**Unsaponifiable and phenolic fractions from virgin olive oil prevent
neuroinflammation skewing microglia polarization toward M2 phenotype**

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8   **Running title:** Minor compounds from VOO on microglia activation

ABSTRACT

Inhibiting M1 microglia phenotype while stimulating M2 microglia phenotype has been suggested as potential therapeutic approach for the treatment of neuroinflammation-related diseases. Our aim was to evaluate the anti-neuroinflammatory effects of minor compounds found in unsaponifiable fraction (UF) and in phenolic fraction (PF) of virgin olive oil (VOO) in BV-2 microglial cells and in brain of mice with high-fat diet (HFD)-induced obesity by RT-qPCR, ELISA and flow cytometry techniques. We observed that UF and PF enhance M2 microglia polarization, whereas LPS polarized microglia prone to M1 phenotype. In addition, in contrast to dietary SFAs, dietary VOO primed for a reduced pro-inflammatory profile in the brain of mice. These findings unveil a potential beneficial role for minor compounds of VOO in regulating the plasticity of microglia. These exciting findings open opportunities for developing nutraceutical strategies with olive oil as principal source of fat to prevent development and progression of neuroinflammation-related diseases.

Keywords: olive oil; phenols; unsaponifiable; microglia; neuroinflammation

1. Introduction

The consumption of virgin olive oil (VOO) appears to be a main determinant of the health benefits of Mediterranean diet (Montserrat-de la Paz et al., 2016). Besides high levels of monounsaturated fatty acids (MUFAs), VOO contains a minor, but yet significant, amount of bioactive constituents, such as phenolic and unsaponifiable compounds (Lopez et al., 2014). Studies have demonstrated that the phenolic and unsaponifiable fractions (PF and UF, respectively) from VOO positively influences oxidative stress and inflammation by modulating the expression of several genes involved in these processes (Aparicio-Soto et al., 2018; Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon de la Lastra, 2014).

Neuroinflammation is considered to play an important role in the pathogenesis and progression of neurodegenerative diseases (Yang, Xu, Qian, & Xiao, 2017). Microglia are the major cellular elements with immune function inside the central nervous system (CNS). Similar to periphery macrophages, it is well known that microglia can alter their phenotypes and functions in response to micro-environmental disturbances. Depending on the predominance of secreted factors, microglia have been characterized to express the classical activation phenotype (M1, pro-inflammatory) or the alternative activation phenotype (M2, anti-inflammatory) (Bottcher et al., 2019). The M1 state causes the release of proinflammatory cytokines with increased expression of CD80 and iNOS. The M2 state with expression of the anti-inflammatory cytokines as well as CD200R and arginase-1 (Arg1) (Sousa et al., 2018; Timmerman, Burm, & Bajramovic, 2018; Tang & Le, 2016; Xu et al., 2015).

1 The evidence of microglia polarization in neuroinflammation-related diseases
2 models led to the idea that, compared with merely suppressing microglia
3 activation, the inhibition of M1 microglia polarization along with promoting M2
4 microglia polarization may be a viable treatment strategy for the treatment of
5 inflammation-related diseases (Wu et al., 2015; Olajide, Kumar, Velagapudi,
6 Okorji, & Fiebich, 2014). However, the effect of minor compounds presents in
7 VOO, particularly unsaponifiable compounds, on microglia activation is scarcely
8 known. In this study, we investigated the potential role of UF and PF in
9 regulating microglia polarization and neuroinflammation.

11 **2. Materials and methods**

12 This study was conducted according to Good Clinical Practice Guidelines and in
13 line with the principles outlined in the Helsinki Declaration of the World Medical
14 Association. Informed consent for the study was obtained. All animal protocols
15 received appropriate institutional approval (Animal Care and Use Committee of
16 the University of Seville) and were performed according to the official rules
17 formulated in the Spanish law on the care and use of experimental animals (UE
18 Directive of 2010: 2010/63/UE; RD 53/2013).

20 *2.1. Preparation of UF and squalene from VOO*

21 VOO of Picual variety was used as a matrix for UF extraction. UF was isolated
22 following conventional procedures, as described IUPAC 2401 (Montserrat-de la
23 Paz, Marin-Aguilar, Garcia-Gimenez, & Fernandez-Arche, 2014; Paquot, 1992).
24 In brief, 5 g of VOO was saponified at 80 °C by refluxing with 50 ml of 2 N
25 potassium hydroxide solution in ethanol (Panreac, Barcelona, Spain), boiling

1 gently until the solution became clear, and then for additional 20 min. Heating
2 was stopped by addition of 50 mL distillate water through the top of the
3 condenser and the solution was swirled. After cooling to 30-35 °C, the solution
4 was rinsed several times with water and UF was extracted with diethyl ether
5 (Panreac) used as solvent for evaporation by distillation on a rotary evaporator
6 (B-480 model, Büchi Labortechnik, Essen Germany) at 30 °C under vacuum.
7 Extracted material was dried in oven at 103 ± 2 °C for 15 min. Sample yields
8 were between 1.3% and 1.4%. Quantitative analysis of UF aliphatic alcohols,
9 sterols, and triterpenic dialcohols was performed according to European
10 Regulation EEC/2568/91 for olive oil. Unsaponifiable matter was extracted as
11 mentioned above and fractioned by thin layer chromatography on a silica gel
12 plate (Kieselgel 60 F254, Merck, Germany) with a mixture of hexane and
13 ethylether (65:35 v/v). Aliphatic alcohols, sterols, and triterpenic dialcohols were
14 identified by their retention factor. The triterpenic dialcohols, erythrodiol and
15 uvaol, recovered from the plate were transformed into trimethylsilyl ethers by
16 adding 200 µL of a mixture of 9:3:1v/v/v of pyridine-hexamethyldisilazane-
17 trimethylchlorosilane and analysed by gas chromatography (GC) using an HP
18 5890 series II gas chromatograph equipped with a flame ionization detector and
19 a 30 m, 0.32 mm i.d., Tracsil TRB-5 (95% dimethylpolysiloxane, 5% diphenyl,
20 film thickness 0.25 µm) capillary column (Teknokroma, Barcelona, Spain). The
21 chromatographic conditions were as follows: injector 300 °C, isothermal column
22 275 °C, and detector 300 °C. The split ratio was 1:50 and the hydrogen flow
23 rate of 1.0 ml/min, 130 Kpa. The chromatographic conditions for alcohol
24 determinations were the same as those mentioned above for sterols and
25 triterpene diols, except that oven temperature was 215 °C (5 min) and then 3

1 °C/min increased to 290 °C, and held for 2 min. The quantifications were based
2 on the peak areas identified by their relative retention time. The quantification of
3 tocopherols was based on the comparison of the peak areas with those of an
4 external standard curve of R-tocopherol and identified by HPLC (Montserrat-de
5 la Paz, Fernandez-Arche, Angel-Martin, &. Garcia-Gimenez, 2014). The test
6 sample was prepared as a solution to 10% by weight in hexane (Panreac) and
7 analysed in an HPLC system (Hewlett–Packard, Minnesota, US 1050) equipped
8 with a fluorescence detector (Shimadzu RF-535) using an excitation wavelength
9 set at 290 nm, an emission wavelength at 330 nm, and an HPLC analytical
10 column silica (250 mm x 4 mm i.d. x 5 µm) (Merck, Superspher Si60) at a
11 temperature of 30 °C. A flow rate of 1 mL/min, 400 bar was used. Total
12 concentrations were calculated as the sum of individual concentrations. Results
13 were expressed as mg/100 mg UF (**Table 1**).

14 The quantification of squalene (SQ) was based on the comparison of the peak
15 areas with those of an external standard curve and identified by GC. VOO (0.5
16 g) with 1 mL internal SQ standard (Sigma-Aldrich, St Louis, MO, USA) (5
17 mg/mL) were weighed and mixed; then, 2 mL of heptane and 400 mL of
18 methanolic solution of potassium hydroxide 2 N were added and the mix was
19 shaken vigorously. When the upper solution became clear, 1 µL was injected
20 into a GC. GC analysis of SQ content was performed using an HP5890
21 (Hewlett-Packard) gas chromatograph fitted with a flame ionization detection
22 system and a split injection system (split ratio 1:50). Separations were carried
23 out on a fused silica capillary column SPB-5 (30 m x 0.25 mm i.d.) coated with
24 5% phenyl-95% methylsilicone, with a thickness of 0.25 µm (Supelco). The
25 operating conditions were as follows: injector temperature, 280 °C; detector

1 temperature, 300 °C; oven programming temperatures, initial 240 °C for 6 min
2 and then rising at 5 °C/min to 280 °C. A hydrogen flow rate of 1 mL/min, 130
3 Kpa was used. Total concentration was calculated as the sum of individual
4 concentrations.

6 *2.2. Preparation of PF and hydroxytyrosol (HTyr) from VOO*

7 PF was obtained using the method described by Vazquez-Roncero *et al*
8 (Vazquez-Roncero, Janer del Valle, & Janer del Valle, 1973). with some
9 modifications. An amount of 50 g of VOO was extracted with 125 mL of
10 methanol:water (80:20, v/v). The resultant was mixed with a vortex at 5000 g for
11 1 min and then sonicated for 15 min. After decantation, the methanolic phase
12 was concentrated in vacuum under a stream of nitrogen at a temperature lower
13 than 35 °C until it reached a syrupy consistency. PF was lyophilized and stored
14 at –80 °C until its use. Quantitative and qualitative analysis of PF was
15 performed according to the COI/T20/29. The method is based on direct
16 extraction of the phenolic minor polar compounds by means of a methanol
17 solution and subsequent quantification by HPLC. After direct extraction, an
18 aliquot of the supernatant phase was taken, filtered, and injected into a HPLC
19 system equipped with C18 reverse-phase column (25 cm x 4.6 mm), type
20 Spherisorb ODS-2 (5 mm) and a spectrophotometric UV detector at 280 nm.
21 The content of phenols was expressed in mg of tyrosol per kg of oil and was
22 calculated by measuring the areas of the related chromatographic peaks. The
23 composition of the isolated PF is detailed in **Table 2**. To *in vitro* treatments, both
24 HTyr and SQ were purchased by Sigma-Aldrich (Madrid, Spain).

2.3. BV-2 microglial cell culture

BV-2 microglial cells were purchased from the Instituto de la Grasa (Seville, Spain). Cells were routinely cultured in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM, #L0104, Biowest, Nuaillé, France) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Culture conditions were 5 % CO₂ at 37 °C. Subcultures were made every 3 days using 0.25% trypsin-EDTA. To maintain cells in an undifferentiated state, they were passaged before obtaining confluence. Only BV-2 microglial cells at culture passages 5 to 10 were used for the experiments. Before the treatment, BV-2 microglial cells were seed at a density of 5x10⁵ cells/mL in a 12-well plates for 24 h in high glucose DMEM supplemented with 1 % heat-inactivated FBS and 1 % P/S.

2.4. M1/M2 microglia polarization

For M1 or M2 polarization, BV-2 microglia cells were exposed to LPS (100 ng/mL) and IFN γ (20 ng/mL) or to IL-4 (20 ng/mL), respectively, for additional 24 h. To assess the effect of minor compounds from VOO on microglia polarization, BV-2 microglial cells were incubated with LPS (100 ng/mL) and IFN γ (20 ng/mL) in the absence or presence of UF and PF from VOO at 25 and 50 μ g/mL, SQ at 50 μ M, or HTyr at 41 μ M for 24 h.

2.5. Cell viability assay (MTT)

BV-2 microglial cells were seeded at a density of 10⁴ cells/well in 96 well plates and incubated with UF and PF up to 100 μ g/mL, and SQ and HTyr up to 100 μ M for 24 h. Afterward, the MTT solution (Sigma) was incubated in the well until

1 a purple precipitate was visible. MTT-formazan crystals were solubilized with
2 DMSO (Sigma), and after 10-20 min in dark at room temperature, were
3 measured with a microplate reader at 570 nm corrected to 650 nm. Cell survival
4 was expressed as the percentage of absorbance compared with that obtained
5 in control, non-treated cells (any minor compound or fraction added to the
6 culture medium for 24 h did not induce cytotoxicity, data not shown).

7 8 *2.6. RNA isolation and qRT-PCR analysis*

9 Total RNA was extracted by using Trisure Reagent (Bioline), as instructed by
10 the manufacturer. A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer
11 (Thermo Scientific, Madrid, Spain) was used to determinate RNA quality.
12 Momentarily, RNA (1 μ g) was subjected to reverse transcription (iScript, Bio-
13 Rad, Madrid, Spain). An amount of 10 ng of the resulting cDNA was used as a
14 template for real-time PCR amplifications. The mRNA levels for specific genes
15 were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA
16 template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad)
17 containing the primer pairs for either gene or for glyceraldehyde 3-phosphate
18 dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1
19 (HPRT) as housekeeping genes (**Supplementary Table 1**). All amplification
20 reactions were performed in triplicate and average threshold cycle (Ct) numbers
21 of the triplicates were used to calculate the relative mRNA expression of
22 candidate genes. The magnitude of change of mRNA expression for candidate
23 genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were
24 normalized to endogenous reference (GAPDH and HPRT) gene content and
25 expressed as percentage of controls.

2.7. Measurement of cytokine release

The levels of TNF- α , IL-1 β , and IL-6 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diacclone, Besancon, France). The cytokine concentrations were expressed in ng or pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

2.8. Animals and diets

C57BL/6J (The Jackson Laboratory) were maintained in light/dark- (12 h light/12 h dark), temperature- (22 ± 1 °C) and humidity- (50%–60% relative humidity) controlled room, fed with standard chow diet (CD, SAFE A04-10 Panlab) *ad libitum* and had free access to drinking water at the animal facilities of the School of Medicine (University of Seville). Male animals at the age of 10-12 weeks were divided in 4 experimental groups ($n = 10/\text{group}$). As shown in **Table 3**, mice were fed chow diet (3% energy as fat from soybean oil, A04-10) or high-fat diets (HFDs) based on the above A04-10 diet with additional 21% energy as fat from milk cream (HFD-saturated fatty acids, SFAs), refined olive oil (ROO, HFD-ROO) or VOO (HFD-VOO) for 8 weeks (**Table 4**). **Body weight was recorded weekly (Supplementary Figure 1).** Diets and drinking water were refreshed every week. All diets, which had low cholesterol (0.01%), were prepared by Panlab and presented as pellets to the animals. At the end of the experiments, animals were sacrificed at the beginning of the light cycle and after 10 h of food deprivation, brain tissue was then removed, and frozen at -80 °C for further analysis.

2.9. Immunostaining Analysis by FACS

Brain tissue samples were collected after 8 weeks of diet. Microglia membrane expression of CD45 (PE-Cy7 anti-mouse CD45, BD), CD11b (PerCP-Cy5.5 anti-mouse CD11b, BD), F4/80 (APC anti-mouse APC, Miltenyi), CD80 (FITC anti-mouse FITC, Miltenyi), and CD200R (PE anti-mouse CD200R, Miltenyi) on brain tissue was assessed by flow cytometry. According to the manufacturer's instructions, the tissue was homogenate and cells were incubated with antibodies at room temperature, in the dark, for 15 minutes. Fluorescence intensity was measured by FACSCanto II (BD) and calibrating using FACSCanto II analyser software (BD). Mean fluorescence intensity (MFI) of 10^4 counted cells was measured in each sample. Myeloid cells were gated as side scatter (SSC)-CD45⁺ cells. Microglial cells, in myeloid cells, were gated as CD11b⁺F4/80⁺ cells. In microglial cells, M1 and M2 phenotypes were gated as CD80⁺ and CD200R⁺ cells, respectively. Expression levels were presented as MFI corrected for non-specific binding of isotype control antibodies.

2.10. Statistical analysis

All values are expressed as arithmetic means $\pm$ standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as post hoc test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. UF, SQ, PF, and HTyr favor a M2 activation status in BV-2 microglial cells

To study the role of minor compounds from VOO on microglia plasticity, we observed that LPS particularly promoted the transcriptional activity of M1 microglia gene markers (CD80 and iNOS) in BV-2 microglial cells (Fig. 1A and 1B, respectively). In contrast, UF, SQ, PF, and HTyr not only reduced the mRNA levels of CD80 and iNOS genes, compared to LPS, but also promoted the transcriptional activity of M2 microglia gene markers (CD200R and Arg-1) in BV-2 microglial cells (Fig. 1C and 1D, respectively).

3.2. Minor compounds from VOO modulate cytokine gene expression and secretion in BV-2 microglial cells

To further explore the possible role of minor compounds on microglia activation status, gene expression and secretion of TNF- α , IL-1 β , and IL-6 were analyzed (Fig. 2). We observed that LPS-induced release of pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) and expression of genes encoding these cytokines were diminished by UF, SQ, PF, and HTyr in LPS-treated BV-2 microglial cells. In the case of UF and PF, these effects were dose-dependent.

3.3. Minor compounds from VOO modulate M1/M2 microglia subsets in brain tissue of obese mice

As shown in Figure 3, microglial cells (CD11b⁺F4/80⁺) were analyzed in myeloid lineage (CD45⁺) from brain tissue of mice fed with chow diet and HFDs. Total microglial cell population was significantly increased in brain tissue of mice fed with HFD-SFAs compared to those fed with chow diet or those fed with

HFD-ROO and HFD-VOO (**Fig. 3A**). In addition, HFD-SFAs showed an increase in CD80 (M1 phenotype) protein levels and a decrease in CD200R (M2 phenotype) protein levels compared to the chow diet, HFD-ROO, and HFD-VOO (**Fig. 3B** and **Fig. 3C**, respectively). Fully consistent, brain tissue of mice on HFD-ROO had higher mRNA levels of the M1 marker CD80 and that of mice on HFD-VOO and HFD-VOO had higher mRNA levels of the M2 markers CD200R (**Fig. 4**).

3.4. VOO-enriched diet promotes neuroprotection in obese mice

To explore immune-related events that follow microglia activation, we analyzed the local inflammation in brain tissue of mice fed with chow diet and HFDs (**Fig. 5**). Brain tissue gene expression levels of pro-inflammatory TNF- α (**Fig. 5A**), IL-1 β (**Fig. 5C**), and IL-6 (**Fig. 5E**), and the secretion of these cytokines into brain tissue homogenates (**Fig. 5B**, **5D**, and **5F**, respectively) were higher with HFDs but these levels were significantly less pronounced with HFD-VOO than with HFD-SFAs and with HFD-ROO.

4. Discussion

Herein, we elucidate for first time the underlying mechanisms by which UF and PF from VOO attenuates neuroinflammation development by regulating microglia macrophage plasticity.

Pharmacological treatments for neurodegenerative diseases currently available have the potential to delay the progression or even reduce the symptoms at a certain level. In this context and due to the limited efficacy of pharmacological treatment and the multifactorial nature of these disorders, a multifaceted

1 approach seems appropriate. Diet interventions are a promising approach to
2 prevent and delay the onset and progression (Hornedo-Ortega et al., 2018).

3 The potential of Mediterranean Diet in preventing neurodegenerative disorders
4 has been mainly related with its high content in plant foods: fruits and
5 vegetables and virgin olive oil, sources of a wide of bioactive compounds
6 (Angeloni, Malaguti, Barbalace, & Hrelia, 2017; Dominguez-Perles, Auñon,
7 Ferreres, & Gil-Izquierdo, 2017; Lopez et al. 2017). Bioactive compounds
8 comprise a heterogeneous group of thousands of molecules present mainly in
9 plant foods and also known as phytochemicals. The composition of VOO
10 includes minor compounds (UF and PF) that could range from 1-3% of the oil.
11 The constituents of minor compounds are present in low concentrations but
12 they are responsible for their flavor, nutritional and biological properties of VOO
13 (aldehydes, alcohols, esters, hydrocarbons, ketones, furans, and others)
14 (Montserrat-de la Paz et al., 2016).

15 In the present paper we have determined that acute activation of inflammation
16 in BV-2 microglial cells by treatment with LPS and IFN γ result in an increase in
17 the relative proinflammatory M1 microglia subset. Microglial polarization
18 depends on different microenvironment and signals that promote specific
19 phenotypes. Classical M1-activated microglia secrete proinflammatory
20 cytokines (TNF- α , IL-1 β , and IL-6) and, alternatively, M2-activated microglia is
21 able to repair small damage, have an anti-inflammatory phenotype, contributing
22 to trophic support of neurons, possess the ability to degrade toxic aggregates
23 and increase the neuroprotective functions thanks to anti-inflammatory
24 interleukin production (Papa, Caron, Rossi, Veglianesse, 2016). Many
25 researchers have found immunomodulatory compounds presents in food, for

1 example lutein highly present in parsley, was able to reduce LPS-induced
2 neuroinflammation in BV-2 microglial cells, by significantly inhibiting iNOS (Wu
3 et al., 2015), another example is fucosterol, a phytosterol contained in brown
4 seaweed, that inhibits the production of nitric oxide and the expression of iNOS
5 (Jung, Jin, Ahn, Lee, & Choi, 2013). While effects of minor compounds from
6 VOO on inflammation and polarization in the periphery are well studied, any
7 reports have investigated effects of UF and PF on neuroinflammation and
8 microglial cells that are the major immunological mediators of this process in the
9 CNS. In our study, we find that dietary UF, SQ, PF, and HTyr can change
10 microglial activation status ratio regard to LPS+IFN γ . That means that M1
11 markers (CD80 and iNOS) and proinflammatory cytokine mRNA levels and
12 secretion are decreased in BV-2 microglial cells, meanwhile M2 markers
13 (CD200R and Arg-1) are enlarged when they are exposed at minor compound
14 from VOO.

15 An excessive or prolonged neuroinflammatory response to HFD, insults may
16 have the unintended consequence of generating bystander neuronal cell death.
17 Similarly, the presence of a chronic, low-grade neuroinflammatory condition,
18 such as obesity and metabolic syndrome, may also cause eventual bystander
19 cell death with cumulative and serious consequences over time (Frank-Cannon,
20 Alto, McAlpine, & Tansey, 2009). Consumption of a diet rich in SFAs, may
21 chronically activate neuroinflammatory responses of microglia. For a deeper
22 understanding of the influence that dietary VOO exerts on neuroinflammation
23 during obesity and metabolic syndrome in mice, we analyzed the number of
24 microglial cells (CD11b and F4/80 positive cells) found increased in brain of
25 mice on different HFDs. As lower is the cellularity and thereby the activation of

M1 microglia subset, the lower is the inflammatory state and the better is the brain health status in mice on HFD-VOO. This is crucial to avoid the advance of neuroinflammation and neurodegeneration under sustained conditions of obesity and metabolic syndrome. Herein, we found that, in contrast to the HFD-SFAs, olive oil-based HFDs had a dramatic lower influence on microglial cells positive to CD80, which is a marker for M1 microglial activation. This also concurs with a decrease in the gene expression of CD80. Still, olive oil-based HFDs promoted the relative abundance of microglial cells positive to CD200R and with upregulated of gene expression, a marker that resemble M2 activation. By concordant polarization patterns, lower transcriptional activities and secretion of pro-inflammatory cytokines in brain of mice fed with olive oil-based HFDs were found.

5. Conclusion

Our study further pointing to a low neuroinflammation and thereby the safeguard of brain homeostasis when, the predominant dietary fat during HFD-induced obesity and metabolic syndrome is VOO. Importantly, our data emphasize the benefits of dietary minor compounds from VOO as nutritional strategies for reduction of activation and inflammation of microglia cells to prevent neuroinflammatory and neurodegenerative diseases.

Conflicts of interest

The authors state no conflict of interest.

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Abbreviations

Arg, arginase; **CD**, cluster of differentiation; **CNS**, central nervous system; **FBS**, fetal bovine serum; **HFD**, high fat diet; **HTyr**, hydroxytyrosol; **IFN**, interferon; **IL**, interleukin; **iNOS**, inducible nitric oxide synthase; **LPS**, lipopolysaccharide, **MUFA**, monounsaturated fatty acid; **PF**, phenolic fraction; **ROO**, refined olive oil; **SFA**, saturated fatty acid; **SQ**, squalene; **UF**, unsaponifiable fraction; **VOO**, virgin olive oil, **TNF**, tumor necrosis factor.

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8 **References**

9 Angeloni, C., Malaguti, M., Barbalace, M.C., & Hrelia, S. (2017). Bioactivity of
10 olive oil phenols in neuroprotection. *International Journal of Molecular Science*,
11 18, E2230.

12 Aparicio-Soto, M., Montserrat-de la Paz, S., Sanchez-Hidalgo, M., Cardeno, A.,
13 Bermudez, B., Muriana, F.J.G., & Alarcon-de la Lastra, C. (2018). Virgin olive oil
14 and its phenol fraction modulate monocyte/macrophage functionality: a potential
15 therapeutic strategy in the treatment of systemic lupus erythematosus. *British*
16 *Journal of Nutrition*, 120, 681-692.

17 Bottcher, C., Schlickeiser, S., Sneeboer, M.A.M., Kunkel, D., Knop, A., Paza,
18 E., ... & Priller, J. (2019). Human microglia regional heterogeneity and
19 phenotypes determined by multiplexed single-cell mass cytometry. *Nature*
20 *Neuroscience*, 22, 78-90.

21 Cardeno, A., Sanchez-Hidalgo, M., Aparicio-Soto, M., & Alarcon-de la Lastra, C.
22 (2014). Unsaponifiable fraction from extra virgin olive oil inhibits the
23 inflammatory response in LPS-activated murine macrophages. *Food Chemistry*,
24 147, 117-123.

- 1 Dominguez-Perles, R., Auñón, D., Ferreres, F., & Gil-Izquierdo, A. (2017).
2 Physiological linkage of gender, bioavailable hydroxytyrosol derivatives, and
3 their metabolites with systemic catecholamine metabolism. *Food & Function*, 8,
4 4570-4581.
- 5 Frank-Cannon, T.C., Alto, L.T., McAlpine, F.E., & Tansey, M.G. (2009) Does
6 neuroinflammation fan the flame in neurodegenerative diseases? *Molecular*
7 *Neurodegeneration*, 4, 47.
- 8 Hornedo-Ortega, R., Cerezo, A.B., De Pablos, R.M., Krisa, S., Richard, T.,
9 Garcia-Parrilla, M.C., & Troncoso, A.M. (2018). Phenolic Compounds
10 Characteristic of the Mediterranean Diet in Mitigating Microglia-Mediated
11 Neuroinflammation. *Frontiers in Cellular Neuroscience*, 12, 373.
- 12 Jung, H.A., Jin, S.E., Ahn, B.R., Lee, C.M., & Choi, J.S. (2013). Anti-
13 inflammatory activity of edible brown alga *Eisenia bicyclis* and its constituents
14 fucosterol and phlorotannins in LPS-stimulated RAW264.7 macrophages. *Food*
15 *Chemistry & Toxicology*, 59, 199-206.
- 16 Lopez, S., Bermudez, B., Montserrat-de la Paz, S., Jaramillo, S., Varela, L.M.,
17 Ortega-Gomez, A., Abia, R., & Muriana F.J.G. (2014). Membrane composition
18 and dynamics: A target of bioactive virgin olive oil constituents. *Biochimica et*
19 *Biophysica Acta-Biomembranes*, 1838, 1638-1656.
- 20 Lopez, S., Montserrat-de la Paz, S., Lucas, R., Bermudez, B., Abia, R.,
21 Morales, J.C., & Muriana, F.J.G. (2017). Effect of metabolites of hydroxytyrosol
22 on protection against oxidative stress and inflammation in human endothelial
23 cells. *Journal of Functional Foods*, 29, 238-247.

- 1 Montserrat-de la Paz, S., Bermudez, B., Cardelo, M.P., Lopez, S., Abia, R., &
- 2 Muriana F.J.G. (2016). Olive oil and postprandial hyperlipidemia: implications
- 3 for atherosclerosis and metabolic syndrome. *Food & Function*, 7, 4734-4744.
- 4 Montserrat-de la Paz, S., Marin-Aguilar, F., Garcia-Gimenez, M.D., &
- 5 Fernandez-Arche, M.A. (2014). Hemp (*Cannabis sativa* L.) Seed Oil: Analytical
- 6 and Phytochemical Characterization of the Unsaponifiable Fraction. *Journal of*
- 7 *Agricultural and Food Chemistry*, 62, 1105-1110.
- 8 Montserrat-de la Paz, S., Fernandez-Arche, M.A., Angel-Martin, M., & Garcia-
- 9 Gimenez, M.D. (2014). Phytochemical characterization of potential nutraceutical
- 10 ingredients from Evening Primrose oil (*Oenothera biennis* L.). *Phytochemistry*
- 11 *Letters*, 8, 158-162.
- 12 Olajide, O.A., Kumar, A., Velagapudi, R., Okorji, U.P., & Fiebich, B.L. (2014).
- 13 Punicagin inhibits neuroinflammation in LPS-activated rat primary microglia.
- 14 *Molecular Nutrition & Food Research*, 58, 1843-1851.
- 15 Papa, S., Caron, I., Rossi, F., & Veglianese, P. (2016). Modulators of microglia:
- 16 a patent review. *Expert Opinion on Therapeutic Patents*, 26, 427-433.
- 17 Paquot, C. (1992). Determination of the unsaponifiable matter. Method 2401.
- 18 IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives. 7th ed.,
- 19 Revised and Enlarged. Oxford: Blackwell.
- 20 Sousa, C., Golebiewska, A., Poovathingal, S.K., Kaoma, T., Pires-Afonso, Y.,
- 21 Martina, S., ... & Michelucci, A. (2018). Single-cell transcriptomics reveals
- 22 distinct inflammation-induced microglia signatures. *EMBO Reports*, 19, e46171.
- 23 Tang, Y., & Le, W. (2016). Differential Roles of M1 and M2 Microglia in
- 24 Neurodegenerative Diseases. *Molecular Neurobiology*, 53, 1181-1194.

- 1 Timmerman, R., Burm, S.M., & Bajramovic, J.J. (2018). An Overview of in vitro
- 2 Methods to Study Microglia. *Frontiers in Cellular Neuroscience*, 12, 242.
- 3 Vazquez-Roncero, A., Janer del Valle, M.I., & Janer del Valle, C. (1973).
- 4 Determination of total phenols in olive oil. *Grasas y Aceites*, 24, 350-355.
- 5 Wu, W., Li, Y., Wu, Y., Zhang, Y., Wang, Z., & Liu, X. (2015). Lutein suppresses
- 6 inflammatory responses through Nrf2 activation and NF-κB inactivation in
- 7 lipopolysaccharide-stimulated BV-2 microglia. *Molecular Nutrition & Food*
- 8 *Research*, 59, 1663-1673.
- 9 Xu, Y., Xu, Y., Wang, Y., Wang, Y., He, L., Jian, Z., ... & Pang, T. (2015).
- 10 Telmisartan prevention of LPS-induced microglia activation involves M2
- 11 microglia polarization via CaMKKβ-dependent AMPK activation. *Brain, Behavior*
- 12 *and Immunity*, 50, 298-313.
- 13 Yang, X., Xu, S., Qian, Y., & Xiao, Q. (2017). Resveratrol regulates microglia
- 14 M1/M2 polarization via PGC-1α in conditions of neuroinflammatory injury. *Brain,*
- 15 *Behavior and Immunity*, 64, 162-172.

FIGURE LEGENDS

Fig. 1. Expression of M1 and M2 microglia gene markers in BV-2 microglial cells. Cells were treated with IL-4 (M2 microglia) or LPS+IFN γ (M1 microglia) in the absence or presence of UF (at 25 and 50 μ g/ml), SQ (at 50 μ M), PF (at 25 and 50 μ g/ml) or HTyr (at 41 μ M) for 24 h. M1 gene markers: (A) CD80 and (B) iNOS. M2 gene markers: (C) CD200R and (D) Arg-1. Values are presented as means $\pm$ SD (n = 6) and those marked with different letters are significantly different ($P < 0.05$).

Fig. 2. Expression and secretion of proinflammatory cytokines in BV-2 microglial cells. Cells were treated with IL-4 (M2 microglia) or LPS+IFN γ (M1 microglia) in the absence or presence of UF (at 25 and 50 μ g/ml), SQ (at 50

1 μM), PF (at 25 and 50 $\mu\text{g/ml}$) or HTyr (at 41 μM) for 24 h. (A) TNF- α mRNA
2 expression and (B) cytokine secretion, (C) IL-1 β mRNA expression and (D)
3 cytokine secretion, and (E) IL-6 mRNA expression and (F) cytokine secretion.
4 Values are presented as means $\pm$ SD ($n = 6$) and those marked with different
5 letters are significantly different ($P < 0.05$).

6
7 **Fig. 3.** (A) FACS analysis of total microglia subsets by means of (B) CD80 (M1
8 microglia subset) and (C) CD200R (M2 microglia subset) surface marker
9 expression (relative values) in the brain tissue of C57BL/6J mice fed on a CD,
10 HFD-SFAs, HFD-ROO, and HFD-VOO for 8 weeks. Values are presented as
11 means $\pm$ SD ($n = 10$ mice/group) and those marked with different letters are
12 significantly different ($P < 0.05$).

13
14 **Fig. 4.** (A) CD80 and (B) CD200R mRNA expression in the brain tissue of
15 C57BL/6J mice fed on a CD, HFD-SFAs, HFD-ROO, and HFD-VOO for 8
16 weeks. Values are presented as means $\pm$ SD ($n = 10$ mice/group) and those
17 marked with different letters are significantly different ($P < 0.05$).

18
19 **Fig. 5.** (A) TNF- α mRNA expression and (B) cytokine concentration, (C) IL-1 β
20 mRNA expression and (D) cytokine concentration, and (E) IL-6 mRNA
21 expression and (F) cytokine concentration in the brain tissue of C57BL/6J mice
22 fed on a CD, HFD-SFAs, HFD-ROO, and HFD-VOO for 8 weeks. Values are
23 presented as means $\pm$ SD ($n = 10$ mice/group) and those marked with different
24 letters are significantly different ($P < 0.05$).

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SUPPLEMENTARY FIGURE LEGENDS

Sup. Fig. 1. (A) Body weight monitored over time on each diet and (B) body weight gain in C57BL/6J mice fed on a CD, HFD-SFAs, HFD-ROO, and HFD-VOO for 8 weeks. Values are presented as means $\pm$ SD (n = 10 mice/group) and those marked with different letters are significantly different ($P < 0.05$).

Supplementary Table 1. Sequences of RT-qPCR primers for gene expression analysis.

Target	No. GenBank	Direction	Sequence (5'→3')
GAPDH	NM_001289726	Forward	AACTTTGGCATTGTGGAAGG
		Reverse	ACACATTGGGGGTAGGAACA
HPRT	NM_013556.2	Forward	TATGTCCCCCGTTGACTGAT
		Reverse	TGCTCGAGATGTCATGAAGG
CD80	NM_009855.2	Forward	GGGAAAGTGTACGCCCTGTA
		Reverse	GCTACTTCTGTGCCCAACCAT
iNOS	NM_010927	Forward	CACCTTGGAGTTCACCCAGT
		Reverse	ACCACTCGTACTTGGGATGC
CD200R	NM_021325.3	Forward	GTTGCCCTCCTATCGCATTA
		Reverse	TGGAAATTCCCATCAGGTGT
ARG1	NM_007482	Forward	GTGAAGAACCCACGGTCTGT
		Reverse	CTGGTTGTCAGGGGAGTGTT
TNF-α	NM_001278601	Forward	AGCCCCCAGTCTGTATCCTT
		Reverse	CTCCCTTTGCAGAACTCAGG
IL-1β	NM_008361	Forward	GCCCATCCTCTGTGACTCAT
		Reverse	AGGCCACAGGTATTTTGCG
IL-6	NM_031168	Forward	AGTTGCCTTCTTGGGACTGA
		Reverse	TCCACGATTTCCCAGAGAAC

Table 1. Main composition of UF from VOO.

Component	mg/Kg (ppm)
Squalene	5681
Total sterols	1289
Campesterol	40.7
β -sitosterol	7.8
Δ 5-Avenasterol	69.7
Total methylsterols	69.8
Total triterpenic alcohols	568
Total aliphatic alcohols	163
C24	40.8
C26	71.9
α -tocopherol	87

Table 2. Main composition of PF from VOO using COI/T20/29doc.

Component	µM Phenol (50 µg PF/mL)
Hydroxytyrosol	41.07
Tyrosol	43.09
Vanillic acid	5.09
p-Coumaric acid	3.42
Decarboxymethyl oleuropein aglycone (dialdehyde)	10.27
Tyrosyl acetate	4.97
Decarboxymethyl ligstroside aglycone (dialdehyde)	11.33
Pinoresinol	6.21
Cinnamic acid	6.89
Acetoxy-pinoresinol	6.22
Oleuropein aglycone, aldehyde form	39.18
Ligstroside aglycone, dialdehyde form	26.20
Luteolin	4.18
Apigenin	0.88

Table 3. Macronutrients composition of diets.

Macronutrients	CD	HFD-SFAs	HFD-ROO	HFD-VOO
Fat (%)	3	24	24	24
Carbohydrate (%)	77.5	56.5	56.5	56.5
Protein (%)	19.5	19.5	19.5	19.5

Values are expressed as the percentage of energy derived from fat, carbohydrate or protein. Chow diet (CD), high-fat diet rich in saturated fatty acids (HFD-SFAs), high-fat diet rich in refined olive oil (HFD-ROO), and high-fat diet rich in virgin olive oil (HFD-VOO).

Table 4. Fatty acid composition of dietary fats for corresponding HFD.

	HFD-SFAs	HFD-ROO	HFD-VOO
Fatty acid	g/100 g of fatty acid $\pm$ SD		
4:0, butyric	0.83 $\pm$ 0.09	-	-
6:0, caproic	0.25 $\pm$ 0.01	-	-
8:0, caprylic	0.61 $\pm$ 0.04	-	-
10:0, capric	2.47 $\pm$ 0.08	-	-
12:0, lauric	3.09 $\pm$ 0.24	-	-
14:0, myristic	10.87 $\pm$ 0.53	-	-
16:0, palmitic	35.54 $\pm$ 0.47	20.42 $\pm$ 0.51	20.21 $\pm$ 0.43
16:1(n-7), palmitoleic	3.60 $\pm$ 0.18	0.97 $\pm$ 0.10	1.04 $\pm$ 0.09
18:0, stearic	11.49 $\pm$ 0.43	5.70 $\pm$ 0.06	5.91 $\pm$ 0.12
18:1(n-9), oleic	25.33 $\pm$ 0.41	61.96 $\pm$ 0.71	60.86 $\pm$ 0.93
18:2(n-6), linoleic	4.27 $\pm$ 0.47	7.97 $\pm$ 0.38	7.90 $\pm$ 0.42
18:3(n-3), α -linolenic	0.39 $\pm$ 0.03	1.04 $\pm$ 0.08	1.24 $\pm$ 0.06
Others	0.96 $\pm$ 0.24	2.05 $\pm$ 0.62	2.93 $\pm$ 0.75
Total-SFAs	63.46 $\pm$ 1.07 ^a	26.11 $\pm$ 0.59 ^b	26.12 $\pm$ 0.53 ^b
Total-MUFAs	28.93 $\pm$ 1.48 ^b	62.83 $\pm$ 0.78 ^a	61.90 $\pm$ 0.91 ^a
Total-PUFAs	4.66 $\pm$ 0.46 ^b	9.01 $\pm$ 0.42 ^a	9.14 $\pm$ 0.38 ^a

The values are expressed as the mean $\pm$ SD (n = 3). An ANOVA followed by Tukey's post-hoc test was performed. Different superscript letters (a-c) denote that mean values in a row with different letters are significantly different ($p < 0.05$). High-fat diet rich in saturated fatty acids (HFD-SFAs), high-fat diet rich in refined olive oil (HFD-ROO), and high-fat diet rich in virgin olive oil HFD-VOO).

A **Supplementary Figure 1**
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Figure 1
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Figure 2
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Figure 3
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A
 Total microglial cells
 (CD11b⁺F4/80⁺ cells)
 (Relative to chow diet control)

B
 M1 microglial cells
 (CD80⁺ cells)
 (Relative to chow diet control)

C
 M2 microglial cells
 (CD200R⁺ cells)
 (Relative to chow diet control)

Figure 4

[Click here to download Figure: Figure 4.pdf](#)

Figure 5
[Click here to download Figure: Figure 5.pdf](#)

Unsaponifiable and phenolic fractions from virgin olive oil prevent neuroinflammation skewing microglia polarization toward M2 phenotype

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Conflict of Interest File

Declarations of interest: none

Ethics Statements File

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained. All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville, AGL2012-40247-C02-01) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).