and that of linoleic acid (18:2, n-6) higher in POMACE. The main TG molecular species were composed of the main fatty acids (Table 2), with trioleoyl-glycerol (OOO) accounting for almost half of the TG determined in both oils. Only slight differences were found in the TG molecular species composition of OLIVE and POMACE. Whereas OLIVE was richer in palmitoyl-dioleoyl-glycerol (POO) and stearoyl-oleoyl-linoleoyl-glycerol (SOL), a higher concentration of dioleoyl-linoleoyl-glycerol (OOL) and oleoyl-dilinoleoyl-glycerol (OLL) was observed in POMACE. In any case, although significant, differences were lower than 4%.

3.2. Unsaponifiable fraction composition of POMACE and OLIVE.

More important differences were found among the components of the unsaponifiable fraction (Table 3). The concentration of sterols in POMACE doubled that of OLIVE and the concentration of tocopherols was nearly 5 times higher in POMACE than in OLIVE, with α-tocopherol as the main species. The concentration of the triterpenic alcohols erythrodiol and uvaol was nearly 30 times higher in POMACE. Likewise, a high difference was found in the content of waxes (fatty alcohols) between the two oils. In contrast, the squalene content was similar in both oils.
3.3. Fatty acid composition of postprandial triglyceride-rich lipoproteins

Table 4 shows the fatty acid composition of postprandial TRL at 2, 4 and 6h after the intake of the experimental meals. The intake of POMACE-rich meal caused a higher presence of oleic acid in the particles at 2h compared to the intake of OLIVE. While the content of this fatty acid in POMACE-TRL was not modified throughout the postprandial period, in TRL obtained after the intake of OLIVE its concentration was higher at 4 and 6h compared to 2h. The linoleic acid content was also higher at 2h after the intake of POMACE and, alike oleic acid but not, it did not change at 4 and 6h after the intake of this oil. No modifications in the linoleic acid concentrations of OLIVE-TRL were observed at any time point. The palmitic and stearic acid content was reduced in TRL during the postprandial period after the OLIVE-rich meal was administrated but not after the intake of POMACE. It is noteworthy that the stearic acid content in postprandial TRL at 2h after POMACE was approximately half of that observed in the particles after OLIVE was ingested.

3.4. Triglyceride molecular species composition of postprandial triglyceride-rich lipoproteins

Table 5 shows the TG molecular species composition of postprandial TRL at 2, 4 and 6h after the intake of the experimental meals. TG molecular species concentrations did not vary significantly during the postprandial period and differences were only significant for minor TG (<1 mg/100mg). In contrast, significant differences were found when the concentrations of TG molecular species analyzed from TRL obtained after administration of POMACE or OLIVE were compared at each experimental time point. The intake of POMACE resulted in higher concentrations of linoleic acid-containing TG, such as OLL, and OOL at 2h, and at 4
and 6h a higher presence of in palmitoyl-dilinoleoyl-glycerol (PLL), stearoyl-dilinoleoyl-
glycerol (SLL) and palmitoyl-oleoyl-linoleoyl-glycerol (POL) was observed. Conversely,
consumption of OLIVE resulted in higher concentrations of TG rich in oleic acid and SFA,
such as OOO, POO, myristoyl-dipalmitoyl-glycerol (MPP) and stearoyl-dioleoyl-glycerol
(SOO). All these TG were present in higher concentrations at 2h after the intake of OLIVE,
but the oleic acid-rich TG (OOO, POO and SOO) were found in higher concentrations also at
4 and 6h (except for OOO).

3.5. Triglyceride/apolipoprotein B ratios

Apo B concentration in TRL was significantly lower at 2h and 6h after the intake of
POMACE compared to OLIVE (Fig. 1). These concentrations were used to calculate the TG
to apo B ratios (Fig. 2A, 2B, 2C and 2D) to estimate the TG molecular species clearance from
the particle during the postprandial period. For the four main TG (OOO, POO, POL, OOL),
the TG/apo B ratio was significantly higher at 2h after the intake of POMACE compared to
OLIVE. However, at 4 and 6h only OOL/apo B and POL/apo B ratios remained higher in
POMACE-TRL. In any case, for the four main TG, the TG/Apo B ratio was more drastically
reduced if they formed part of TRL obtained after the intake of POMACE compared to
OLIVE. This effect was highlighted when the variations of TG concentrations (Fig. 3A) and
TG/Apo B ratios (Fig. 3B) between 2h and 4h were plotted against the equivalent carbon
number (ECN) of TG, taken as an estimation of their polarity. Both TG concentrations and
TG/Apo B ratios correlated negatively with ECN regardless of the TRL origin, POMACE or
OLIVE.
4. DISCUSSION

POMACE, which is obtained from the residues of virgin olive oil extraction, is specially rich in lipophilic minor components that may have important roles in the composition of postprandial and fasting TRL [10]. In the present study, we report the fatty acid and TG molecular species composition of postprandial TRL obtained from healthy males at 2, 4 and 6h after the intake of meals rich in POMACE or OLIVE. These dietary oils have similar fatty acid and TG profiles but considerable differences in the content of minor components from the unsaponifiable fraction.

The fatty acid composition of postprandial TRL obtained after the intake of POMACE was similar to that of particles obtained after administration of OLIVE. Significant differences were found only for stearic, oleic and linoleic acids and mainly at 2h after the intake of the oils. This weak effect was also reflected in the TG molecular species composition of postprandial TRL. Out of 26 TG species quantified, only 9 were different between POMACE-TRL and OLIVE-TRL and among these, only 3 were significantly different at the three time points studied (2, 4 and 6 h). Consequently, differences were very modest and related to the slight differences in TG molecular species composition of the experimental oils. This is consistent with previous observations using virgin olive oil [15,16]. In these studies, the effects on TRL composition of virgin olive oil supplemented in minor components was compared to the effect of non-supplemented virgin olive oil; hence, with exactly the same TG molecular species composition. From these findings, we suggested that postprandial TRL-TG profile was mainly dependent upon the TG molecular species composition and not the unsaponifiable fraction of virgin olive oil.
Nevertheless, minor components of virgin olive oil have been related to modifications of the TG molecular species composition of TRL. In a randomized cross-over controlled trial, consumption of olive oil with different concentrations of phenolic compounds (refined, common and virgin olive oils) resulted in important effects on the TG composition of VLDL [17]. After the intake of the olive oil with the highest phenolic content (virgin olive oil), VLDL presented higher concentrations of linoleic acid–containing TG and lower concentrations of those TG containing palmitic acid. Interestingly, a significant positive correlation between the phenolic content in the oil and linoleic acid-rich moieties was observed. This phenomenon was associated with a possible effect of phenolics on the modulation of the gene and protein expression or activities of key enzymes involved in TG transport and metabolism, like LPL [18], apo B48, microsomal TG transfer protein (MTP) [19], or receptors involved in the uptake of VLDL by the liver [20].

The TG composition of TRL is decisive for their clearance through hydrolysis by LPL and/or liver uptake. Previous work has shown that the TRL uptake by the liver is modulated by the fatty acid composition of the TG in the particle [21]. Sato et al. [5, 22] suggested that modifications in lipoprotein fluidity by means of changing the polarity of the TG present in TRL might modulate the affinity between the particles and LPL. In a previous work [16], we observed that the rate of hepatic uptake of high-oleic sunflower oil-TRL was significantly higher than that of TRL derived from virgin olive oil, probably due to the up-regulation of mRNA expression for LDL receptor-related protein (LRP), one of the main receptors involved in lipoprotein uptake by the liver. However, the observed effect might also involve changes in the TG molecular species of TRL causing differential interaction with the receptors, and/or differences in the content of the unsaponifiable fraction.
TRL clearance also depends on particle size. Karpe et al. [23] demonstrated that large TRL are cleared from the plasma at a faster rate than small VLDL-sized TRL. In a previous study, compared to OLIVE, POMACE led to higher TG/Apo B-48 and TG ratio/Apo B-100 ratios in TRL obtained at 2h after the intake of the oils, leading to larger particles [10]. This effect was associated with faster clearance rates and was attributed to the influence of the minor components present in POMACE [10]. The higher estimated size (TRL/Apo B ratio) of POMACE-TRL, compared to OLIVE-TRL was confirmed in the present study for all TG molecular species (data shown for the main TG only, OOO, POO, POL and OOL). We also plotted the variation of the TG concentrations and TG/Apo B ratios from 2h to 4h (TRL clearance) against the ECN of each TG molecular species analyzed, finding a very significant correlation. The ECN is used in chromatography as a determinant of the elution order of TG and, thus, of their polarity [24]. Because this parameter is inherent to TG molecules, the ECN can also be used as an indicator of TG polarity in lipoproteins, which can, in turn, determine their propensity to be hydrolyzed by LPL. The correlation showed that TG with higher ECN values (more apolar TG, containing mainly SFA) disappear more rapidly from each TRL particle compared to those with lower ECN values (more polar TG, rich in MUFA and PUFA). This is in agreement with previous observations in rats by Sato et al. [5], who demonstrated that the V-max of LPL for chylomicrons and VLDL increased linearly with the increased palmitic acid content in the lipoprotein TG, hence with more apolar TG molecular species. These authors had previously suggested that lipoprotein catalysis by LPL is modulated by the palmitic acid content of the lipoprotein triglyceride, which reduces the fluidity of lipoproteins, enhancing the LPL-lipoprotein contact, compared to oleic and linoleic acids [22]. Unfortunately, they did not include stearic acid-rich TG in the comparison. In
addition, we found that the variation of TG concentrations between 2h and 4h was higher in the postprandial TRL obtained after the intake of POMACE for all TG molecular species, which suggests that the high presence of minor components might be exerting an effect in TG clearance. We observed this phenomenon for all TG molecular species, which indicates that the effect the minor components is not selective.

In conclusion, the unsaponifiable fraction of POMACE influenced the clearance rate of TG molecular species in postprandial TRL, which was related to their size, estimated as TG/Apo B ratio. This conclusion is consistent with the hypothesis postulated at the beginning of the study. Importantly, TG were cleared from TRL in a polarity-dependent manner, with the most apolar TG molecular species being removed in the first place. Nevertheless, the study has some limitations. Firstly, despite being in agreement with similar postprandial studies [7,8,10-12], the number of participants was rather low and focused in one gender only. Secondly, TRL particle size was estimated from the Apo B and TG content. Direct methods, such as dynamic light scattering [25], can give a more precise estimation of particle size. Finally, the ECN is not a continuous variable for which it should not be used to predict the actual TG clearance from TRL.

ACKNOWLEDGMENT

This work was supported by funds from Comisión Interministerial de Ciencia y Tecnología (CYCIT, AGL2011-23810). The authors declare no conflicts of interest.
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Fig. 1 Apolipoprotein B (Apo B) concentrations in triglyceride-rich lipoproteins collected at 2, 4 and 6 hours after the intake of refined olive oil (OLIVE, black bar) or pomace olive oil (POMACE, grey bar). Statistical significance of differences between the effects of both experimental oils was evaluated by paired t-test analyses. *: \( p < .05 \), **: \( p < .01 \), vs. OLIVE. Data are expressed as means ± SD, \( n = 10 \).

Fig. 2 Triacylglycerol molecular species to apolipoprotein B ratio (TG/Apo B) in triglyceride-rich lipoproteins collected at 2, 4 and 6 hours after the intake of refined olive oil (OLIVE, black bar) or pomace olive oil (POMACE, grey bar). Statistical significance of differences between the effects of both experimental oils was evaluated by paired t-test analyses.*: \( p < .05 \), **: \( p < .01 \), ***: \( p < .001 \), vs. OLIVE. 2A: trioleoyl-glycerol, OOO; 2B: palmitoyl-dioleoyl-glycerol, POO; 2C: palmitoyl-oeloyl-linoleoyl-glycerol, POL; 2D: dioleoyl-linoleoyl-glycerol, OOL. Data are expressed as means ± SD, \( n = 10 \).

Fig. 3 Variations from 2h to 4h after the intake of pomace olive oil (POMACE, black dots) or refined olive oil (OLIVE, white dots) of triglyceride (TG) concentrations (3A) and TG to apolipoprotein B ratios (TG/Apo B, 3B) against the equivalent carbon number (ECN). Correlations between variables were assessed using Pearson’s correlation coefficients.