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FULL ARTICLE

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Minor compounds from virgin olive oil attenuate LPS-induced inflammation via visfatin-related gene modulation on primary human monocytes

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Abstract

We have analyzed the effects of minor compounds found in the unsaponifiable fraction (UF) and in the phenolic fraction (PF) of virgin olive oil (VOO) on LPS-induced inflammatory response via visfatin modulation in human monocytes. For this purpose, monocytes were incubated with UF and PF at different concentrations and the pro-inflammatory stimulus LPS for 24 hr; squalene (SQ) and hydroxytyrosol (HTyr), the main components in UF and PF, respectively, were also used. The relative expression of both pro-inflammatory and anti-inflammatory genes, as well as other genes related to the NAD+-biosynthetic pathway was evaluated by RT-qPCR; and the secretion of some of these markers was assessed by ELISA procedures. We found that UF, SQ, PF, and HTyr prevented from LPS-induced dysfunctional gene expression and secretion via visfatin-related gene modulation in human monocytes. These findings unveil a potential beneficial role for minor compounds of VOO in the prevention of inflammatory-disorders.

Practical application

In this project, potential health benefits of VOO micronutrients (unsaponifiable and phenolic compounds) were confirmed through anti-inflammatory assays. Our results reveal new interesting researching goals concerning nutrition by considering the role of bioactive VOO compounds in the prevention and progress of diseases related to inflammation.

KEYWORDS

NAMPT, olive oil, phenols, unsaponifiable, visfatin

Abbreviations: AMPK, AMP-activated protein kinase; **CCR2**, Chemokine receptor type 2; **COX**, Cyclooxygenase; **GAPDH**, Glyceraldehyde-3-phosphate dehydrogenase; **HTyr**, Hydroxytyrosol; **LPS**, Lipopolysaccharide; **MCP-1**, Monocyte chemoattractant protein-1; **MUFA**, Monounsaturated fatty acid; **NAD**, Nicotinamide adenine dinucleotide; **NAMPT**, Nicotinamide phosphoribosyltransferase; **NF-κB**, Nuclear factor-kappa B; **NMNAT**, Nicotinamide mononucleotide adenyltransferase; **NOS**, Nitric oxide synthase; **PPARγ**, Peroxisome proliferator-activated receptor gamma; **SIRT**, Sirtuin; **SQ**, Squalene; **TNF**-α, Tumor necrosis factor α; **UF**, Unsaponifiable fraction.

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1 | INTRODUCTION

Traditional Mediterranean includes the intake of virgin olive oil (VOO). Moreover, VOO is responsible of the remarkable healthy benefits of this diet (Montserrat-de la Paz, Bermudez, et al., 2016). Regarding its composition, virgin olive oil contains large amounts of monounsaturated fatty acids (MUFAs) as well as several minor phenolics and other bioactive compounds included in the unsaponifiable fraction (UF) (Lopez et al., 2014). It has been previously shown that both the phenolic fraction (PF) and UF from VOO modulate gene expression involved in oxidative stress and inflammation (Aparicio-Soto et al., 2018; Cardeno, Sanchez-Hidalgo, & Alarcon-de la Lastra, 2013; Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon-de la Lastra, 2014). Additionally, it is known that PF and UF from VOO decrease the expression of both cyclooxygenase *(COX)-2* and inducible nitric oxide synthase (*iNOS*) involving nuclear factor kappa B (NF-κB)-mitogen-activated protein kinase (MAPK) signaling pathway in lipopolysaccharide (LPS)-activated murine macrophages (Cardeno et al., 2014; Rosignoli, Fuccelli, Fabiani, Servili, & Morozzi, 2013; Scoditti et al., 2014). It has also been established the anti-atherogenic properties of PF and UF from VOO not only in vivo but also in vitro conditions (Meza-Miranda et al., 2016; Montserrat-de la Paz, Naranjo, et al., 2016). PF and UF from VOO act through different inflammatory pathways modulating immune system cells. VOO diet prevented the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT), MAPKs signaling pathways and upregulated nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and haem oxygenase (HO-1) protein expressions (Aparicio-Soto, Sanchez-Hidalgo, Rosillo, Castejon, & Alarcon-de la Lastra, 2016).

It is known that visfatin or nicotinamide phosphoribosyltransferase (*NAMPT*) is secreted by activated leukocytes as a pre-B-cell colony-enhancing factor in bone marrow stroma (Samal et al., 1994). In the rescue route of nicotinamide adenine dinucleotide (NAD^+) generation, intracellular NAMPT (*iNAMPT*) appears to be the rate-limiting enzyme (Montserrat-de la Paz, Naranjo, et al., 2017). Previous studies have proved the significance of NAMPT-mediated NAD^+recycling, in cooperation with NAD^+-dependent protein deacetylases (sirtuins), directly on the expression of peroxisome proliferator-activated receptors (*PPARs*), *NF-κB*, and endothelial NOS (*eNOS*); many data also provide evidence of an indirect role in metabolism, aging, inflammation, cell proliferation, and differentiation (Bermudez et al., 2017). Cells also produce *NAMPT* to create an extracellular supply (eNAMPT) with cytokine-like activity (Dahl, Holm, Aukrust, & Halvorsen, 2012). Our previous data revealed that within atherosclerotic carotid plaques, the expression of *NAMPT* is increased and eNAMPT causes inflammatory and matrix degrading responses (Dahl et al., 2012, 2007). Considering useful effects of VOO secondary compounds, this study focusses on their potential to modulate LPS-mediated activation of primary human monocytes via visfatin/*NAMPT*-related gene modulation, which is related to several diseases, such as atherosclerosis.

2 | MATERIAL AND METHODS

2.1 | Preparation of UF and squalene (SQ) from VOO

For UF extraction, we used VOO of Picual variety. According to IUPAC 2401, using standard methods, UF was isolated (Cardeno et al., 2014; Paquot, 1992). Briefly, a temperature of 80°C saponified 5 g of VOO refluxing with a volume of 50 ml of a 2 N solution of potassium hydroxide prepared in ethanol (Panreac, Barcelona, Spain), with gently boiling and 20 min after the suspension turned clear. The addition of 50 ml of distillate water through the top of the condenser stopped heating and then the mixture started rotating movement. The solution was washed many times with water when cooled to 30–35°C, and UF was finally extracted by distillation using a rotary evaporator (B-480 model, Büchi Labortechnik, Essen Germany) and the solvent diethyl ether (Panreac) for evaporation at 30°C under vacuum conditions. In oven, the fraction extracted was dried for 15 min at 103 ± 2°C. Sample production was between 1.3% and 1.4%. The quantitative evaluation of the aliphatic alcohols, sterols, and triterpenic dialcohols contained in the UF was determined in accordance with European Regulation EEC/2568/91 for olive oil. As described previously, the unsaponifiable matter was extracted and then fractioned using TLC (thin layer chromatography) with a 65:35 mixture of hexane and ethylether on a silica gel plate (Kieselgel 60 F254, Merck, Germany). Components such as triterpenic dialcohols, aliphatic alcohols, and sterols, were characterized by their retention factor. Then the triterpenic dialcohols recovered from the plate were converted from erythrodiol and uvaol to trimethylsilyl ethers; first of all adding a volume of 200 μl of a 9:3:1 v/v/v mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane and then analyzing by GC (gas chromatography). The HP 5890 series II gas chromatograph included a 30 m, 0.32 mm i.d., Tracsil TRB-5 (95% of dimethylpolysiloxane, 5% of diphenyl, film thickness 0.25 μm) capillary column and a flame ionization detector (Teknokroma, Barcelona, Spain). Settings for the gas chromatograph were both injector and detector 300°C and isothermal column 275°C; split ratio 1:50 and hydrogen flow rate 1.0 ml/min and 130 Kpa. For alcohol chromatographic measurement, the protocol was similar to that for sterols and triterpene diols, but the oven temperature was as follows: 215°C during 5 min, then 3°C/min raising to 290°C and kept 2 min more. The relative retention time let us identify the peak areas to quantify. For tocopherols, we used an R-tocopherol standard curve to perform quantification comparing peak areas and identification by HPLC (high-performance liquid chromatography) (Montserrat-de la Paz, Fernandez-Arche, Angel-Martin, & Garcia-Gimenez, 2014). An HPLC system (Hewlett-Packard, Minnesota, US 1050) was used to examine the solution 10% weight in hexane (Panreac) test sample. The equipment conditions were: 290 nm excitation and 330 nm emission wavelengths with a fluorescence detector (Shimadzu RF-535), as well as an analytical column silica (250 mm × 4 mm i.d. × 5 μm) (Merck, Superspher Si60) at 30°C and 1 ml/min, 400 bar flow rate. Table 1 includes results as mg/100 mg UF calculating total concentrations as the sum of individual concentrations.

For squalene (SQ) quantification, we used an external standard curve to compare the peak areas and carried out a GC identification. Briefly, 1 ml of internal SQ standard (Sigma-Aldrich, St Louis, MO, USA) (5 mg/ml) was mixed with 0.5 g of VOO. Then both a volume of 400 ml of 2 N of potassium hydroxide in methanol and 2 ml of heptane were added and the whole mixture was agitated vigorously. After becoming clear, the upper solution, 1 µl was injected into an HP5890 (Hewlett-Packard) GC to perform SQ quantification. The equipment included a split injection system (split ratio 1:50) and a flame ionization detector. Components were separated on a fused silica capillary column SPB-5 (30 m × 0.25 mm i.d.) coated with 5% of phenyl-95% methylsilicone, 0.25 µm of thickness (Supelco). The equipment conditions were: 1 ml/min hydrogen flow rate, 130 Kpa; 280°C injector temperature, 300°C detector temperature; oven temperatures were 240°C for 6 min initially and then rising at 5°C/min to 280°C. Results are expressed as mg/100 mg UF and total concentration is calculated as the sum of individual concentrations.

2.2 | Preparation of PF and hydroxytyrosol (HTyr) from VOO

PF was obtained using the method described by Vazquez-Roncero, Janer del Valle, and Janer del Valle (1973). An amount of 50 g of VOO was extracted with 125 ml of methanol:water (80:20, v/v). First the resultant was mixed using a vortex during 1 min at a speed of 5,000 g followed by sonication during 15 min. Methanolic phase was concentrated after decantation in vacuum conditions under a stream of nitrogen at a temperature lower than 35°C until it reached a syrupy consistency. PF was then lyophilized and stored at −80°C. Both quantitative and qualitative analysis of PF were carried out according to the COI/T20/29doc. This protocol is based on direct extraction and quantification of the phenolic minor polar compounds using a methanol solution and HPLC. Once extracted, an aliquot of the supernatant phase was taken, filtered, and injected into a HPLC system which included C18 reverse-phase column (25 cm × 4.6 mm), type Spherisorb ODS-2 (5 mm) and a spectrophotometric UV detector at 280 nm. Phenols content was quantified after measurement of areas of the related chromatographic peaks and expressed in µM of phenols in 50 µg PF/ml. Table 2 includes detailed data concerning the composition of the isolated PF. To in vitro treatments, both HTyr and SQ were purchased by Sigma-Aldrich (Madrid, Spain).

TABLE 1 Main composition of UF from EVOO

Component	mg/kg (ppm)
Squalene	5,681
Total sterols	1,289
Campesterol	40.7
β-sitosterol	7.8
Δ5-Avenasterol	69.7
Total methylsterols	69.8
Total triterpenic alcohols	568
Total aliphatic alcohols	163
C24	40.8
C26	71.9
α-tocopherol	87

2.3 | Blood collection and isolation of human monocytes

To perform this study, we proceeded according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Healthy male blood donors aged <35 declared to be non-smokers and not taking any medication. Participants agreed to give informed consent at the University Hospital Virgen del Rocio, Seville. From a large antecubital vein, peripheral blood samples were taken and then collected into K3EDTA-containing tubes (Becton Dickinson, NJ, USA). Centrifugation over a Ficoll-Histopaque (Sigma) gradient isolated peripheral blood mononuclear cells (PBMCs) from peripheral blood samples. Then using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) according to the manufacturer's instructions, monocytes were finally isolated from PBMCs. Flow cytometry (FACScanto II flow cytometer and FACSDiva software, BD) showed a purity of the CD14 monocytes isolations >90% routinely. Isolated monocytes were finally resuspended in RPMI 1640 cell-culture medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin.

2.4 | Cell treatments

Monocytes (5×10^5 cells/ml) were incubated with LPS (100 ng/ml) followed or not of 24 hr treatment in 24-well plates with UF at 25 and 50 µg/ml, SQ at 50 µM, PF at 25 and 50 µg/ml, or HTyr at 41 µM from

TABLE 2 Composition of PF from EVOO using COI/T20/29doc

Component	µM Phenol (50 µM 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

VOO. SQ and HTyr concentration and HTyr concentration in human monocytes was selected based on previous studies and these concentration are equivalent to the concentration of SQ in UF at 50 μg/ml and of HTyr in PF at 50 μg/ml. Cells incubated only with LPS were considered as positive control and untreated cells were considered as negative control. Supernatants were removed after the treatments, and then stored at −20°C. Cells were used to RNA extraction.

2.5 | RNA isolation and quantitative real-time PCR analysis

Trisure Reagent (Bioline) was used to extract total RNA. In a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), RNA quality was evaluated by A_{260}/A_{280} ratio. Reverse transcription was performed using 1 μg RNA (iScript, BioRad). About 20 ng of the cDNA obtained was used as template for real-time polymerase chain reaction amplifications. Amplification of each specific gene product was performed using a CFX96 system (BioRad). Every PCR reaction contained brilliant SYBR green QPCR Supermix (BioRad), the primer pairs for the corresponding gene and cDNA template. As housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used. Table 3 shows both the sequence and the information for the primers. Every reaction was done in triplicate. To estimate the relative mRNA expression of analyzed genes, the average threshold cycle (Ct) values of the triplicates were used. With the standard $2^{-(\Delta\Delta Ct)}$ method, the magnitude of change of mRNA expression for candidate genes was assessed. Results were normalized using *GAPDH* expression and showed as percentage of control samples.

2.6 | Measurement of cytokine release

To determine levels of visfatin, IL-1β, IL-6, TNF-α, and IL-10 in culture supernatants the enzyme-linked immunosorbent assay (ELISA) was used, according to the instructions of the manufacturer (Diaclone, Besancon, France). The levels of visfatin, IL-1β, IL-6, TNF-α, and IL-10 in culture supernatants were measured by ELISA, following the indications of the manufacturer (Diaclone, Besancon, France). Using calibration curves from serial dilution of human recombinant standards in each assay, the concentrations of cytokine were estimated and expressed in pg/ml.

2.7 | Statistical analysis

Data were expressed as arithmetic mean ± *SD* (standard deviations). Statistical analyses were done using Graph Pad Prism Version 5.01

TABLE 3 Sequences of RT-qPCR primers for gene expression analysis

Target	No. *GenBank*	Direction	Sequence (5′ --> 3′)
Visfatin/NAMPT	NM_005746.2	Forward	GCCAGCAGGGAATTTTGTTA
		Reverse	TGATGTGCTGCTTCCAGTTC
NMNAT-1	NR_022787.3	Forward	TCTCCTTGCTTGTGGTTCATTC
		Reverse	TGACAACTGTGTACCTTCCTGT
NMNAT-2	NM_015039.3	Forward	GTAAAACGACGGCCAGT
		Reverse	TAATACGACTCACTATAGG
NMNAT-3	NM_001200047.2	Forward	GTAAAACGACGGCCAGT
		Reverse	TAATACGACTCACTATAGG
SIRT-1	NM_012238.4	Forward	CAGACCCTCAAGCCATGTTT
		Reverse	TAATACGACTCACTATAGG
PPARγ	NM_001330615.1	Forward	CTGAATGTGAAGCCCATTGAA
		Reverse	GATCCTTTGGATTCCTGAAA
MCP-1	NM_011333.3	Forward	ACTGAAGCTCGTACTCTC
		Reverse	CTTGGGTTGTGGAGTGAG
CCR2	NM_001123041.2	Forward	AGAGGCATAGGGCAGTGAGA
		Reverse	GCAGTGAGTCATCCCAAGAG
TNF-α	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IL-1β	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
IL-6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
IL-10	NM_001289746	Forward	ATGCACAGCTCAGCACTGC
		Reverse	GTTTCGTATCTTCATTGTCATGTAGG
GAPDH	NM_002982.3	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT

software (San Diego, CA, USA). Significance of parameter variations was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as post hoc test. Those *P* values fewer 0.05 were determined statistically significant.

3 | RESULTS

3.1 | Effects of minor compounds from VOO on visfatin/eNAMPT secretion in LPS-treated human monocytes

The effects of UF, SQ, PF, and HTyr of VOO on secretion of visfatin (eNAMPT) in human primary monocytes are showed in Figure 1. LPS caused an increase in the concentration of visfatin (eNAMPT) in the medium of human primary monocytes compared to untreated control cells. Interestingly, this LPS-induced secretion of visfatin was prevented by UF at 50 μg/ml, PF at 25 and 50 μg/ml and HTyr at 50 μM but not by UF at 25 μg/ml or SQ at 41 μM. In the case of PF, the effect was dependent on concentration (50 μg/ml > 25 μg/ml, $p < 0.05$). These observations suggest that minor compounds in VOO, probably phenolics, such as HTyr, may have a beneficial role in regulating visfatin secretion in activated human monocytes.

3.2 | Effects of minor compounds from VOO on expression of NAMPT- and inflammatory-related genes in LPS-treated human monocytes

Genes involved in the NAD^+ biosynthetic salvage pathway, including *NAMPT* (Figure 2A), *NMNAT-1* (Figure 2B), and NMNAT-3 (Figure 2D), were downregulated by LPS in human primary monocytes; however, *NMNAT-2* gene was unaffected (Figure 2C). When LPS-treated cells were incubated with UF, PF, HTyr, and SQ not only the effect of LPS on the above genes was abrogated (e.g., NAMPT gene with UF at 25 μg/ml and 50 μg/ml and SQ at 50 μM) but also their transcriptional activity was upregulated. This was particularly prominent with PF and HTyr, and concentration-dependent (50 μg/ml > 25 μg/ml, $p < 0.05$) with PF on *NMNAT-1* and *NMNAT-2* genes. In addition, the expression of genes encoding NAD^+-dependent deacetylase *SIRT-1* (Figure 2E) and its downstream target PPARγ (Figure 2F) was repressed by LPS but upregulated in the presence of VOO-derived fractions or compounds, with notable effects of PF and HTyr and with the exception of SQ that only retained mRNA of *PPARγ* gene to a level similar to that of untreated control cells. We also observed that LPS-induced upregulation of *MCP- 1* gene (Figure 2G) (which encodes an important factor for the recruitment of cells within foci of inflammation) and *CCR2* gene (Figure 2H) (which encodes the receptor for *MCP-1*) was abolished by either UF, PF, HTyr, or SQ, reaching mRNA levels lower than those found in the untreated control cells by PF at 50 μg/ml or HTyr at 41 μM. These observations suggest that minor compounds in VOO, probably SQ and phenolics such as HTyr, may have a beneficial role in regulating NAD^+ biosynthetic pathway and the inflammatory response in activated human monocytes.

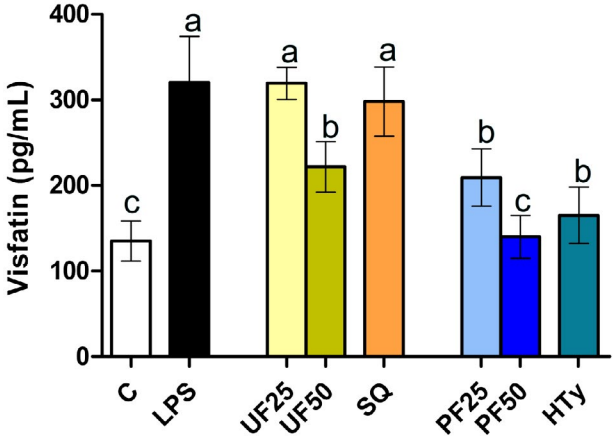

FIGURE 1 Effects of UF, SQ, PF, and HTyr from VOO on visfatin/eNAMPT secretion in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml), or HTyr (at 41 μM) for 24 hr. The concentration of visfatin in culture supernatants was measured by ELISA. Values shown are the mean ± *SD* ($n = 3$) and those marked with different lowercase letters are significantly different ($p < 0.05$)

3.3 | Effects of minor compounds from VOO on gene expression and of pro- and anti-inflammatory cytokines in LPS-treated human monocytes

To gain insight into the effects of VOO-derived fractions and compounds in human primary monocytes upon LPS treatment, we analyzed secretion and gene expression of both pro-inflammatory (*IL-1β*, *IL-6*, and *TNF-α*) and anti-inflammatory (*IL-10*) cytokines. UF, PF, HTyr, and SQ diminished LPS-induced release of IL-1β (Figure 3A), IL-6 (Figure 3B), and TNF-α (Figure 3C) as well as expression of genes encoding these cytokines (Figure 3E–G). In the case of UF and PF, their effects were concentration-dependent (50 μg/ml > 25 μg/ml, $p < 0.05$). The IL-10 anti-inflammatory cytokine secretion was increased by UF at 50 μg/ml, PF at 25 and 50 μg/ml, and HTyr at 50 μM (Figure 3D) while all tested VOO-derived fractions and compounds increased the transcriptional activity of *IL-10* gene (Figure 3H) above the level of untreated control cells. The effects of UF and PF on IL-10 secretion and of PF on *IL-10* gene expression were concentration-dependent (50 μg/ml > 25 μg/ml, $p < 0.05$). These observations reinforce the notion that minor compounds of VOO, probably SQ and phenolics such as HTyr, may have a role in reducing the inflammatory response in activated human monocytes.

4 | DISCUSSION

"Mediterranean diet" is described as protective preventing cardiovascular diseases, not only by improving classical risk factors, but also by promoting an anti-inflammatory effect, while others like "meat-based" or "Westernized" diets are considered as inductive of inflammatory conditions (Naranjo et al., 2016). The activation

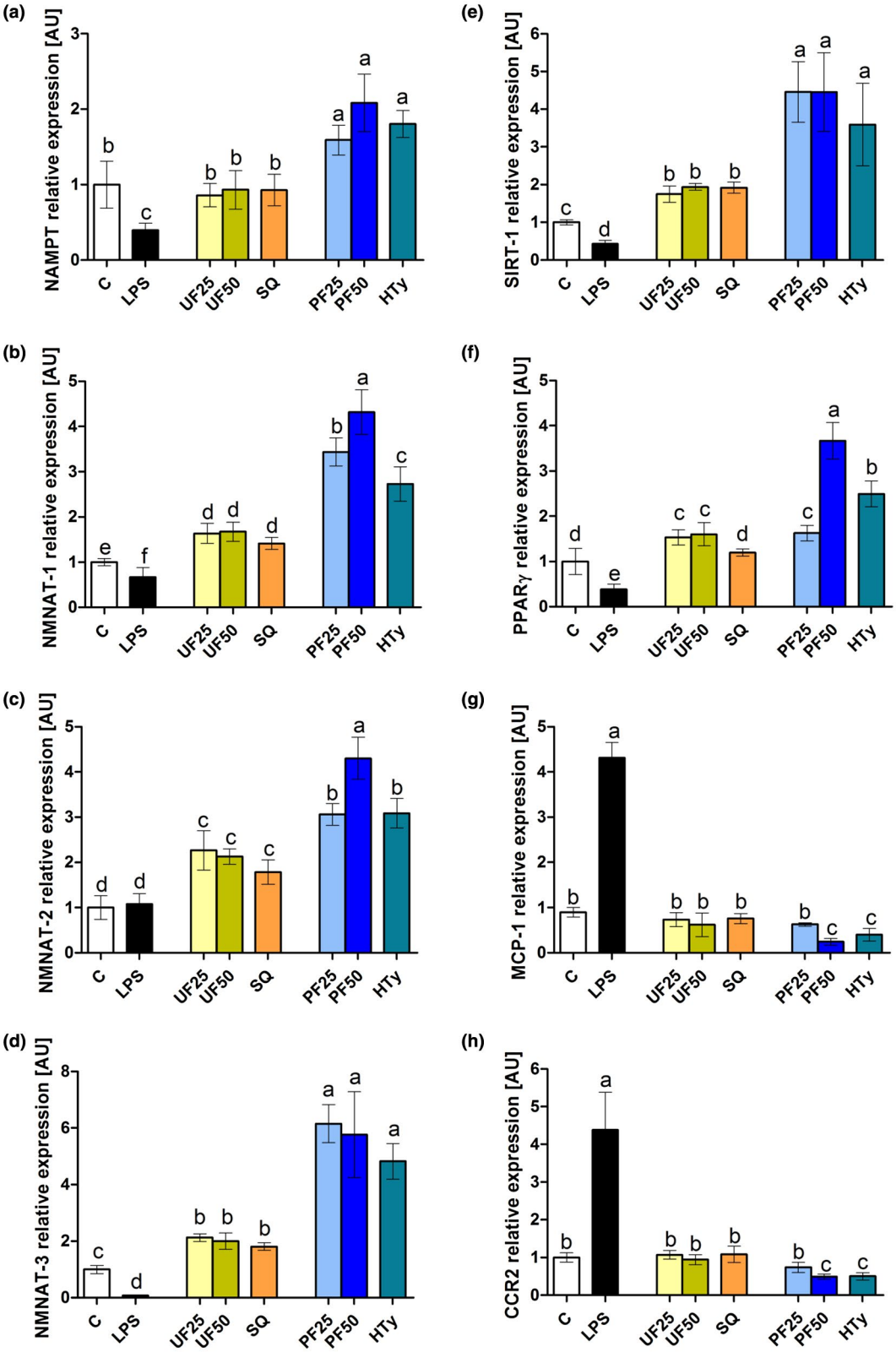

FIGURE 2 Effects of UF, SQ, PF, and HTyr from VOO on expression of NAMPT- and inflammatory-related genes in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml), or HTyr (at 41 μM) for 24 hr. The expression of *NAMPT* (A), *NMNAT-1* (B), *NMNAT-2* (C), *NMNAT-3* (D), *SIRT-1* (E), *PPARγ* (F), *MCP-1* (G), and *CCR2* (H) genes was measured by qPCR. Values shown are the mean ± *SD* (n = 3) and those marked with different lowercase letters are significantly different ($p < 0.05$)

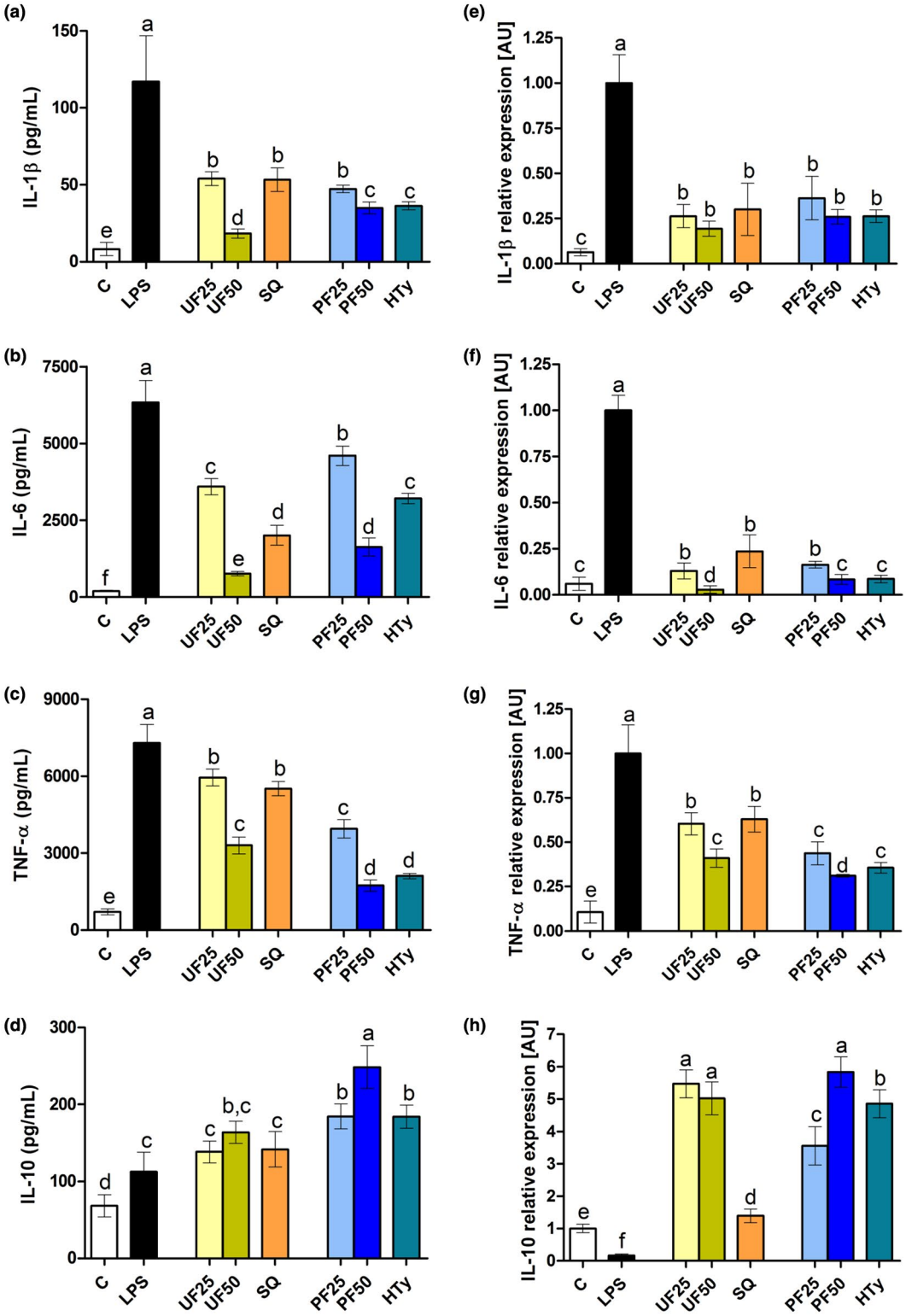

FIGURE 3 Effects of UF, SQ, PF, and HTyr from VOO on secretion and gene expression of pro- and anti-inflammatory cytokines in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml), or HTyr (at 41 μM) for 24 hr. The concentration of IL-1β (A), IL-6 (B), TNF-α (C), and IL-10 (D) in culture supernatants was measured by ELISA, whereas the expression of *IL-1β* (E), *IL-6* (F), *TNF-α* (G), and *IL-10* (H) genes was measured by qPCR. Values shown are the mean ± *SD* (n = 3) and those marked with different lowercase letters are significantly different ($p < 0.05$)

of circulating leukocytes is known to be a remarkable process during inflammation (Montserrat-de la Paz, Rodriguez, et al., 2017). Among the leukocytes, when injury occurs, the earliest and quickest immune cells to react are the monocytes, and its activation causes the increase and perpetuation of the inflammatory state (Libby, Nahrendorf, & Swirski, 2016). Herein, we aimed to address the relevance of UF from VOO in modulating LPS-mediated inflammatory response in human primary monocytes. Our study shows that UF and PF, as well as HTyr from VOO could reduce the inflammatory response via downregulation of visfatin. This protein has a potential involvement in inflammation and several other disorders, including myocardial failure, atherosclerosis, metabolic syndrome, malignancies, neurodegeneration, and aging (Montserrat-de la Paz, Lopez, et al., 2014). There is recent evidence of LPS targeting visfatin as a biomarker of inflammation (Iwasa et al., 2016). However, the role of visfatin/*NAMPT* is controversial. Recent studies have been focused on eNAMPT, which is thought to act as a pro-inflammatory cytokine (Laiguillon et al., 2014). However, Lin et al. (2015) and our research group also reported that *iNAMPT* may have beneficial anti-inflammatory properties (Bermudez et al., 2017). In the present study, this dual role of eNAMPT (visfatin) and *iNAMPT* has been explored by analyzing the concentration of visfatin/eNAMPT in the medium of cells and the relative mRNA levels of *NAMPT/iNAMPT*. Other compounds in the unsaponifiable of olives, such as oleanolic acid and related triterpenoids, have been shown to inhibit visfatin secretion in murine 3T3-L1 adipocytes (Rodriguez-Rodriguez, 2015). The effects on visfatin secretion that we observed were dependent on the concentration of UF and PF from VOO, suggesting that these fractions, probably due to HTyr, have anti-inflammatory properties through regulation of visfatin exit from cells. The results or our study agree with a previous study in which UF of VOO exhibited antioxidant and inhibitory activity of NF-κB-mediated signaling in murine peritoneal macrophages treated with LPS (Cardeno et al., 2014). It is noteworthy that a nutritional intervention demonstrated that the ingestion with VOO (25 ml/day, 577 mg of phenolic compounds/kg) for 4 weeks reduced serum levels of visfatin/eNAMPT in 30 patients with type-2 diabetes (Santangelo et al., 2016). This effect was accompanied with an improvement in the metabolic control of glucose and in the profile of circulating inflammatory adipokines. Previously, Gesing and cols (2017) show that circulating levels of eNAMPT and iNAMPT protein expression in PBMC were selectively increased in patients with acute infections and symptomatic chronic inflammatory conditions compared to healthy controls and also compared to patients with asymptomatic chronic inflammatory diseases, allergic manifestations, or obesity.

Our study is the first in showing a role for UF, PF, SQ, and HTyr from virgin olive oil in the regulation of genes involved in NAD^+ biosynthesis, such as *NAMPT (iNAMPT)*, *NMNAT-1*, *NMNAT-2*, and *NMNAT-3*. *NMNAT-1* has been recently reported to exhibit protection of neuronal function against tau-induced inflammation and atrophy in a mouse model of Alzheimer´s disease (Musiek et al., 2016). A possible role of *NMNAT* family members in the biology of innate immune cells and in the inflammatory response mediated by monocytes remains to be established. The fact that LPS downregulated the gene encoding *NAMPT* suggests that *iNAMPT* is not involved in the inflammatory response induced by LPS in human monocytes. These findings could be indicative of contrary activities of visfatin/eNAMPT and *iNAMPT* in inflamed tissues (Benito-Martin et al., 2014).

Because *NAMPT* is a key NAD^+ biosynthetic enzyme and *SIRT1* requires NAD^+ for its enzymatic activity that includes the abolishment of inflammatory mediator production and cell recruitment (Busch, Mobasheri, Shayan, Stahlmann, & Shakibaei, 2012; Dong et al., 2014), we also analyzed the impact of the UF, PF, SQ, and HTyr from VOO in the gene regulation of *SIRT1* and its downstream target *PPARγ* (Qu et al., 2016). In a recent study, *SIRT1* has been described as a critical immune regulator whose expression can be downregulated in human macrophages following the release of inflammatory cytokines upon cell stimulation with LPS (Jia et al., 2016). In contrast, the deficiency of *SIRT1* in human monocytes and mononuclear cells has been associated to systemic and possibly vascular wall inflammation, leading to atherogenesis (Kitada, Ogura, & Koya, 2016). In human monocytes, we found that all tested fractions or compounds of VOO reversed the reduction of *SIRT1* gene expression caused by LPS. Our results match with those referred to the polyphenol resveratrol in TNF-α treated human endothelial cells (Liu et al., 2017). Furthermore, we observed that the gene expression profile for *PPARγ* was similar as to *SIRT1*; the UF, PF, and HTyr not only restoring but also potentiating *PPARγ* gene expression in LPS-treated human monocytes. PPARγ agonists were reported to suppress inflammation by inhibiting NF-κB signaling pathways in endothelial cells (Marcone, Haughton, Simpson, Belton, & Fitzgerald, 2015). Therefore, our study suggests that compounds in the UF of VOO may counteract LPS-induced pro-inflammatory response by restoring mRNA levels of *SIRT1* and *PPARγ* genes in human monocytes.

To evaluate more deeply into the anti-inflammatory action of UF, PF, SQ, and HTyr from VOO in human monocytes, we investigated whether these fractions or compounds played a role in the regulation of *MCP-1* and *CCR2*. *MCP-1*, also known as *CCL2*, is mainly produced by monocytes/macrophages in response to many stimuli and conditions for example cytokines, oxidative stress or growth factors (Deshmane, Kremlev, Amini, & Sawaya, 2009). After binding this chemokine to the receptor *CCR2*, both migration and infiltration of many cell systems including monocytes, natural killer cells, T lymphocytes, and memory cells, are facilitated (Vakilian, Khorramdelazad, Heidari, Rezaei, & Hassanshahi, 2017). We found that all tested fractions or compounds of VOO avoided LPS-induced up-regulation of *MCP-1* and *CCR2* genes in human monocytes. Sanchez-Fidalgo, Cardeno, Sanchez-Hidalgo, Aparicio-Soto, and de la Lastra (2013) and Sanchez-Fidalgo, Cardeno, Sanchez-Hidalgo, Aparicio-Soto, Villegas, et al. (2013) demonstrated that dietary VOO polyphenols and unsaponifiable supplementation reduced *MCP-1* in a chronic colitis in mice. Other natural polyphenols such as curcumin have been shown to exert similar effects on *MCP-1* gene expression in several cell lines (Karimian, Pirro, Majeed, & Sahebkar, 2016). Our findings were consistent with the notion that UF

and PF, and notably HTyr of VOO may be useful for preventing recruitment and chemotaxis of monocytes across the vascular endothelium.

These observations were consistent with the decrease in the medium and prevention of LPS-induced gene expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, and *TNF-α*) in human monocytes. All the fractions (in a dose-dependent manner), SQ, and HTyr exerted these beneficial effects. Of note, they also promoted the release and gene expression of the anti-inflammatory cytokine *IL-10*. Our study is complementary to a previous one in which squalene was shown to reduce mRNA levels of pro-inflammatory cytokines in human monocytes and neutrophils (Cardeno et al., 2015). This is in line with evidence of anti-inflammatory properties of UF of virgin olive oil (Amiot, 2014; Cardoso, Catarino, Semiao, & Pereira, 2014).

5 | CONCLUSIONS

This study unveils new beneficial effects of UF, including SQ, PF, and HTyr, from VOO and establishes the potential use of these fractions/compounds in the prevention and treatment of inflammatory disorders. Within this context, the consumption of VOO and its bioactive components have shown a broad range of promising activities in different inflammatory and autoimmune diseases and it may be a relevant approach in the prevention and management of these disorders.

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CONFLICTS OF INTEREST

The authors state no conflict of interest.

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