

# Virgin olive oil rich in phenolic compounds modulates the expression of atherosclerosis-related genes in vascular endothelium

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## Abstract

**Purpose** Previous studies have shown the anti-inflammatory and antioxidant properties of phenolic compounds of virgin olive oil (VOO). However, the effect of bioavailable phenolic compounds on the vascular endothelium is unknown. We aimed to evaluate the effect of the consumption of virgin olive oil rich in phenolic compounds on the vascular endothelium.

**Methods** We treated HUVEC with human serum obtained in fasting state and after the intake of a breakfast

prepared with VOO with a high or low content of phenolic compounds.

**Results** Treatment of HUVEC with serum obtained 2 h after the intake of the high-phenol VOO-based breakfast decreased *p65* and *MCP-1* gene expression ( $p < 0.001$  and  $p = 0.002$ , respectively) and increased *MT-CYB*, *SDHA* and *SOD1* gene expression ( $p = 0.004$ ,  $p = 0.012$  and  $p = 0.001$ , respectively), as compared with the treatment of HUVEC with the serum obtained 2 h after the intake of the low-phenol VOO-based breakfast. The treatment with serum obtained 4 h after the intake of the high-phenol VOO-based breakfast decreased *MCP-1* and *CAT* gene expression ( $p < 0.001$  and  $p = 0.003$ , respectively) and increased *MT-CYB* gene expression ( $p < 0.001$ ), as compared to the treatment with serum obtained 4 h after the intake of the low-phenol VOO-based breakfast.

**Conclusion** Our results suggest that the consumption of virgin olive oil rich in phenolic compounds may reduce the risk of atherosclerosis development by decreasing inflammation and improving the antioxidant profile in the vascular endothelium.

**Keywords** Phenolic compounds · Virgin olive oil · Vascular endothelium · Gene expression · HUVEC

Francisco Pérez-Jiménez and Antonio Camargo have contributed equally to this article.

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## Introduction

Metabolic syndrome is a disorder associated with a high risk of cardiovascular diseases (CVD) and characterized by hypertriglyceridemia, low HDL cholesterol levels, insulin resistance, abdominal obesity and hypertension [1, 2].

Atherosclerosis, the pathogenic substrate for CVD in patients with metabolic syndrome, is an inflammatory disease closely related to endothelial dysfunction (ED) [2],

which is one of the first steps in the development of arteriosclerosis [3] and is associated with and predicts CVD [4]. The early stages of atherosclerosis development are characterized by the transport of LDL particles across the endothelium wall, which triggers several endothelium responses, such as the expression of adhesion molecules and the release of cytokines, which leads to the tethering, activation and attachment of lymphocytes and macrophages. These phagocytize LDL particles, which causes the generation of reactive oxygen species (ROS), and then, they become foam cells and form the fatty streak and further the atherosclerotic plaque [5, 6].

Oxidative stress is known to be a key factor in the pathogenesis of CVD [7, 8], as it has been shown that it plays a role in the process of endothelial dysfunction [9]. Additionally, it is acknowledged that high concentrations of serum inflammatory markers are associated with CVD [10], which is particularly important in a number of pathological conditions such as obesity and metabolic syndrome, which are characterized by a low-grade inflammation that favors the development of atherosclerosis [11].

However, the processes involved in the development of atherosclerosis are sensitive to modulation by diet, which therefore influences the development of CVD. In fact, the low rate of cardiovascular mortality in Mediterranean countries could be explained on the basis of the anti-atherogenic effects associated with consumption of the Mediterranean diet [12, 13]. In particular, virgin olive oil (VOO), the major fatty component of the Mediterranean diet, and whose beneficial effects are attributed to its minor components such as phenolic compounds, is considered the key component of the Mediterranean diet and its healthy benefits [14–16].

In fact, our group has previously shown that the consumption of VOO with a high content of phenolic compounds improves endothelial dysfunction and reduces oxidative stress plasma parameters [17–19]. Moreover, it has also been described how phenolic compounds have a positive effect on the inflammatory response through a lower activation of nuclear transcription factor *NF- $\kappa$ B* in peripheral blood mononuclear cells (PBMC) [20], which repress the expression of genes involved in inflammation and modulate positively the expression changes of cancer-related genes, DNA damage, oxidative stress, apoptosis and lipid metabolism [21–23].

In addition, *in vitro* studies have shown that olive oil phenolic compounds repress the gene expression of pro-atherogenic adhesion molecules via inactivation of *NF- $\kappa$ B* in endothelial cells [24–26] and that they show a powerful scavenging ROS activity in the presence of high oxidative stress, prevent oxidative damage to DNA and enhance antioxidant activity in endothelial cells and PBMC [27–29].

However, the main limitations of most of the studies *in vitro* lie in the fact that they were performed by using non-bioavailable phenolic compounds, while most of the phenolic compounds are modified after the intake of olive oil, and their derived metabolites are found in plasma [30, 31]. Therefore, the objective of this study was to fill this gap by evaluating the effect of the consumption of virgin olive oil rich in phenolic compounds on the vascular endothelium, by treating HUVEC with serum obtained after the intake of high-phenol and low-phenol VOO-based breakfasts.

## Materials and methods

### Clinical study description and serum sample collection

Serum from a population of twenty patients with metabolic syndrome was used [22]. All subjects fulfilled three or more of the criteria proposed by the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) for metabolic syndrome. These patients received two virgin olive oil-based (VOO) breakfasts with high (398 ppm) and low (70 ppm) content of phenolic compounds following a double-blinded random crossover design. All of them gave their informed consent and underwent a comprehensive medical history, physical examination and clinical chemical analysis before enrollment. The experimental protocol was approved by Human Investigation Review Committee at Reina Sofia University Hospital according to the Helsinki Declaration. Venous blood samples were obtained at 0, 2 and 4 h after consumption of each breakfast. Samples from fasting and postprandial states were collected in BD Vacutainer® Serum Tube (Cat. No. 367624) and stored in containers with iced water and kept in the dark. Special care was taken to avoid exposure to air, light and ambient temperature. Serum was separated from whole blood by low-speed centrifugation at 1500×g for 20 min at 20 °C within 1 h after extraction. Serum samples were pooled according to the time and breakfast: fasting and at 2 and 4 h after the intake of the breakfast prepared with VOO with a high content of phenolic compounds, and fasting and at 2 and 4 h after the intake of the breakfast prepared with VOO with a low content of phenolic compounds. No significant differences between the VOO-based breakfasts with different phenolic contents were observed in the main metabolic variables in plasma (total cholesterol, triglycerides, LDL-c, HDL-c, glucose and insulin) in the postprandial state. The breakfasts were similar with the exception of the oils, which had a similar composition of fat and micronutrients, with the exception of phenolic compounds content (70 vs 398 ppm). Thus, the differences in the antioxidant levels

between the serums used to treat the cells come from the different content in phenolic compounds of the oils. To prepare the low-phenol olive oil, other antioxidants present in olive oil, such as tocopherols, were left in the oil after physical extraction of most phenolic compounds from the high-phenol olive oil [32].

The phenolic compounds or metabolites derived from phenolic compounds found in plasma, oleuropein, hydroxytyrosol monoglucuronide, tyrosol glucuronide, luteolin, apigenin, homovanillic acid sulfate, hydroxytyrosol monosulfate, tyrosol sulfate, syringic acid, ferulic acid, caffeic acid, vanillic acid, o-coumaric acid, p-coumaric acid, hydroxytyrosol and vanillin have been previously described [32].

### Cell culture

Human umbilical vein endothelial cells (HUVEC; 5th passage) (Cambrex Bio Science Walkersville, Inc) were cultivated until confluence was reached (within 3–4 days) in EMB medium supplemented with an EMB-bullet kit (Lonza Walkersville, Inc) containing 10 % FBS, hEGF, hydrocortisone, GA-1000, VEGF, hFGF- $\beta$ , R<sup>3</sup>-IGF-1 and heparin, in a humidified atmosphere (37 °C, 5 % CO<sub>2</sub>). The growth and proliferation of the cells were monitored daily under a microscope to ensure that they continued as endothelial cells. The culture medium was changed every 2 days. The cells were detached using trypsin–EDTA (Lonza Walkersville, Inc).

Once the HUVEC reached confluence, the medium was replaced by one supplemented with 10 % of human serum pool (5.4 ml of EBM media not supplemented and 0.6 ml of serum pool for each time point and oil). The cell treatment with human serum was performed for 24 h under the same environmental conditions mentioned above. The treatment was repeated three times, and the results are presented as a mean result. Cell viability was checked every time through 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay.

### RNA isolation

Total RNA was extracted from the endothelial cells with TRI Reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) and purified with RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). Recovered RNA was quantified using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology®, Cambridge, UK).

### qRT-PCR for gene expression analysis

Retrotranscription reaction was performed with 1  $\mu$ g of total RNA using the commercial kit iScript® cDNA

Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), following the manufacturers' instructions. Real-time PCRs were carried out using the OpenArray™ NT Cyclor system (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturers' instructions. The gene expression analysis was performed in samples from the participants by duplicate at basal time, 2 and 4 h after intake of the breakfasts. Primer pairs were selected from the database TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA) <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267>, for the following genes: inflammatory genes: *IKKa* (*Hs00175141\_m1*), *IKKb* (*Hs00233287\_m1*), *p65* (*Hs00153294\_m1*), *Ikb* (*Hs00153283\_m1*), *MCP-1* (*Hs00234140\_m1*), *IL-6* (*Hs00174131\_m1*) and *CXCL1* (*Hs00236937\_m1*), pro-oxidant enzymes and respiratory chain genes: *p22<sup>phox</sup>* (*Hs03044361\_m1*), *gp91<sup>phox</sup>* (*Hs00166163\_m1*), *COX4II* (*Hs00971639\_m1*), *MT-CYB* (*Hs02596867\_s1*), *NADH* (*Hs00942568\_m1*) and *SDHA* (*Hs00188166\_m1*), and antioxidant enzymes: *SOD1* (*Hs00916176\_m1*), *SOD2* (*Hs00167309\_m1*), *CAT* (*Hs00156308\_m1*), *Gpx1* (*Hs00829989\_g1*), *Gpx4* (*Hs00989766\_g1*) and *TXNRD1* (*Hs00917067\_m1*). We used as housekeeping genes: beta-2-microglobulin (*Hs99999907\_m1*), glyceraldehyde-3-phosphate dehydrogenase (*Hs99999905\_m1*) and hypoxanthine phosphoribosyltransferase 1 (*Hs99999909\_m1*). Ct values for these were combined by the software Bestkeeper [33] to obtain a more stable reference value than could be obtained for each one independently. Gene expression values were obtained as a relative expression of the target gene versus the Bestkeeper value (relative expression =  $2^{-(Ct_{\text{Target gene}} - Ct_{\text{Bestkeeper value}})}$ ). The data set was analyzed using OpenArray® Real-Time qPCR Analysis software (Applied Biosystems, Carlsbad, CA, USA).

### Statistical analysis

All the data presented are expressed as mean values  $\pm$  SEM. PASW Statistics, version 18 (Chicago, IL, USA) was used for the statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA). Post hoc statistical analysis was completed by using Bonferroni's multiple comparison tests. A probability of less than 0.05 was considered significant.

## Results

### Effect of phenolic compounds on the expression of inflammatory genes

The treatment of HUVEC with serum obtained at 2 h after the intake of high-phenol VOO-based breakfast decreased

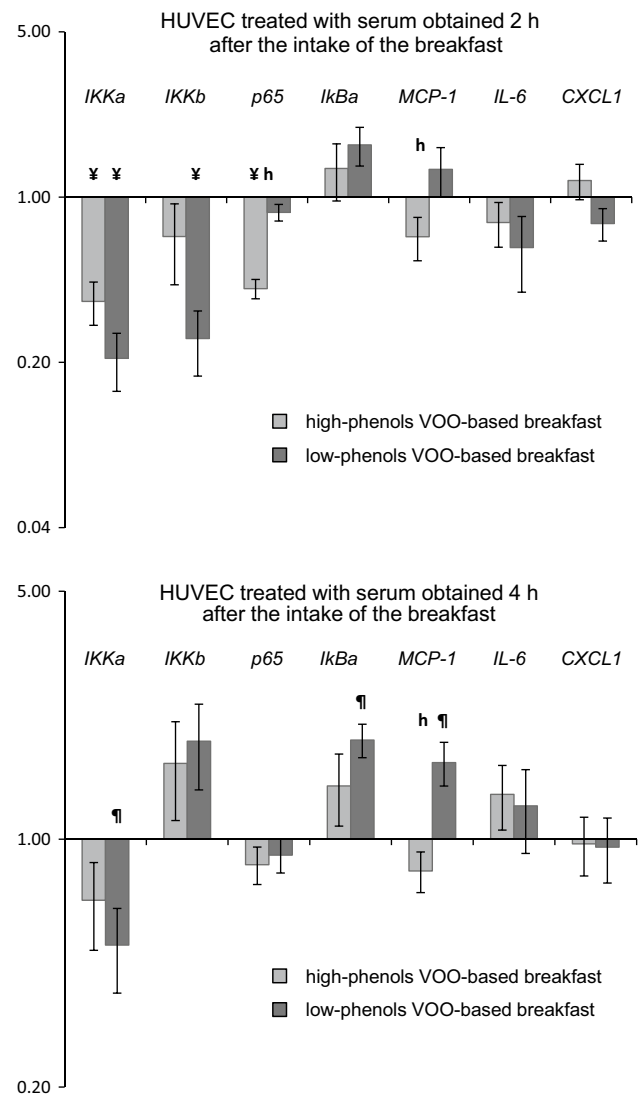
the *IKKa*, and *p65* and gene expression ( $p < 0.001$  in both cases) and tended to reduce *MCP-1* gene expression, although it did not reach statistical significance, as compared to the cells treated with fasting serum. However, when the cells were treated with serum obtained at 2 h after the intake of low-phenol VOO-based breakfast, we observed a decrease in the expression of *IKKa* and *IKKb* genes ( $p < 0.001$  in both cases) as compared to the cells treated with fasting serum.

Moreover, when we treated the HUVEC with serum obtained at 4 h after the consumption of the high-phenol VOO-based breakfast, we observed an increase in the *IkBa* and *MCP-1* gene expression ( $p = 0.002$  and  $p = 0.024$ , respectively) and a decreased *IKKa* gene expression ( $p = 0.006$ ) as compared to the cells treated with fasting serum. No statistical significant gene expression changes were observed when we treated the HUVEC with serum obtained at 4 h after the consumption of the high-phenol VOO-based breakfast as compared to the cells treated with fasting serum (Fig. 1).

#### Effect of phenolic compounds on the expression of pro-oxidant enzymes and respiratory chain genes

The treatment of HUVEC with serum obtained at 2 h after the intake of the breakfasts decreased the *p22<sup>phox</sup>* and *gp91<sup>phox</sup>* gene expression as compared to the cells treated with fasting serum, irrespective of the phenolic compound content of the oil used to prepare the breakfast ( $p < 0.001$  and  $p = 0.004$ , respectively, for the serum obtained after the intake of the high-phenol VOO-based breakfast;  $p < 0.001$  and  $p = 0.018$ , respectively, for the serum obtained after the intake of the low-phenol VOO-based breakfast, respectively). In addition, the treatment of HUVEC with serum obtained at 4 h after the consumption of the breakfasts also decreased the *p22<sup>phox</sup>* gene expression as compared to the cells treated with serum obtained in fasting state, irrespective of the phenolic compound content of the oil used to prepare the breakfast ( $p = 0.005$  and  $p = 0.004$ , respectively) and decreased the *p91<sup>phox</sup>* gene expression when the cells were treated with the serum obtained after the intake of the low-phenol VOO-based breakfast ( $p = 0.033$ ), and it tended to decrease when the cells were treated with the serum obtained after the intake of high-phenol VOO-based breakfast, although it did not reach statistical significance.

Moreover, we observed an increase in the *MT-CYB* gene expression when HUVEC were treated with serum obtained at 2 h ( $p = 0.002$ ) and at 4 h ( $p < 0.001$ ) after the intake of the high-phenol VOO-based breakfast, as compared to the treatment of the HUVEC with serum obtained in the fasting state. The expression of this gene remained unchanged when cells were treated with serum obtained after the intake of the low-phenol VOO-based breakfast



**Fig. 1** Expression of inflammatory genes in HUVEC. Values are mean  $\pm$  SEM. mRNA levels are expressed as fold change (FC) between the gene expression of HUVEC after treatment with the serum obtained from patients at 2 or 4 h after intake of breakfasts as compared to the gene expression of HUVEC after the treatment with serum obtained in the fasting state. One-way ANOVA statistical analysis. §  $p < 0.05$  2 h versus fasting state. ¶  $p < 0.05$  4 h versus fasting state. h,  $p < 0.05$  decrease with serum of VOO with high content of phenolic compounds as compared with serum of VOO low in phenolic compounds

as compared to the treatment of the HUVEC with serum obtained in the fasting state.

We also observed that the expression of the *SDHA* gene increased when the HUVEC were treated with serum obtained at 2 h after the intake of the high-phenol VOO-based breakfast, as compared to the treatment of the HUVEC with serum obtained in fasting state ( $p = 0.030$ ), whereas it decreased when the HUVEC were treated with serum obtained at 2 h after the intake of the low-phenol

VOO-based breakfast, as compared to the treatment of the HUVEC with serum obtained in the fasting state ( $p = 0.006$ ). No statistically significant gene expression changes were observed when we treated the HUVEC with serum obtained 4 h after the intake of the breakfast (Fig. 2).

#### Effect of phenolic compounds on the expression of antioxidant enzyme genes

The treatment of HUVEC with serum obtained at 2 h after the consumption of the high-phenol VOO-based breakfast increased the *SOD1* gene expression ( $p = 0.009$ ) and decreased the *SOD2* and *CAT* gene expression, as compared to the cells treated with fasting serum ( $p = 0.005$  and  $p = 0.006$ , respectively). Otherwise, the treatment of HUVEC with serum obtained at 2 h after the consumption of the low-phenol VOO-based breakfast decreased the *SOD1*, *Gpx4* and *TXNRD1* gene expression compared to the cells treated with fasting serum ( $p = <0.001$ ,  $p = 0.005$  and  $p < 0.001$ , respectively).

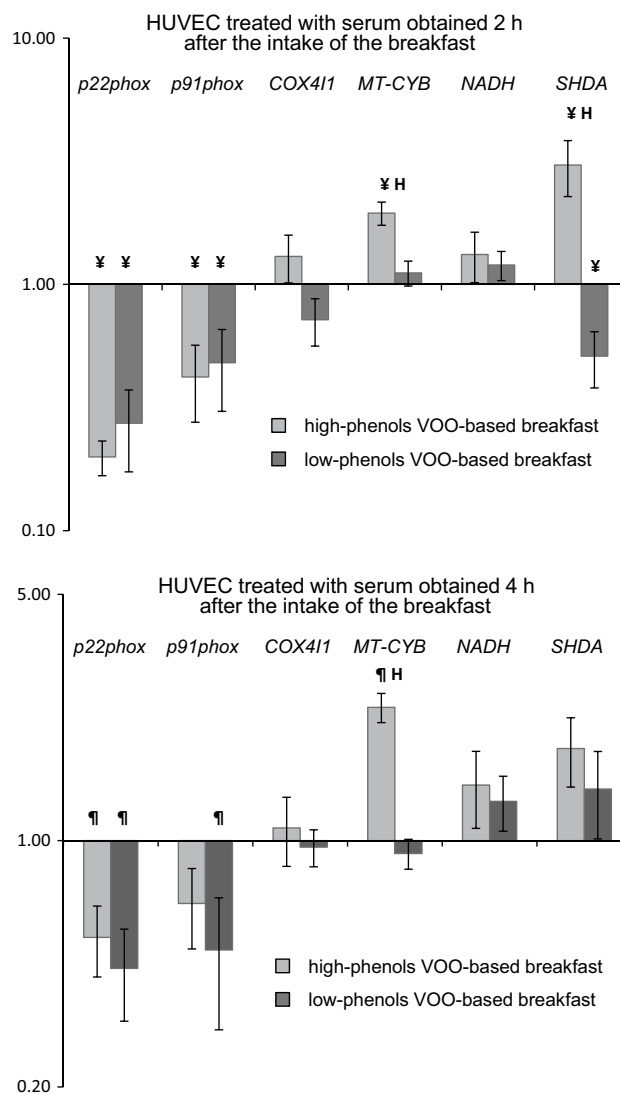
Moreover, the treatment of HUVEC with serum obtained at 4 h after the consumption of the high-phenol VOO-based breakfast decreased the *CAT* and *Gpx4* gene expression as compared to the cells treated with fasting serum ( $p = 0.011$  and  $p = 0.016$ , respectively). No statistically significant gene expression changes were observed after the treatment with serum obtained at 4 h after the consumption of the low-phenol VOO-based breakfast (Fig. 3).

#### Effect of content of phenolic compounds in VOO on the postprandial state

We have also compared the differences between the gene expression after the treatment of HUVEC with the serum obtained after the intake of the breakfast prepared with VOO with a high and low content of phenolic compounds (Figs. 1, 2, 3).

We found that the treatment of HUVEC with the serum obtained at 2 h after the intake of the high-phenol VOO-based breakfast decreased the expression of *p65* and *MCP-1* ( $p < 0.001$  and  $p = 0.002$  respectively) and increased the expression of *MT-CYB*, *SDHA* and *SOD1* ( $p = 0.004$ ,  $p = 0.012$  and  $p = 0.001$ , respectively) as compared to the treatment of the HUVEC with the serum obtained 2 h after the intake of the high-phenol VOO-based breakfast.

Additionally, we also observed that treatment of HUVEC with the serum obtained 4 h after the intake of the high-phenol VOO-based breakfast decreased the expression of *MCP-1* and *CAT* ( $p = <0.001$  and  $p = 0.003$ , respectively) and increased the expression of *MT-CYB* ( $p < 0.001$ ) as compared to the treatment of HUVEC with the serum obtained 4 h after the intake of the low-phenol VOO-based breakfast.

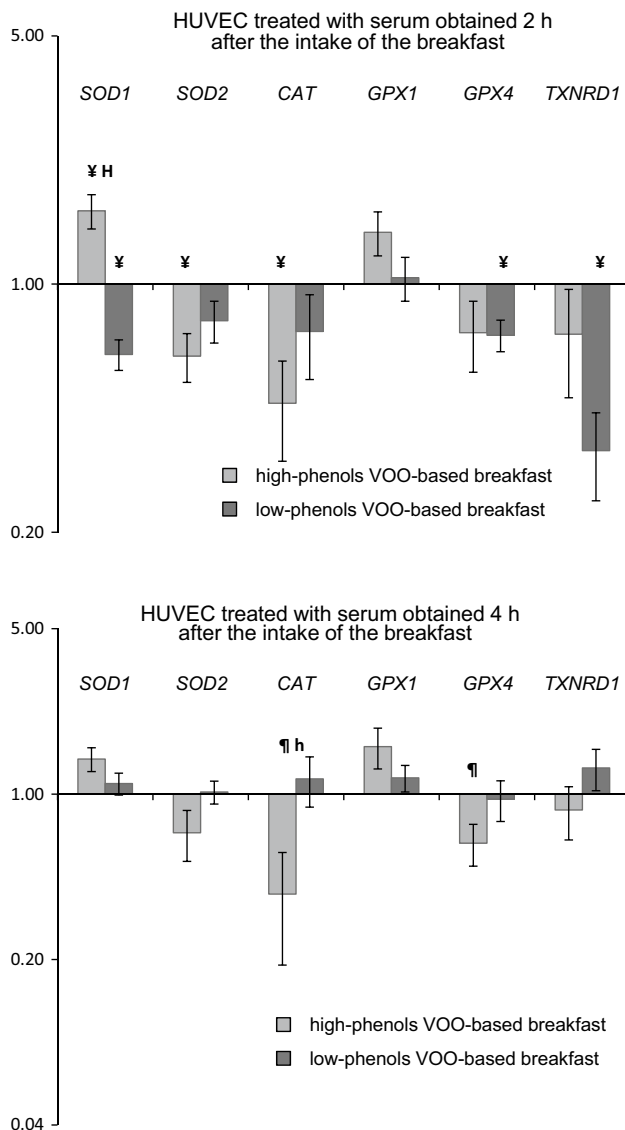


**Fig. 2** Expression of pro-oxidant enzymes and mitochondrial respiratory chain component genes in HUVEC. Values are mean  $\pm$  SEM. mRNA levels are expressed as fold change (FC) between the gene expression of HUVEC after treatment with the serum obtained from patients at 2 or 4 h after intake of breakfasts as compared to the gene expression of HUVEC after the treatment with serum obtained in the fasting state. One-way ANOVA statistical analysis. §  $p < 0.05$  2 h versus fasting state. ¶  $p < 0.05$  4 h versus fasting state. H,  $p < 0.05$  increase with serum of VOO with high content of phenolic compounds as compared with serum of VOO low in phenolic compounds

#### Discussion

Our study evaluated the effect of the consumption of two breakfasts prepared with VOO with high and low phenolic compounds in an in vitro model of vascular endothelium. In fact, once in the organism, the phenolic compounds are modified or conjugated to molecules such as sulfate or glucuronide acid, and the concentration of these bioavailable phenolic compounds in plasma is proportional to their





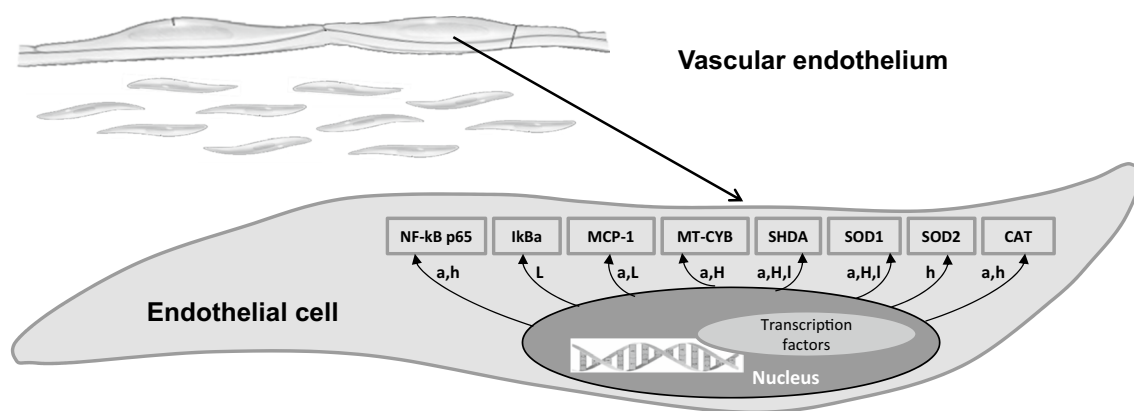
**Fig. 3** Expression of antioxidant enzyme genes in HUVEC. Values are mean  $\pm$  SEM. mRNA levels are expressed as fold change (FC) between the gene expression of HUVEC after treatment with the serum obtained from patients at 2 or 4 h after intake of breakfasts as compared to the gene expression of HUVEC after the treatment with serum obtained in the fasting state. One-way ANOVA statistical analysis. ¥  $p < 0.05$  2 h versus fasting state. §  $p < 0.05$  4 h versus fasting state. H,  $p < 0.05$  increase with serum of VOO with high content of phenolic compounds as compared with serum of VOO low in phenolic compounds. h,  $p < 0.05$  decrease with serum of VOO with high content of phenolic compounds as compared with serum of VOO low in phenolic compounds

concentration in the VOO ingested [30]. We observed that the treatment of HUVEC with serum obtained after the intake of a high-phenol VOO-based breakfast decreased the expression of inflammatory and oxidative stress-related genes, as compared to the cell treated with serum obtained

after the intake of a low-phenol VOO-based breakfast (Fig. 4).

Atherosclerosis, the pathogenic substrate responsible for CVD in patients with metabolic syndrome, is an inflammatory disease, and chronic inflammation is one of the major pathophysiological characteristics of these patients, which makes them very susceptible to developing atherosclerotic plaques [34]. In our study, we found that the treatment of HUVEC with the serum obtained 2 h after the intake of the high-phenol VOO-based breakfast reduced the expression of the *p65* gene, a subunit of the *NF- $\kappa$ B* transcription factor which is involved, among others, in the pathogenesis of atherosclerosis and whose activation leads to the expression of inflammatory genes [35]. The treatment of HUVEC with the serum obtained 2 h after the intake of this breakfast also tended to reduce the expression of *MCP-1*, a pro-inflammatory cytokine that plays a critical role in atherosclerotic plaque formation by initiating fatty streak formation [36, 37]. Additionally, we observed an increase in the expression of *MCP-1* when cells were treated with serum obtained 4 h after the intake of the low-phenol VOO-based breakfast. Moreover, the treatment with this serum also increased the expression of *I $\kappa$ B $\alpha$*  gene, a member of *I $\kappa$ B* family (inhibitors of  $\kappa$ B), which is involved in the *NF- $\kappa$ B* inactivation by sequestering in the cytoplasm and induced as negative feedback when the inflammatory response is activated [38]. These results agree with a previous in vivo study in which we demonstrated the postprandial repression of several pro-inflammatory genes in PBMC after the intake of a high-phenol VOO-based breakfast, as compared to the intake of a breakfast prepared with the same olive oil but with a low content in phenolic compounds [22]. Additionally, other studies have demonstrated that a Mediterranean diet based on VOO with a high content of phenolic compounds also decreases the postprandial expression of the *NF- $\kappa$ B* *p65* subunit and *MCP-1* genes in PBMC [39]. Interestingly, we observed that the expression of both subunits *IKK $\alpha$*  and *IKK $\beta$*  of the I $\kappa$ B kinase complex was reduced when cells were treated with serum obtained 2 h after the intake of the low-phenol VOO-based breakfast and also *IKK $\beta$*  when the serum was obtained 4 h after the intake of this breakfast. These observations suggest that a reduction in the expression of the kinase involved in inflammatory response occurs when this kinase is activated as a negative feedback regulatory loop [38, 40].

Our results also suggest a reduction in oxidative stress in HUVEC after their treatment with the serum obtained after the intake of the high-phenol VOO-based breakfast. In response to oxidative stress, cells attempt to increase their antioxidant defenses. Among them, the antioxidant enzymes *SOD*, *CAT* and *Gpx* are considered to be the first line of defense during oxidative stress [41]. *SOD* converts



**Fig. 4** Scheme showing the effect of phenolic compounds on the vascular endothelium. <sup>a</sup>Decrease with serum of VOO with a high content of phenolic compounds as compared with serum of VOO low in phenolic compounds. <sup>h</sup>Increase with serum of VOO with high content of phenolic compounds as compared with serum at the fasting state.

<sup>h</sup>Decrease with serum of VOO with a high content of phenolic compounds as compared with serum at the fasting state. <sup>L</sup>Increase with serum of VOO low in phenolic compounds as compared with serum at the fasting state. <sup>I</sup>Decrease with serum of VOO with low content of phenolic compounds as compared with serum at the fasting state

$\cdot\text{O}_2^-$  into  $\text{H}_2\text{O}_2$ , which is further detoxified into water or molecular oxygen by either *CAT* or *Gpx* [42].

Our results showed that the treatment of HUVEC with serum obtained 2 h after the intake of the high-phenol VOO-based breakfast decreased the expression of the mitochondrial isoform of *SOD*, the *SOD2* gene and the *CAT* gene, which are both involved in the detoxification of ROS from  $\text{O}_2^-$  up to water [42], suggesting a lower oxidative stress under this condition. By contrast, the expression of *GPx1*, the main enzyme involved in turning  $\text{H}_2\text{O}_2$  into molecular oxygen, was not modulated by any treatment. Moreover, the expression of the *SOD1* gene, the cytoplasmic isoform of *SOD*, increased after the treatment with serum obtained 2 h after the intake of the high-phenol VOO-based breakfast, whereas it decreased when the cells were treated with serum obtained 2 h after the intake of the low-phenol VOO-based breakfast. These have been shown to protect the vasculature against dysfunction, besides decreasing the superoxide levels that can be produced by the mitochondria itself in accordance with the basis of mitochondrial origin of most of the reactive oxygen [42].

This notion is also supported by an increase in the expression of *MT-CYB* and *SDHA* genes, both involved in the mitochondrial electron transport chain, when cells were treated with serum obtained 2 h after the intake of the high-phenol VOO-based breakfast, since their up-regulation prevents the production of pro-oxidant intermediates by a higher consumption of  $\cdot\text{O}_2^-$  [43], while the expression of other sources of ROS such as NADPH oxidase complex (*p22<sup>phox</sup>* and *gp91<sup>phox</sup>*) [44] seems to not be affected by the content of phenolic compounds in the VOO.

Moreover, our results agree with several human studies which showed that the ingestion of high-phenol VOO can partly ameliorate the deleterious effects of oxidative stress

on the ED, an early predictor of arteriosclerosis [17, 45]. In fact, endothelial dysfunction is one of the first steps in the development of arteriosclerosis [3] and is associated with pathophysiological alterations such as inflammation and oxidative stress, which are closely interrelated processes [46, 47]. Endothelial dysfunction is characterized by an increased expression of adhesion molecules in response to pro-inflammatory cytokines [48], a decrease in the availability of nitric oxide in blood vessels and an excessive increase production of ROS that exceeds the capacity of the antioxidant defenses of the endothelium [49, 50].

In addition, comparing the treatments, we found that the treatment of HUVEC with serum obtained after the intake of the high-phenol VOO-based breakfast decreased significantly the gene expression of *p65* and *MCP-1*, increased the gene expression of *MT-CYB*, *SDHA* and *SOD1* at 2 and at 4 h and decreased the *MCP-1* and *CAT* gene expression, while the gene expression of *MT-CYB* remained high. These results are consistent with those found in other studies, where it has shown that a high concentration of phenolic compounds have postprandial anti-inflammatory effects [51] and reduces the gene expression of genes related to inflammation and oxidative stress [21, 23].

Taken together, these data suggest that the consumption of a high-phenol VOO-based breakfast can modulate positively the pathophysiological mechanisms that underlie the early atherosclerosis in the vascular endothelium (Fig. 4), such as the expression of inflammatory genes [52, 53] and also oxidative stress, which elicits an inflammatory response. However, although the serum used in the cell culture showed no differences in lipid levels, the serum obtained after the intake of the low-phenol VOO-based breakfast contained higher levels of pro-inflammatory

cytokines [32], which represents the real condition in which endothelial cells are exposed in the organism.

Our study has limitations, one of which lies in the fact that we performed only a genomic approach, in which we analyzed the oxidative stress (pro-oxidant and anti-oxidant) and inflammation processes at gene expression levels. In addition, although we also studied the effect of the consumption of the high-phenol VOO on a model of vascular endothelium, and we identified several phenolic compounds in plasma, our study did not identify which one of the phenolic compounds, or a synergistic effect of phenolic compounds, are responsible for the observed effect.

In conclusion, our results suggest that the consumption of virgin olive oil rich in phenolic compounds may reduce the risk of developing atherosclerosis in metabolic syndrome patients by decreasing inflammation and improving the antioxidant profile in the vascular endothelium. These results provide further evidence of the reduction in the risk factors for developing cardiovascular disease observed in Mediterranean regions, where the main source of dietary fat is virgin olive oil.

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