ATTENUATION OF INFLAMMATORY PROCESSES ASSOCIATED WITH ALZHEIMER'S DISEASE. AFTER CONSUMPTION OF OLIVE POMACE OIL

FINAL ACTIVITY REPORT

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1. BACKGROUND AND HYPOTHESIS

This project is based on the previous demonstration that triglyceride-rich lipoproteins (TRL) are capable of activating the microglia, a phenomenon associated with neurodegenerative diseases such as Alzheimer's disease (AD) which is involved in the inflammatory process associated with this disease. In addition, the bioactive compounds in olive pomace oil were shown to be capable of attenuating this activation, which was demonstrated by reduced production of inflammatory markers and enhanced antioxidant defense systems. However, these studies were performed using artificial TRL, created in the laboratory with controlled concentrations of the bioactive compounds in the olive pomace oil. Therefore, the hypothesis of this research project aimed to establishes whether **human TRL generated during the postprandial period after olive pomace oil ingestion incorporates the fat-soluble bioactive components of the oil and thus retains its protective capacity against microglial activation and inflammatory response.**

If this hypothesis holds true, the evidence obtained would lay the foundations for the development of new health applications of olive pomace oil, based on the influence of TRL formed in the postprandial period and which could be used to prevent and treat Alzheimer's disease.

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2. OBJECTIVES

To test the above hypothesis, two specific objectives will be addressed:

1. To obtain and characterize postprandial human TRL after ingestion of olive pomace oil rich in its bioactive components.
2. To assess the attenuating effect of microglial activation by postprandial TRL obtained after olive pomace oil ingestion.

3. METHODS

3.1. Selection of volunteers and administration of the oils in the experiment

The study was designed as a randomized, crossover trial in postprandial phase. Healthy female volunteers, with similar characteristics, were divided into two groups, and administered olive pomace oil (OPO) or high oleic sunflower oil (HOSO) as a control. The olive pomace oil was supplied by Laura Bravo's group (ICTAN- CSIC), from previous projects with ORIVA, while the high oleic sunflower oil was purchased from a retailer.

The postprandial trial was performed in accordance with the basic principles from the Declaration of Helsinki, the Council of Europe Convention and the UNESCO Universal Declaration and took place in the emergency department at the Hospital Virgen del Rocío in Seville. 18 healthy, average weight adult female volunteers (aged 18-26) without any digestive or metabolic disorders were selected. Before taking part, the volunteers signed their consent to a protocol approved by the Institutional Bioethics Committee of the CSIC, after being informed about the experimental procedure in spoken and written form.

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The anthropometric characteristics of the volunteers were determined using standardized measuring tapes, stadiometer and bioimpedance analysis (Tanita) (Table 1). In addition, basal biochemical tests were conducted to ensure the health status of the participants (Table 2).

The volunteers in the study were randomly assigned to each intervention group (OPO or HOSO). On the day of the trial, and after a 12-hour overnight fast, a blood sample was drawn from each participant by puncturing the ulnar vein. They then received a dose of OPO or HOSO, which they ingested as part of a breakfast that included skimmed milk, toast with whole-grain bread and tomato. The composition of the meals for the experiment and oils is shown in Tables 3 and 4. After ingestion, aliquots of ulnar blood were drawn from each participant every hour over a 6-hour postprandial period. At 0 (basal), 2 and 4 hours of the postprandial period, a larger volume of blood was collected to isolate TRL. After a 4-week washout period, the postprandial trial was repeated, swapping over the experimental oil so that both groups received both oils and acted as their own controls.

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3.2. Isolation and characterization of human triglyceride-rich lipoproteins (TRL).

Sera were obtained by centrifugation from the blood aliquots obtained at 0, 2 and 4 hours and stored at -80 ºC until use. Lipid, glucose, and insulin concentrations were determined in the blood serum (Table 3).

TRLs were isolated from sera by ultracentrifugation and used for the microglial activation study. The presence of some of the bioactive components of OPO, such as tocopherols and oleanolic acid, was determined by HPLC connected to a fluorescence detector and by gas chromatography connected to mass spectrometry, respectively.

3.3. Microglial activation in human TRL-treated BV-2 cells

In this activity, the mouse cell line BV-2 was used as a microglial cell model. The cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, streptomycin (100 mg/ml) and penicillin (100 IU/ml) and were treated with the TRLs obtained in the postprandial trial, i.e., after ingestion of OPO or HOSO. Treatments were maintained for 24h. The trials were performed on cultures with a viability above 95%.

In the culture supernatant for the cells treated with TRL, the production of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) was determined using commercial ELISA kits according to the manufacturer's protocols. To determine the gene expression of the cytokines of interest, as well as inducible nitric oxide synthase (iNOS), we used the PCR technique in

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real time after retrotranscription to cDNA, using primers designed for this purpose. The fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) was used to determine the intracellular concentration of ROS. The intensity of the emitted fluorescence was measured at 825 nm, after excitation at 485 nm. The determination of the concentration in the culture medium of nitric oxide was conducted via the Griess reaction.

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4. RESULTS

4.1. Composition of the meals and oils in the experiments conducted in the study.

Table 1 shows the composition of the meals used in the experiments for the study, according to the information declared on the package labels. Breakfast consisted of 3 slices of wholegrain bread, a glass of skimmed milk and OPO or HOSO. In addition, any volunteers who wished to do so could add crushed tomato. Oil was clearly the ingredient that contributed the most to the energy content to the breakfast, accounting for 66% of the total calories, while whole-grain bread and milk contributed 22% and 10%, respectively. Similarly, oil contributed 96.7% of the total fat content of the breakfast, so the contribution of the other ingredients was insignificant. The carbohydrate content was principally due to whole-grain bread, mainly in the form of starch, while milk contributed sugars in the form of lactose. The meals used in the experiments did not contain appreciable amounts of cholesterol, but the wholegrain bread did contribute dietary fiber.

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Table 1. Composition of the meals in the experiments

	Bread whole-grain	**Milk skimmed**	**Tomato**	**Oil**	**Total**
Total (g)	71	208	20	50	329
Energy (kcal)	149	71	4	450	674
Energy (kJ)	621	295	15	1875	2806
Protein (g)	6.4	6.2	0.2	0.0	12.8
Carbohydrates (g)	27.7	9.4	0.6	0.0	39.7
Sugar (g)	1.7	9.4	0.0	0.0	11.1
Polyalcohols (g)	0.8	0.0	0.0	0.0	0.8
Fats (g)	1.1	0.6	0.1	50.0	51.8
Cholesterol (g)	0.0	0.0	0.0	0.0	0
Fiber (g)	6.4	0.0	0.3	0.0	6.7

* High-oleic sunflower oil (HOSO) or olive pomace oil (OPO).

Table 2 shows the fatty acid composition of the two oils used in the experiments conducted in the study. Both oils were characterized by a similar fatty acid content, with a majority presence of oleic acid (18:1), slightly higher in the HOSO, as well as palmitoleic acid (16:1), although the presence of the latter was negligible. On the other hand, the palmitic acid content (16:0) was higher in the OPO. The HOSO also had somewhat higher concentrations of stearic acid (18:0) and linoleic acid (18:2). In short, **the composition was very similar, although the monounsaturated fatty acid content was slightly higher in the HOSO and the saturated fatty acid content was slightly higher in the OPO.**

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Table 2. Fatty acid composition of high oleic sunflower oil (HOSO) or olive pomace oil (OPO) (%).

	HOSO	OPO
16:0	4.1	11.6
16:1	1.3	1.0
18:0	4.0	2.8
18:1	77.4	71.0
18:2	14.3	11.5
20:0	0.1	0.5
18:3	0.3	0.8
20:1	0.1	0.3
22:0	0.9	0.2
Others	0.3	0.3

In addition, the composition of some of the best components of interest in the oils was determined. **HOSO showed a total tocopherol content of 393.2 mg/kg, practically identical to that contained in the OPO (389 mg/kg). In contrast, the total sterol content was much higher in OPO (2839mg/kg) compared to HOSO (1497 mg/kg)**, **almost double. There was also a significant presence of Squalene (799 mg/kg), oleanolic acid (187mg/kg) and erythrodiol+uvaol (638.5 mg/kg) in the OPO.**

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4.2. Basal characteristics of the volunteers participating in the study.

The volunteers in the study, with a mean age of just under 21, presented anthropometric data consistent with normal parameters both in terms of weight and height, as well as in body composition and the anthropometric indices usually used to diagnose obesity, such as body mass index (BMI), waist and hip circumferences and the indices calculated from these values (Table 3).

Table 3. Anthropometric characteristics of volunteers

	Mean	SD
Age (years)	20.9	1.8
Weight (kg)	57.2	6.1
Height (cm)	162.3	5.2
Waist circumference (cm)	68.6	5.0
Hip circumference (cm)	94.4	4.2
Fat mass (kg)	15.4	3.2
Fat mass (%)	26.3	4.7
Lean mass (kg)	41.6	3.7
Total water (kg)	30.4	2.7
Visceral fat (kg)	1.5	0.6
Height (m)	1.62	0.05
BMI	21.4	2.1
Waist-to-hip ratio	0.73	0.04
Waist-to-height ratio	0.42	0.03

BMI: body mass index. SD: standard deviation. N=18

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In addition, biochemical determinations in blood plasma were performed to ensure the suitability of the volunteers participating in the study (Table 4). Basal plasma lipid concentrations, as well as glucose and insulin concentrations, were found to be within normal reference ranges.

Table 4. Plasma biochemical characteristics of the female volunteers

	Mean	**SD**
Glucose (mg/dL)	69.8	6.6
Cholesterol (mg/dL)	177.8	34.6
Triglycerides (mg/dL)	78.4	17.7
HDL-cholesterol (mg/dL)	65.6	16.7
LDL-cholesterol (mg/dL)	95.5	26.1
Insulin (µUI/mL)	7.8	3.5

SD: standard deviation. N=18

4.3. Effect on postprandial biochemical parameters.

Figures 1 to 6 show the evolution of plasma levels of glucose, insulin, triglycerides, cholesterol, LDL-cholesterol, and HDL-cholesterol throughout the postprandial period.

Glucose levels did not change significantly after ingestion of the meals in the experiment during the postprandial period (Figure 1). A slight increase in plasma glucose concentrations was observed in the first hour after ingestion of the breakfasts, due to their carbohydrate content. Afterwards, the levels drop again to remain stable until the sixth hour, when another increase is observed, probably due to the production of

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glucose as a compensatory mechanism for the lack of nutrients after several hours of fasting. No significant differences were found between the two oils studied.

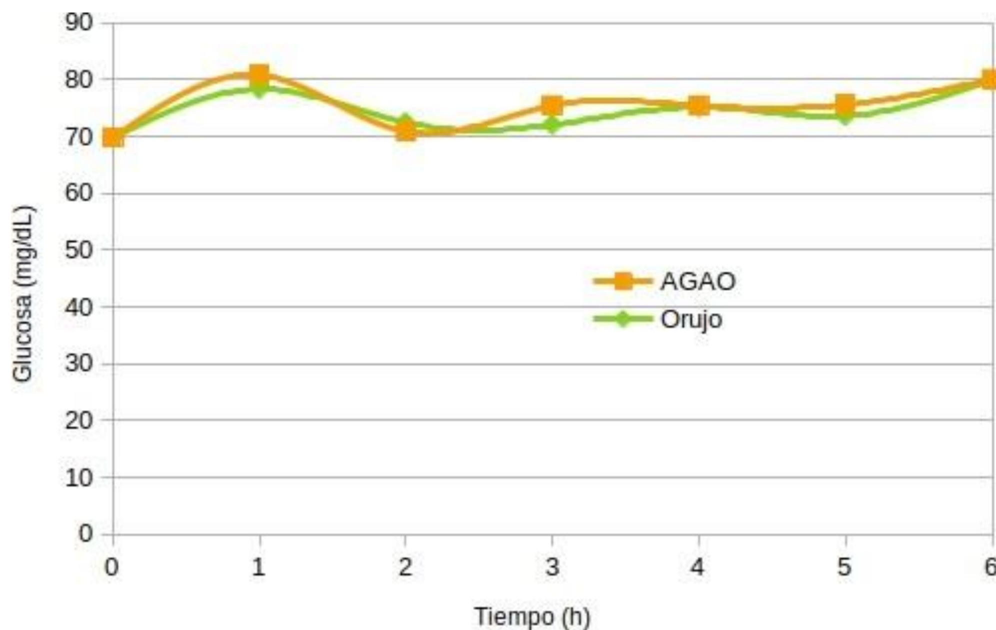

Figure 1. Postprandial plasma glucose concentrations after ingestion of a meal rich in high oleic sunflower oil (HOSO) or olive pomace oil (OPO). N=18.

The food intake in the experiment had a marked effect on plasma insulin levels, with an insulinemic peak being observed in the first hour of the postprandial period (Figure 2). The concentrations of the hormone gradually decreased in the subsequent hours, reaching basal levels in the last hour analyzed. **Consumption of the breakfast containing OPO resulted in a lower postprandial insulinemic response than the breakfast containing HOSO**, which was evident at hour 2 after ingestion. The same result was observed 1 h later, although in this case the difference was not statistically significant. These data point to a greater reduction in postprandial insulin levels, once the maximum peak is reached, when OPO is consumed.

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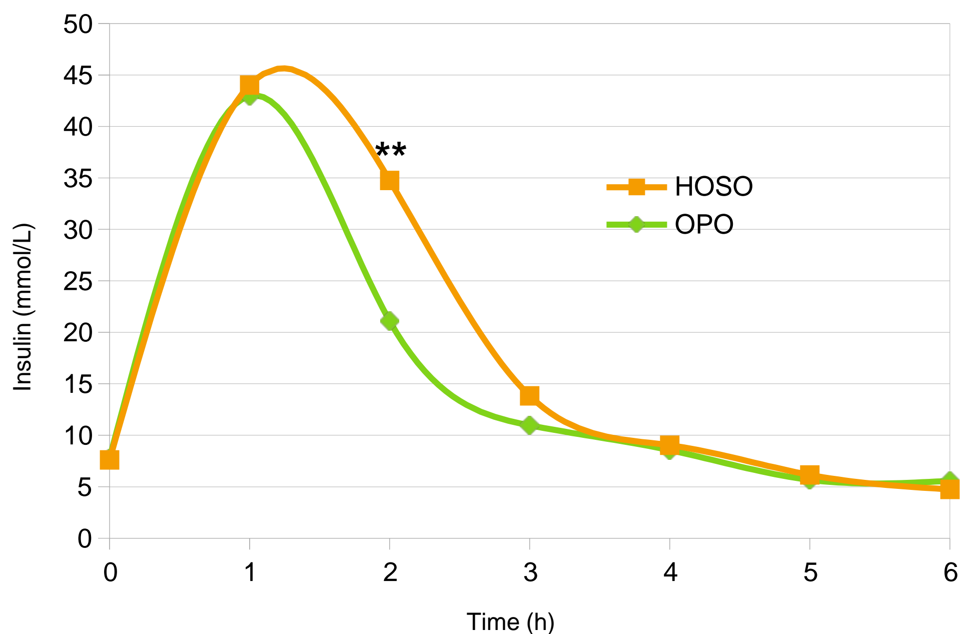

Figure 2. Postprandial plasma insulin concentrations after ingestion of a meal rich in high oleic sunflower oil (HOSO) or olive pomace oil (OPO). **, p<0,01. N=18.

After analysis of plasma triglycerides in the postprandial period, a maximum peak concentration was observed around 2 hours after the breakfasts in the experiment were consumed (Figure 3). In the case of HOSO, the peak was slightly delayed and, although the values returned to basal levels after the fourth hour, they did so more slowly than after the OPO intake, although the difference was not significant. **This result indicates that OPO is slightly more effective at clearing plasma triglycerides than HOSO.**

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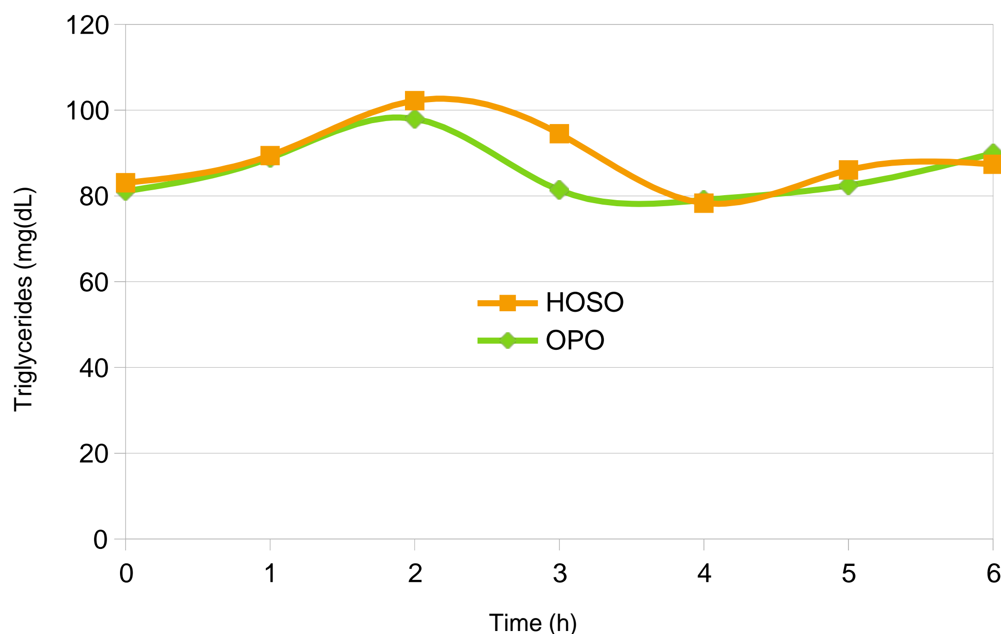

Figure 3. Postprandial plasma triglyceride concentrations after ingestion of a meal rich in high oleic sunflower oil (HOSO) or olive pomace oil (OPO). N=18.

As for plasma concentrations of cholesterol, LDL-cholesterol, and HDL-cholesterol, no significant changes were found throughout the postprandial period after ingestion of either of the two oils in the study (Figures 4, 5 and 6). This result was to be expected since it is known that cholesterol concentrations do not vary significantly in the postprandial phase.

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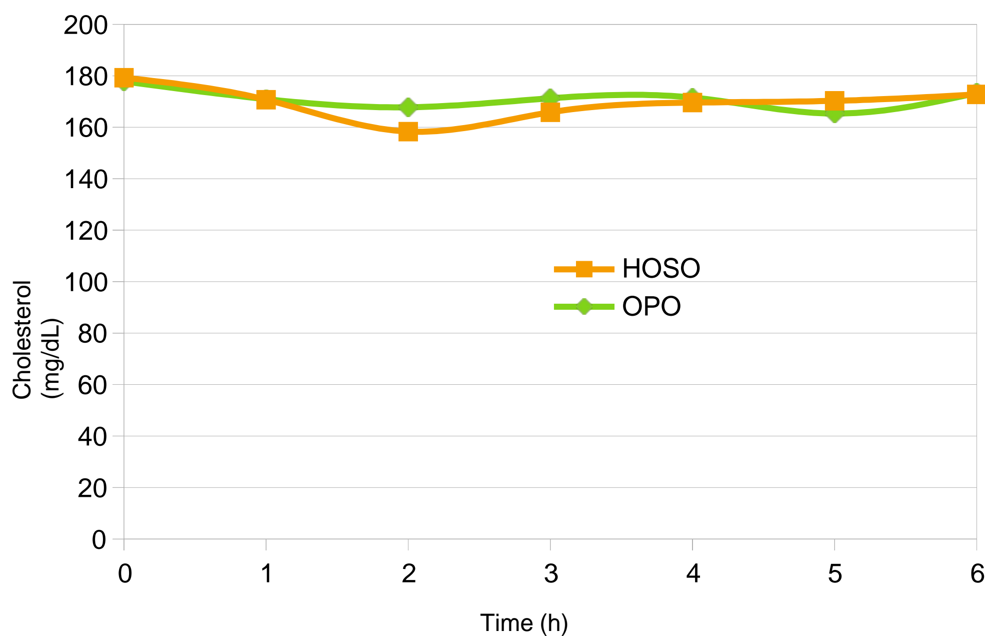

Figure 4. Postprandial plasma cholesterol concentrations after ingestion of a meal rich in high oleicsunflower oil (HOSO) or olive pomace oil (OPO). N=18.

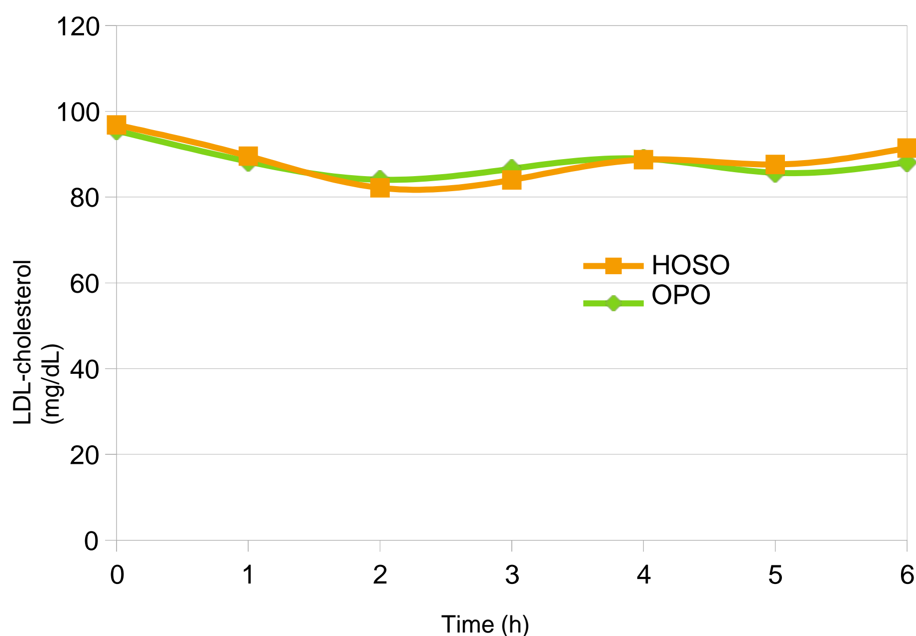

Figure 5. Postprandial plasma concentrations of LDL-cholesterol after ingestion of a meal rich in higholeic sunflower oil (HOSO) or olive pomace oil (OPO). N=18.

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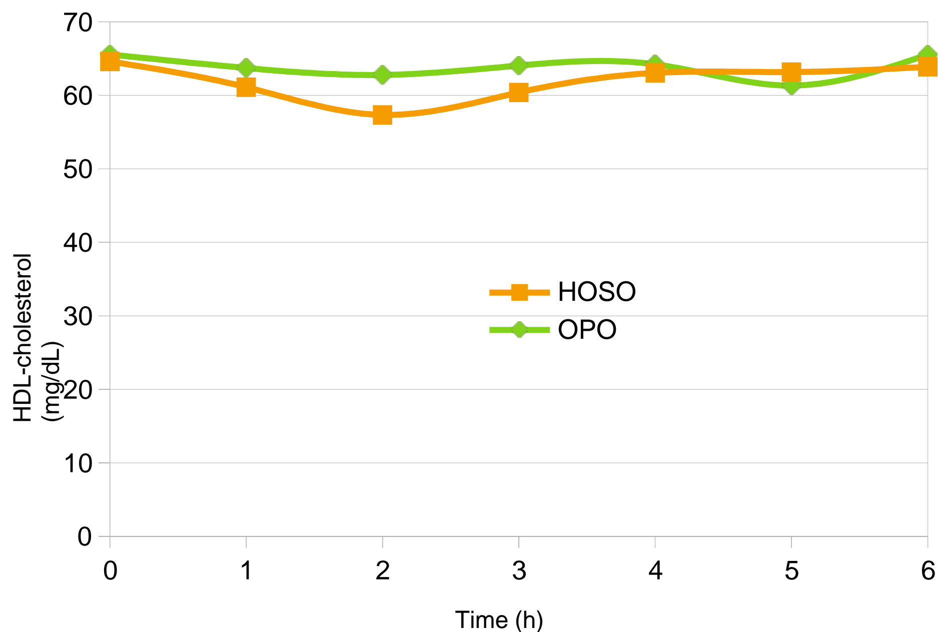

Figure 6. Postprandial plasma HDL-cholesterol concentrations after ingestion of a meal rich in high oleic sunflower oil (HOSO) or olive pomace oil (OPO). N=18.

TRL were isolated from blood sera obtained after ingestion of both oils and their apolipoprotein B composition, which serves as an indicator of TRL particle number, was analyzed. As can be seen in Figure 6, the number of TRL particles decreased throughout the postprandial period, suggesting a gradual clearance. Except for the phase prior to ingestion of the breakfasts, it was observed at all times studied (2 and 4 hours postprandial), that the apolipoprotein B content was higher in the TRL obtained after consumption of HOSO compared to olive pomace oil, although the difference was not statistically significant. **This result suggests that the number of circulating particles after consumption of OPO is lower throughout the postprandial period.**

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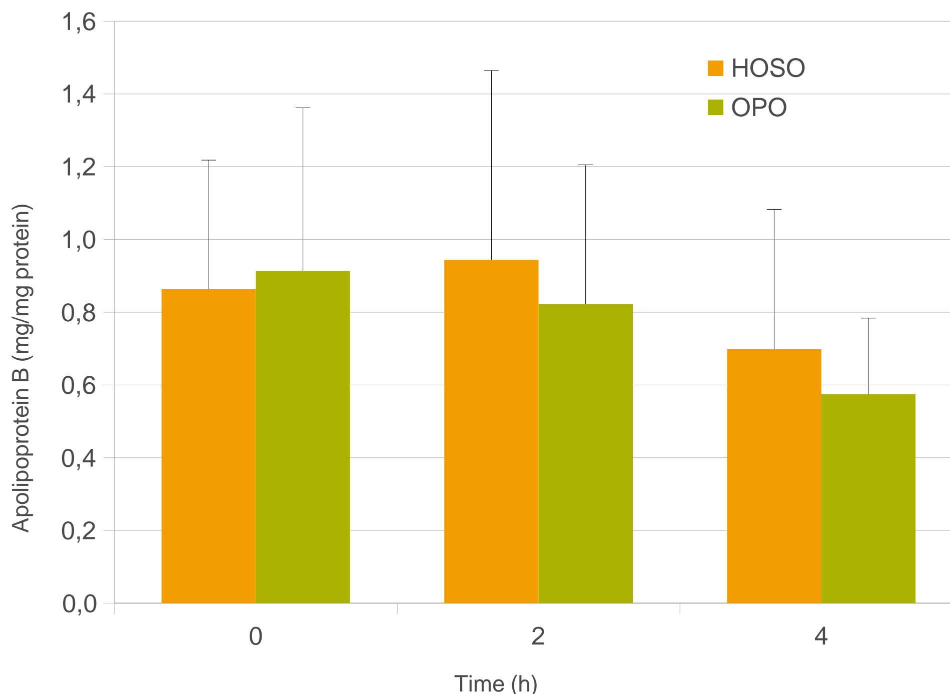

Figure 6b. Postprandial plasma concentrations of apolipoprotein B in triglyceride-rich lipoproteins (TRL) after ingestion of a meal rich in high oleic sunflower oil (HOSO) or olive pomace oil (OPO). N=18.

As mentioned above, the composition of the two oils in the experiment was similar, especially in terms of fatty acid content, particularly oleic acid. In contrast, the olive pomace oil contained higher concentrations of some minority compounds, such as sterols, squalene and triterpenic compounds. These substances are liposoluble or have a certain capacity to dissolve in lipid media, so they must be transported in blood incorporated into TRL. Therefore they would be capable of reaching the encephalic cells and interacting with them, exercising their protective activity.

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This study attempted to determine some of these compounds in human TRL, **which are not described in scientific literature to date due to the methodological difficulties involved in the procedure**, since the quantity of particles obtained from human blood serum is very limited. Specifically, we were able to determine oleanolic acid and α-tocopherol in TRL, by gas chromatography-mass spectrometry and HPLC-fluorescence, respectively, but not β-sitosterol, as the preparative procedure for the latter requires a much larger sample. Except in the TRLs of one of the volunteers, **it was not possible to detect oleanolicacid in these lipoproteins, indicating that this bioactive compound is not transported by this route**. Previous studies, conducted in our research group, demonstrated that the main transport route of oleanolic acid is bound to albumin. As a control of the assay, the presence of cholesterol in TRL was determined. This measurement was performed at 2 h after the breakfasts, since this was the time when the highest TRL was observed, estimated by the plasma triglyceride content and the apolipoprotein B concentration. The concentration of cholesterol was slightly higher in the TRL from the HOSO intake compared to those obtained after the OPO intake (0.16±0.10 vs. 0.12±0.09 mg/mg of protein, respectively), but the differences were not significant.

Determining tocopherols by HPLC-fluorescence allowed the detection and quantification of α-tocopherol and β-tocopherol in TRL obtained 2 hours after ingestion of breakfast. As expected, **no differences were found**

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in α-tocopherol content among TRL obtained after HOSO or pomace oil intake (1.48±1.35 vs. 1.00±0.94 ng/mg protein, respectively). In contrast, **OPO consumption resulted in a higher presence of β-tocopherol in TRL (**0.56±0.16 ng/mg protein) compared to TRL from OUFA intake (0.28±0.14 ng/mg protein).

4.4. BV-2 microglial cell activation by TRL

To determine whether olive pomace oil ingestion can attenuate microglial activation compared to HOSO when its components are transported in the form of postprandial TRL, these particles were incubated with murine BV-2 cells for 24h. After the TRL treatment period, obtained at different times in the postprandial phase, various activation markers were analyzed, such as the presence of pro- inflammatory cytokines and redox state markers.

4.4.1. TRL capture by microglial cells

The capture capacity of TRL by microglial cells was qualitatively determined by oil red O staining of intracellular lipids. As can be seen in Figure 7, in the absence of TRL (control), no intracellular lipid droplets (red color) are observed, whereas in the presence of TRL they are abundant, **indicating that the lipid content of these particles has been incorporated by the cells**.

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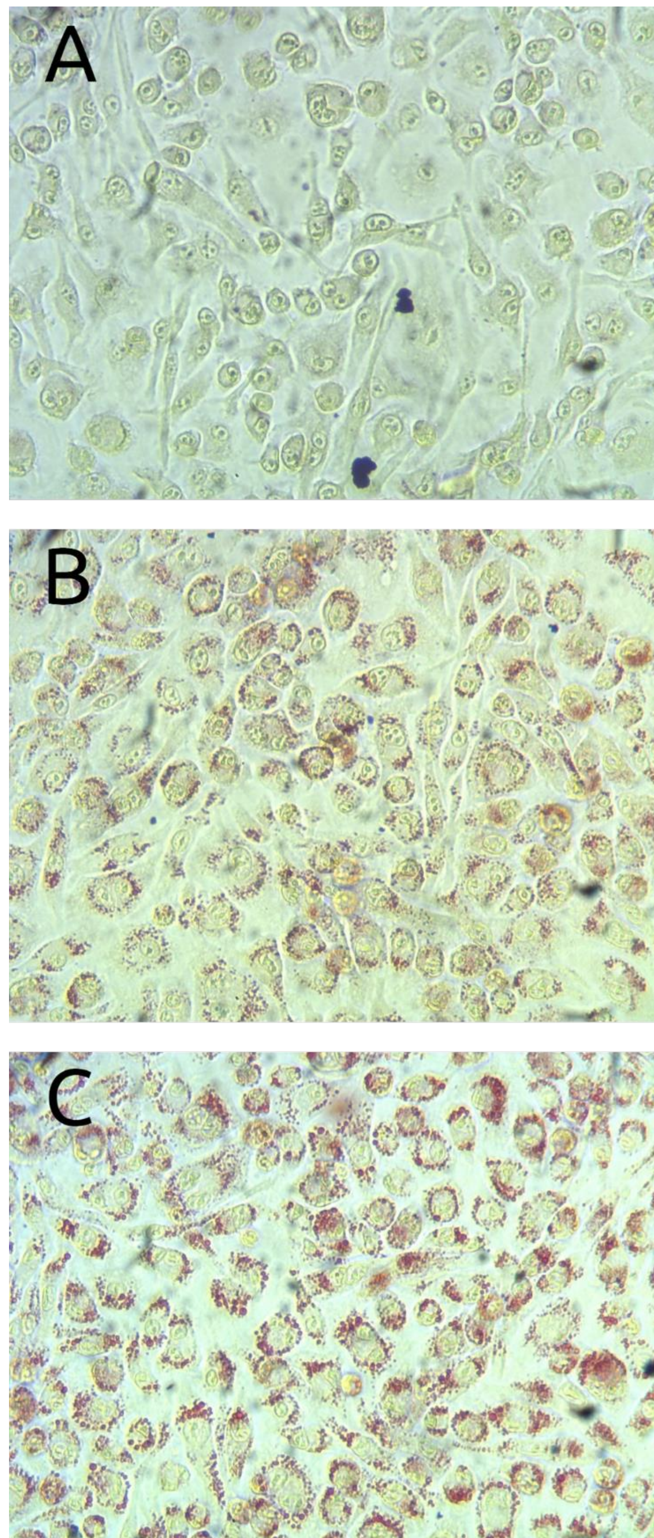

Figure 7. Microphotographs of BV2 cell culture stained with Oil Red O, after being treated for 24h inthe absence (A) or presence of TRL obtained 2h after ingestion of HOSO-rich breakfast (B) or OPO (C).

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4.4.1. Production of pro-inflammatory cytokines

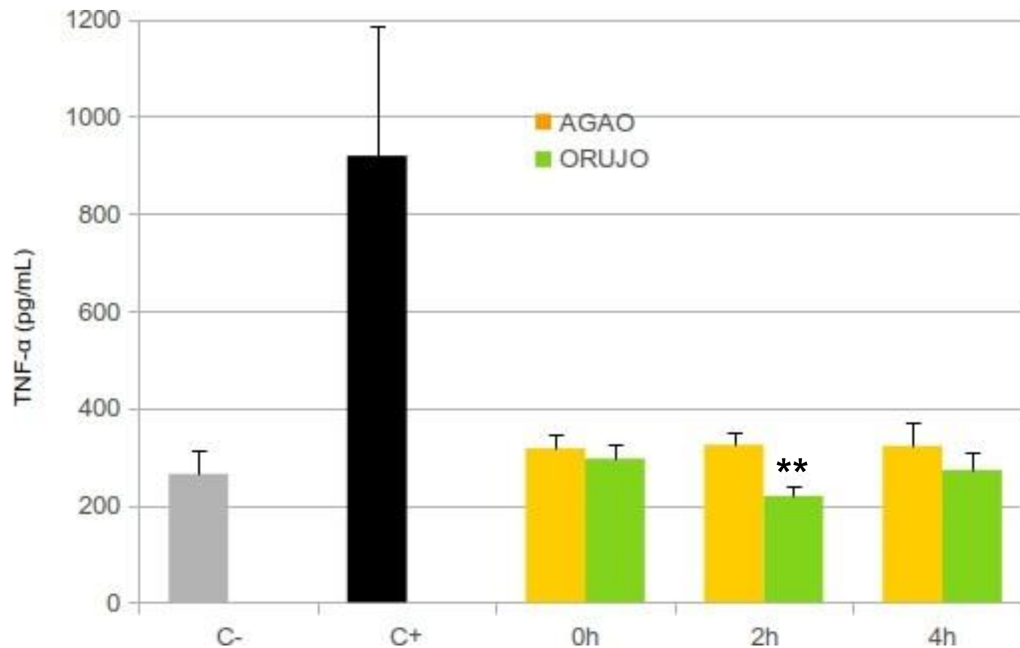

Figure 8. TNF-α concentrations after treatment of BV-2 microglial cells with saline (negative control C-, gray bar), lipopolysaccharide (LPS, positive control C+, black bar) or postprandial TRL from HOSO or pomace oil ingestion. **, p<0,01. Values expressed as mean±SEM.

Cellular activation of microglia is manifested by the release of various inflammatory markers, among which pro-inflammatory cytokines such as TNF-α and IL-6 stand out. Figure 8 shows the effect on TNF-α production after treatment of microglia with TRLs from the ingestion of HOSO or OPO. TRL induced a slight increase in TNF-αrelease by the cells compared to the negative control, but this was much lower than the effect of LPS (positive control). Nonetheless, **the concentrations found were always lower after treatment with TRLs from the intake of olive pomace oil**. The difference was very

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significant at 2 hours, although lower values were also observed at hour 4, with a non-significant difference.

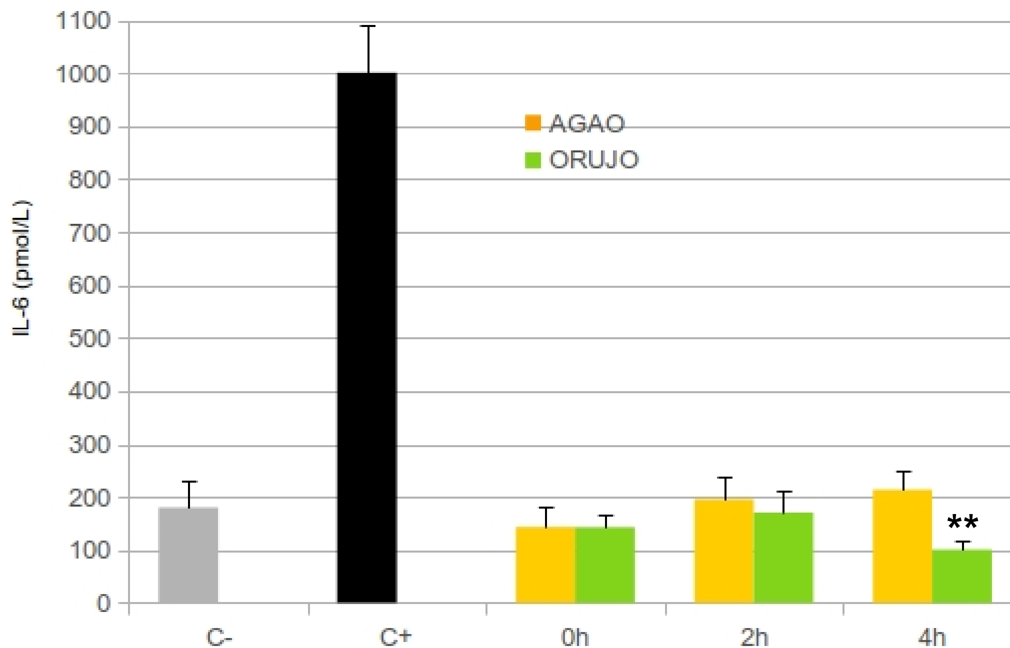

Figure 9. IL-6 concentrations after treatment of BV-2 microglial cells with saline (negative control C-, gray bar), lipopolysaccharide (LPS, positive control C+, black bar) or postprandial TRL from HOSO or pomace oil ingestion. **, p<0,01. Values expressed as mean±SEM.

Figure 9 shows the release of IL-6 after incubation of BV-2 cells with TRL obtained after ingestion of the two assay oils, plus the corresponding negative and positive controls (LPS). As in the case of TNF-α, TRL treatment raised IL-6 concentrations, but to a much lesser extent than LPS. Predictably, at time 0, when the volunteers had not yet consumed the oils, no differences were observed between treatments.

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In contrast, at hours 2 and 4, it was observed that OPO contains the release of this cytokine better, although the difference was only significant at 4h.

4.4.2. Gene expression of proinflammatory cytokines.

To clarify the molecular mechanisms involved in the production of pro- inflammatory cytokines, the expression of genes that regulate their synthesis was studied.

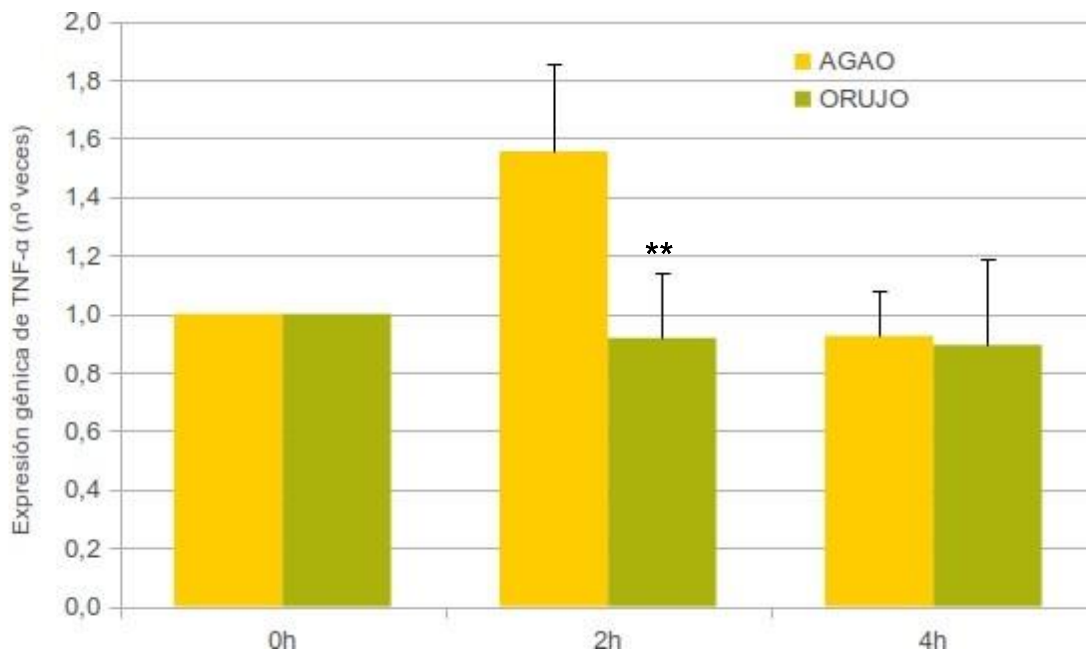

Figure 10. Gene expression of TNF-α after treatment of BV-2 microglial cells with postprandial TRLs from OFA or pomace oil ingestion. Values referred to 0h. **, p<0,01. Values expressed as mean±SEM.

Gene expression values are shown as the number of times a gene is expressed more after one treatment or another, the expression at 0h serving as the reference point, at which time the volunteers had not yet ingested breakfast in the experiment.

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In the case of TNF-α (Figure 10), it is observed that in TRL-treated cells obtained 2 hours after ingestion of the breakfasts in the experiments, **the level of expression is much lower after consumption of the olive pomace oil, which is consistent with the production of the protein** (Figure 8). Therefore, it is highly likely that the observed effect on the release of this inflammatory marker is due interaction of the components of the OPO with the gene coding for this cytokine.

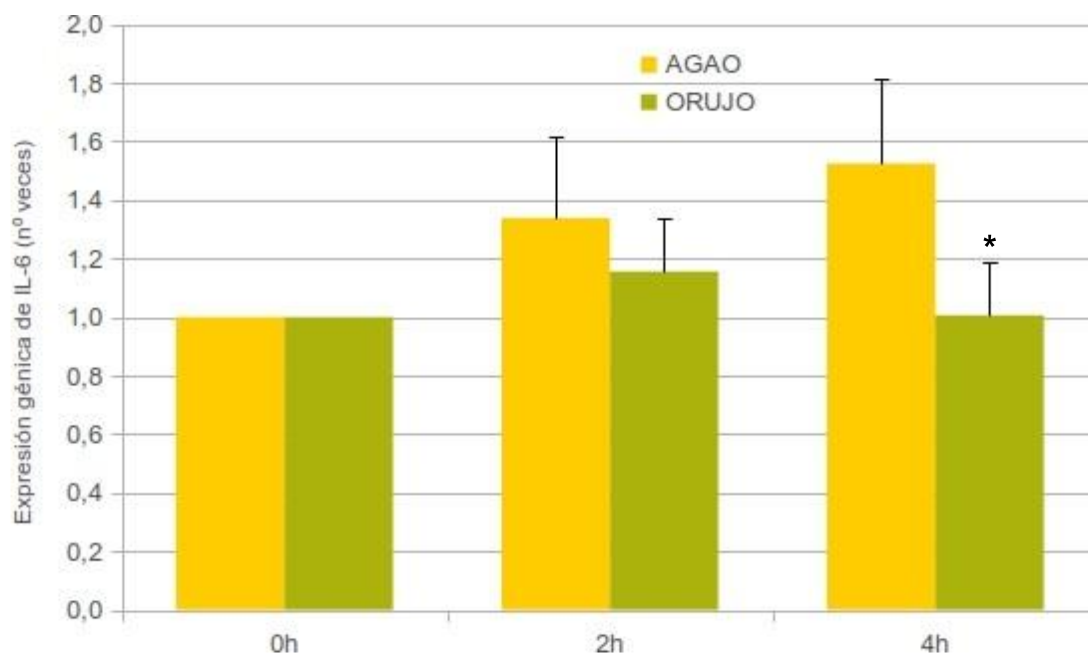

Figure 11. IL-6 gene expression after treatment of BV-2 microglial cells with postprandial TRL from HOSO or OPO ingestion. Values referred to 0h.
*, p<0,05. Values expressed as mean±SEM.

Similarly, **treatment with TRL originating after consumption of olive pomace oil resulted in lower expression of the gene encoding the cytokine IL-6 (**Figure 11), at both hour 2 and 4, although statistical significance was only reached in the latter. The results again indicate that release of this proinflammatory cytokine by BV-2 cells found after consumption of OPO are related to a lower expression of its gene.

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4.4.3. Nitric oxide production and iNOS gene expression

During the inflammatory response following microglia activation, elevated nitric oxide levels have been detected, which are mediated by the the inducible nitric oxide synthase (iNOS) gene expression.

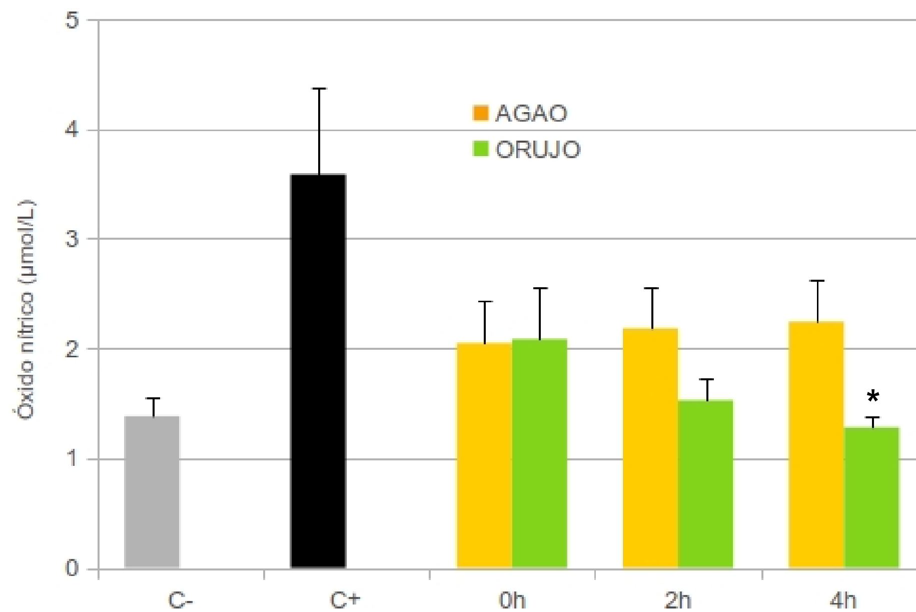

Figure 12. Nitric oxide concentrations after treatment of BV-2 microglial cells with saline (negative control C-, gray bar), lipopolysaccharide (LPS, positive control C+, blackbar) or postprandial TRLs from HOSO or pomace oil ingestion. *: $p<0,05$. Values expressed as mean±SEM.

Figure 12 shows that treatment of BV-2 cells with postprandial TRL resulted in an increase in nitric oxide release by the cells compared to the negative control, but it was not as high as

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that of LPS aggression. **When cells incorporated TRL formed after olive pomace oil ingestion, nitric oxide production was lower compared to TRL generated after HOSO consumption**, the difference being significant at 4h.

However, this effect could not be associated with iNOS gene expression, as although an increase in the postprandial times studied was observed, no significant differences were found.

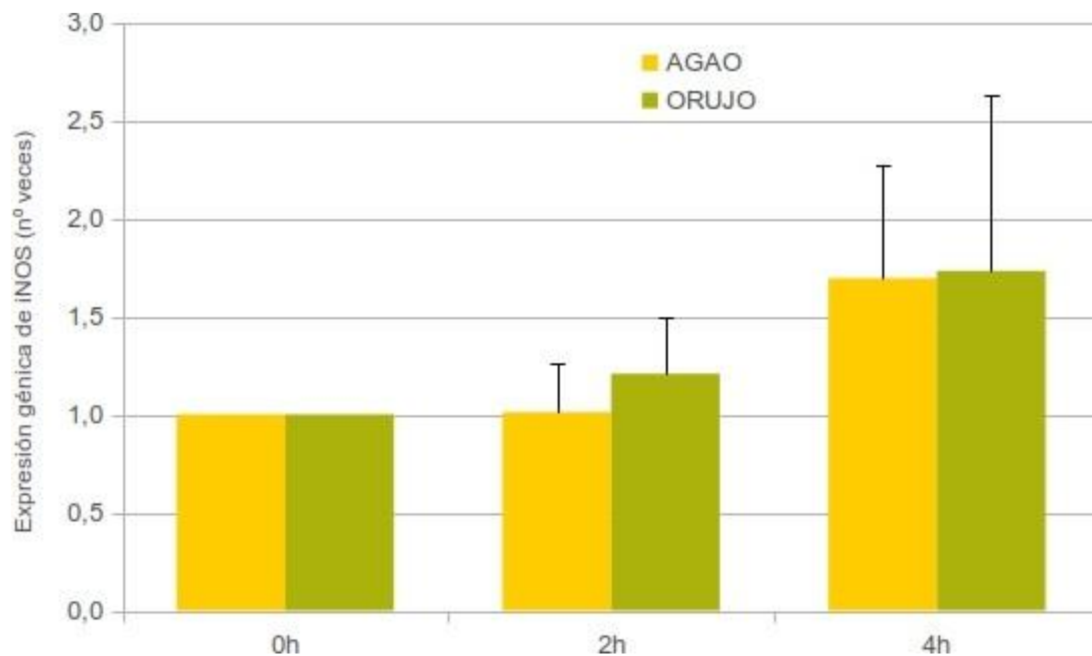

Figure 13. Gene expression of iNOS after treatment of BV-2 microglial cells with postprandial TRLs from HOSO or OPO ingestion. Values referred to 0h. Values expressed as mean±SEM.

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4.4.4. Production of reactive oxygen species

BV-2 cells were stimulated with TRL in the same way as in the previous experiments, but in this case, in addition with hydrogen peroxide and only with particles obtained 0h and 2h after ingestion of the breakfasts in the experiment. The ingestion of both breakfasts resulted in the formation of TRL that caused an increase in cellular production of ROS, **although it was more moderate when the meal contained olive pomace oil.**

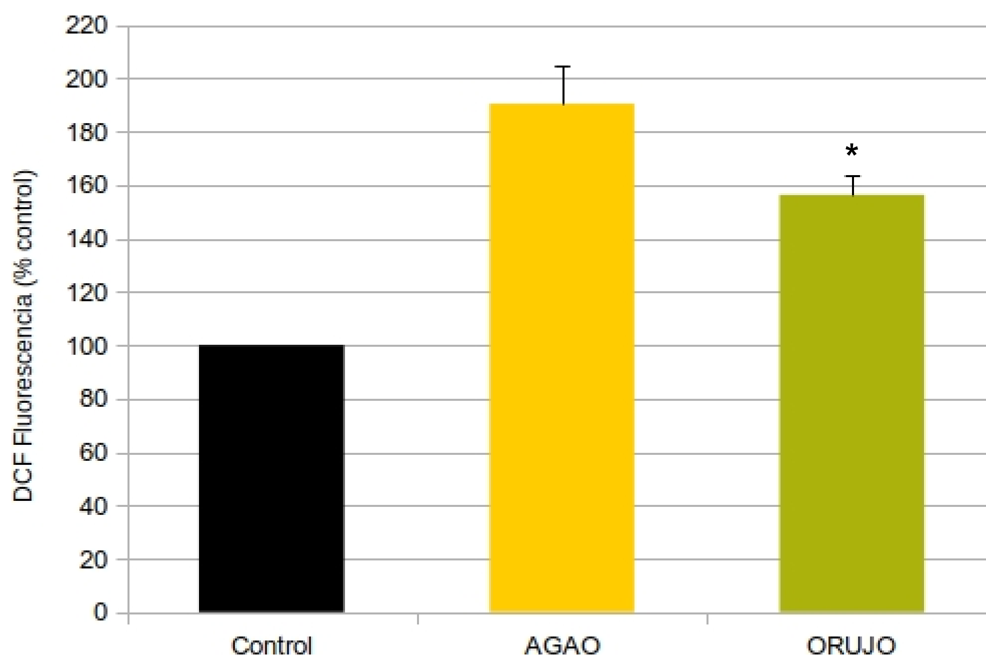

Figure 14. Reactive oxygen species production after treatment of BV-2 microglial cells with postprandial TRLs obtained 2 h after HOSO or OPO ingestion. Values expressed as mean±SEM in percentage with respect to control (TRL obtained at 0 h). *, p<0,05.

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5. INTERPRETATION OF THE RESULTS

In a previous study, we demonstrated that TRLs manufactured in the laboratory are capable of triggering microglial activation, a phenomenon associated with neurodegenerative diseases such as Alzheimer's. In addition, we reported that some of the bioactive compounds in olive pomace oil had the ability to attenuate such activation when added to cells in the form of TRLs. Furthermore, we noted that some of the bioactive compounds in olive pomace oil had the ability to attenuate such activation when added to cells in the form of TRLs. Therefore, the next logical step was to demonstrate that human TRLs generated after olive pomace oil ingestion maintain the same protective capacity and are capable of attenuating the inflammatory response caused by microglial activation.

Olive pomace oil and HOSO share compositional characteristics, since both are rich in oleic acid, its most abundant fatty acid. The oils used in this study had a concentration of more than 70% oleic acid, although the content in HOSO was slightly higher (77.4% vs. 71.0%). On the other hand, these two oils differ in their content of minority components, which, although they represent a small fraction of the total lipid composition, are very significant due to their high biological activity. In this case, both oils presented a similar content in tocopherols. In contrast, the total sterol content was much higher in the olive pomace oil and a significant presence of squalene, oleanolic acid and erythrodiol+uvaol was found. As the rest of the components of the breakfasts in the experiment were identical, the differences observed in the effects of the oils must be attributed to the different composition of the minor components.

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The similarity in the tocopherol content between the two oils was reflected in the concentration of these compounds in the TRL, although the β- tocopherol content was higher after ingestion of the olive pomace oil. Given the methodological difficulties involved in the determination of sterols in TRL, it was not possible to determine them. However, it was possible to analyze the presence of oleanolic acid, although it could not be detected in TRL. This result indicates that its transport does not occur through the TRL route, confirming previous studies that demonstrated albumin-bound transport.

Young (mean age 20.9 years) healthy women were recruited for this study, who stated that they had no metabolic or other diseases that could interfere with the results of the study. The levels of anthropometric and general biochemical parameters were within the normal range. The volunteers in the experiment ingested breakfasts, in which they were given either HOSO or OPO, and blood samples were taken within six hours after ingestion of the oils. These blood samples were used for biochemical analysis and for the isolation of TRL, particles carrying lipid components of the diet.

Postprandial biochemical analyses showed that the intake of the two oils used in the experiment had no effect on plasma levels of cholesterol (total, LDL and HDL) and glucose. This result was expected, since it is known that plasma cholesterol levels do not change throughout the postprandial period, regardless of the content

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fat in the food. Long-term dietary interventions are necessary to observe changes in cholesterol levels. Postprandial plasma glucose concentrations do depend on the composition of the meal in the experiments, but in this case no differences were found, probably because the carbohydrate content of the two meals in the experiment was identical.

In contrast, significant differences were found in the postprandial insulin curves after ingestion of the two oils. **The insulinemic curve corresponding to OPO was significantly lower than that corresponding to HOSO, indicating a lower pressure on the system**. Insulin is a key hormone in the metabolism of carbohydrates, but also of lipids, since, among other functions, it regulates the activity of lipoprotein lipase (LPL). This enzyme is responsible for releasing the fatty acids transported in TRL in the form of triglycerides by hydrolysis to facilitate their incorporation into body tissues. This hydrolytic process results in the reduction of plasma triglycerides and the formation of remaining TRL particles, which are smaller in size. These smaller particles are more dangerous because they can more easily pass through vascular tissue in the case of atherosclerosis. In the case of neurodegenerative diseases, remnant TRLs could more easily come into contact with glial or neuronal cells by crossing the blood-brain barrier.

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On the other hand, **a lower concentration, although not significant, was found in plasma triglycerides after consumption of OPO compared to HOSO**. This reduction could be due to a slowdown in the production of chylomicrons by the intestine or to a greater clearance of the TRL already produced. This clearance can occur in two ways. Firstly, through hydrolysis by LPL, already mentioned, and secondly, through the liver capturing and removing the particles. In any case, lower plasma triglyceride levels in the postprandial phase have been related to a lower cardiovascular risk. There is still little evidence on the influence of postprandial triglycerides on neurodegenerative diseases such as Alzheimer's disease, but studies on experimental animals have shown that the presence of TRL can alter the blood-brain barrier. Although human studies have found the presence of TRL components in the brains of Alzheimer's patients, a postprandial intervention reported no inflammatory effect of a high-fat meal. However, the aforementioned study did not assess the lipid composition of the meal, but only the total amount of fat.

Since TRLs can cross the blood-brain barrier, it is of key interest to assess whether these particles can interact with encephalic cell types, such asmicroglia, and whether their composition can affect the activation of these cells. In a previous study, we demonstrated that murine BV-2 mycoglycan BV-2 cells can capture artificial TRLs created in the laboratory. **In this study, we demonstrate that microglia can also incorporate human TRLs**, as evidenced by the presence of their lipid content in the cellular interior, grouped in fat goticules, as occurs in the macrophages during the atherogenic process.

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This is an unprecedented fact, indicating that TRLs would be able to activate microglia, triggering an inflammatory process.

Microglial cells responded to the TRL incubation insult by releasing markers of inflammation, such as the pro-inflammatory cytokines TNF-α and IL-6, although much more modestly than after treatment with LPS, which served as a positive control for the assay. The effect was observed with TRL obtained in the basal phase (fasting) and at 2 and 4 hours after ingestion of the breakfasts in the experiments, suggesting that both VLDL, which are present only in the fasting period, and chylomicrons, present in the postprandial phase, are capable of activating microglia.

In any case, the most important element is that significant differences were observed between treatments with TRL from HOSO and OPO ingestion. **In the case of TNF-α, a lower release by microglial cells was observed when they were treatedwith TRL obtained 2h after the intake of OPO, compared to HOSO**. A similar effect was found with the incubation of TRL obtained after 4h postprandial period, but the difference was not statistically significant. **Similarly, lower IL-6 production was observed in TRL-treated cells obtained 4h after the intake of OPO.** Again, the same effect was observed at 2h, but without statistical significance.

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It is important to note that the results were reproduced in the same way in the expression of the genes that regulate the production of these two cytokines. That is, the expression of TNF-α and IL-6 was lower when the cells were treated withTRL from olive pomace oil intake, obtained at 2 and 4 hours, respectively, postprandially. **These data indicate that the lower release of inflammatory markers and, therefore, the attenuation effect of microglial activation is due to the activity of the components of pomace oil on the cell nucleus**. In this regard,it is known that some of these components, such as β-sitosterol, squalene and the triterpenes oleanolic acid and probably erythrodiol, have the capacity to modulate the activity of the nuclear transcription factor NF-κB, which is intimately linked to cytokineproduction. These bioactive compounds inhibit the translocation to the cell nucleus of NF-κB, reducing the gene expression of pro-inflammatory cytokines and, therefore, their release into the environment.

Stimulation with TRL resulted in an increase in ROS production. It has been shown that these reactive species can act as triggers of the inflammatory process by activating associated metabolic routes. In our study, we found that **olive pomace oil intake led to the formation of TRL with a lower ROS production capacity, compared to HOSO intake**, which could be associated with the inhibition of cytokine release. This ROS-reducing effect is probably due to the ability of the bioactive compounds in pomace oil to restore the levels of antioxidant defense systems, as we already demonstrated in the previous study.

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Another parameter indicating microglial activation is the increased release of nitric oxide. **The results of this study show a lower production of this substance when the cells were treated with TRL obtained after ingestion of the breakfast containing pomace oil**, although the difference with HOSO was only significant at 4h postprandial period. Contrary to the case of cytokines, the release of nitric oxide was not associated with changes in the expression of the iNOS gene, which is responsible for regulating its production, so it is likely that the effect observed is due to post-transcriptional alterations.

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6. CONCLUSIONS

Neuroinflammation and microglial activation are pathological markers of several neurodegenerative diseases, including Alzheimer's disease. There is scientific evidence to suggest that components of dietary oils can impact inflammatory processes associated with these diseases when transported in the form of TRL. In our previous study we demonstrated that the minority components of pomace oil are capable of inhibiting microglia activation, a process associated with the release of inflammatory markers, both in isolation and as part of TRL. Against this background, this study aimed to assess the attenuating effect of microglial activation by postprandial LTR obtained after olive pomace oil intake in comparison with those produced after HOSO consumption. **The results obtained confirmed the data obtained in the previous study.**

In addition, the assays performed in this study, using human TRL obtained from healthy volunteers after olive pomace oil or HOSO ingestion for the treatment of murine BV-2 microglia cells, allow the following conclusions to be drawn:

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1. **Microglial cells were able to capture human TRLs in the same way as artificial TRLs, accumulating in their cytoplasm the lipid content.**

2. **TRLs obtained 2 and 4 hours after olive pomace oil ingestion resulted in a lower release of pro-inflammatory cytokines than TRLs produced after HOSO ingestion, which was associated with a lower expression of genes involved in their regulation.**

3. **These effects were sustained by lower ROS and nitric oxide production when cells were incubated with TRL isolated after consumption of olive pomace oil.**

In short, our results show that the presence of bioactive compounds in olive pomace oil has an attenuating activity on mycloglial activation after TRL stimulation, which is not observed when HOSO is ingested. Thus, olive pomace oil consumption could have a protective effect against Alzheimer's disease by inhibiting the inflammatory processes associated with this pathology.

Seville, August 5, 2021

Signed by SANCHEZ PERONA JAVIER - 16302852S on day 06/08/2021 with a certificate issued by AC FNMT Users

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