RESEARCH ARTICLE

Squalene ameliorates atherosclerotic lesions through the reduction of CD36 scavenger receptor expression in macrophages

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Scope: Anti-atherogenic features of olive oil (OO) have been attributed, in part, to minor compounds, via diverse mechanisms, although its effects on the CD36 receptor have not been examined. We investigated the effects of minor compounds of OO (squalene (SQ), tyrosol (Tyr) and hydroxytyrosol (OH-Tyr)), on the expression of the CD36 receptor, as well as on monocyte/macrophage differentiation and proliferation.

Methods and results: U937 monocytic cells and macrophages (obtained with 10 nM phorbol-myristate-acetate) were exposed to Tyr, OH-Tyr or SQ at 0, 10, 75 and 200 μM with/without native or oxidised LDL (oxLDL). Flow cytometry was used to achieve the expression of CD36 in both cell types exposed to oxLDL plus antioxidants, as well as the inhibition of monocyte/macrophage differentiation after oxLDL and apoptosis. SQ caused a dose-dependent reduction of CD36 in the presence of native and moderate LDL in monocytes and macrophages. Phenotype-dependent cytotoxic and antiproliferative effects were found for OH-Tyr ($p < 0.05$), while SQ affected neither monocytes nor macrophages ($p < 0.01$).

Conclusion: SQ does not prevent monocyte migration and activation into macrophages, but it would inhibit oxLDL uptake by macrophages, by reducing CD36 expression. This study provides new data about the role of the components of OO in the prevention of atherosclerosis.

Keywords:
Atherosclerosis / Hydroxytyrosol / Minor compounds / Olive oil / Squalene

1 Introduction

Atherosclerosis is a cardiovascular disease that is characterised by the accumulation of lipids in the arterial intima, inflammatory response and oxidative events [1]. Oxidised LDL (oxLDL) is recognised and internalised by the CD36 receptor on the surfaces of macrophages, which are subsequently converted into foam cells [2, 3]. This scavenger receptor plays a significant role in atherogenesis; it contributes to macrophage trapping in the arterial intima and can modulate the migration of macrophages in response to oxLDL [4–9].

Virgin olive oil (OO) is an essential element in the Mediterranean diet, and the benefits derived from its consumption include important anti-cancer and anti-aging effects, together with a decreased risk of obesity, metabolic syndrome, type-2 diabetes and hypertension [10]. Moreover, with respect to cardiovascular disease, the consumption of OO can modify the atherosclerotic process [10,11]. OO is capable of halting the progression of atherosclerotic lesions, by modifying the plasma–lipid profile [12], reducing oxidative stress [13],

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; **LDL**, low density lipoprotein; **OH-Tyr**, hydroxytyrosol; **OO**, olive oil; **oxLDL**, oxidised LDL; **PMA**, phorbol 12-myristate-13-acetate; **PPAR**, peroxisome proliferator activated receptor gamma; **SQ**, squalene; **Tyr**, tyrosol

promoting antibodies against oxLDL [14] or reducing the expression of the CD36 receptor [15]. These anti-atherogenic properties attributed to OO mainly arise from molecular components like MUFA or minor compounds such as squalene (SQ), hydroxytyrosol (OH-Tyr) and tyrosol (Tyr) [16]. These substances are known to prevent LDL from oxidation [12, 17–19] and to decrease the expression of proinflammatory and proatherogenic genes [11, 20, 21]. Nonetheless, little is known about the relationship between these minor compounds and CD36. For this reason, we investigated the effects of SQ, OH-Tyr and Tyr on the expression of the CD36 receptor on the cell surface. In addition, we tested their capacity to inhibit the activation of U937 monocyte-like histiocytic lymphoma cells into macrophages in the presence of oxLDL. Finally, we assayed the cytotoxic role of these antioxidants in both cell types.

2 Materials and methods

2.1 Reagents and antibodies

Anti-human CD14 (sc-1182 fluorescein conjugated), anti-human CD45 (sc-18901 phycoerythrin conjugated) and anti-human CD36 (sc-7309 fluorescein conjugated) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin-V (Annexin-V-FITC) and OH-Tyr (3,4-dihydroxyphenyl ethanol) were obtained from Invitrogen (Carlsbad, CA) and Cayman Chemical (Ann Arbor, MI), respectively. Tyr (2-[4-hydroxyphenyl] ethanol), SQ, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phorbol 12-myristate-13-acetate (PMA), propidium iodide, DMSO, RPMI-1640 medium, foetal bovine serum, EDTA, butylated hydroxytoluene (BHT), PBS, HEPES and the other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Human U937 histiocytic lymphoma cell line was purchased from Scientific Instrument Service, University of Granada (Granada, Spain).

2.2 Cell culture and treatments

Human U937 histiocytic lymphoma cells were grown in RPMI-1640 medium (2 mM L-glutamine, 10% foetal bovine serum, 1 mM pyruvate and 100 units/mL each of penicillin and streptomycin) at 37°C and 5% CO_2. The cells were passaged by trypsinisation and plated at a density of 3×10^4 cells/well in 96-well plates. The cells were then treated with Tyr, OH-Tyr and SQ (0, 10, 75, 200 μM) for 24 h by adding them to culture medium as 100-fold-concentrated stock ethanolic solutions. The cells were exposed to ethanol at the same concentrations as the solutions used for the treatments. No mechanism or end-point under investigation was affected (data not shown).

2.3 Differentiation of monocytes into macrophages with PMA

Human U937 monocyte-like cells were differentiated into macrophages as described [22] with slight modifications. U937 monocytic cells were dispensed into 96-well plates (5×10^4 cells/well) and incubated with 10 nM PMA for 48 h at 37°C. Differentiation into macrophages was assessed by the expression of CD14 and CD45 surface markers. Briefly, 1×10^3 cells were incubated in the presence of 1 μg anti-human CD14 and anti-human CD45 for 30 min at 4°C. Assessment was carried out with a Becton Dickinson FACS Vantage flow cytometer (Becton Dickinson, Mountain View, CA).

2.4 Isolation and oxidation of LDL

LDL isolation, oxidation and dialysation were performed as described previously [23–25]. Briefly, LDL (100 μg/mL protein) isolated from fasting blood samples was oxidised to a mild, moderate or high degree with 20 μM $CuSO_4$ for 24h at 37°C. Oxidation was blocked by adding 200 μM EDTA and 20 μM BHT. All the oxLDL species were dialysed overnight against 0.9% NaCl. LDL oxidation was monitored by measuring conjugated dienes at 234 nm with a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. Highland Park, Vermont) [26].

2.5 Treatment of macrophages with antioxidants in the presence of oxLDL

Macrophages obtained with PMA were placed into 24-well plates (5×10^4 cells/well) and incubated at 37°C for 24 h. The following treatments were subsequently performed: (i) Tyr, OH-Tyr or SQ alone for 24 h (0, 10, 75, 200 μM); (ii) Tyr, OH-Tyr or SQ (0, 10, 75, 200 μM) + native LDL (not oxidised), mild oxLDL or moderate oxLDL (50 μg/mL protein) for 24 h.

2.6 Activation of monocytes into macrophages in the presence of antioxidants and oxLDL

U937 monocytic cells were pipetted into 24-well plates (5×10^4 cells/well) and incubated at 37°C for 24 h. The aim of this assay was to assess the inhibitory activity of SQ, OH-Tyr and Tyr with respect to monocyte/macrophage differentiation. To that end, we exposed U937 monocytes to Tyr, OH-Tyr or SQ at different concentrations (0, 10, 75, 200 μM) + native LDL (not oxidised), mild oxLDL or moderate oxLDL (50 μg/mL protein) for 24 h. The activation of monocytes into macrophages was achieved by determining the expression of CD14 and CD45 surface markers by flow cytometry as described above.

2.7 Expression of CD36 scavenger receptor by flow cytometry

The monocytes were cultured in the presence of Tyr, OH-Tyr or SQ at 0, 10, 75, 200 μM for 24 h. Macrophages were grown in the presence of native LDL, mild or moderate oxLDL for 24 h; they were subsequently treated with minor compounds at the concentrations stated above for 24 h. After incubation, the cells were trypsinised, resuspended in RPMI medium (phenol red and foetal bovine serum free) and then incubated with anti-human CD36 antibody (1:100 dilution) for 30 min at 4°C. Expression of CD36 was evaluated by flow cytometry with the same cytometer as described above.

2.8 Cytotoxicity and proliferation of U937 monocytic cells and macrophages

Cell viability was determined by MTT assay as described previously [27]. U937 monocyte-like cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h at 37°C. After incubation, the culture medium was replaced with fresh medium at different concentrations of Tyr, OH-Tyr or SQ (0, 10, 75 and 200 μM) for 24 h (cytotoxicity) or 72 h (proliferation). Control cells without agents were cultured in parallel using the same conditions with comparable media changes and 0 μM of each compound. After treatment, the culture medium was removed and replaced with a fresh drug-free medium (200 μL/well) and 30 μL MTT (37°C for 4 h). The precipitate was solubilised in DMSO (100 μL/well) and incubated at room temperature for 30 min. Absorbance was determined at 570 nm using a microplate reader.

Macrophages derived from monocytes in the presence of PMA were grown in 96-well plates (1×10^4 cells/well) and treated with Tyr, OH-Tyr or SQ (0, 10, 75 and 200 μM) for 24 h. The MTT assay was performed as described above.

2.9 Analysis of apoptosis and necrosis by flow cytometry

Apoptotic and necrotic cells were measured as previously reported [28]. Briefly, U937 monocytic cells were cultured in 96-well plates (5×10^4 cells/well) for 24 h at 37°C. Then, the culture medium was replaced with fresh medium plus Tyr, OH-Tyr or SQ (0, 10, 75 and 200 μM) for 24 h. The cells were collected, washed with cold PBS and then incubated at room temperature for 15 min with annexin-binding buffer and 0.2-μg annexin-V-FITC. Finally, the cells were resuspended in annexin-binding buffer and 1 μL propidium iodide. Apoptosis and necrosis were assessed by flow cytometry.

2.10 Statistical analysis

Results are presented as means ± SEM. Intergroup comparisons were performed by one-way analysis of variance (ANOVA) and Bonferroni's test. Previously, all the variables were tested for normal and homogeneous variance by Levene's test. When necessary, nonparametrical Kruskall–Wallis' test and Mann–Whitney's post hoc test were performed. A p value of less than 0.05 was considered significant. Data analysis was performed using SPSS version 15.0 software (Statistical Package for Social Sciences, SPSS Inc. Chicago, IL).

3 Results

3.1 Expression of CD36 scavenger receptor

Table 1 summarises the different levels of CD36 after exposure to Tyr, OH-Tyr or SQ in U937 monocytes in the presence of native LDL, mild or moderate oxLDL. The most significant result was obtained for SQ, which caused a dose-dependent decrease of CD36 expression in the presence of native LDL (73.93%, 44.07% and 54.73% at 10, 75 and 200 μM, respectively) and moderate oxLDL (75.00%, 89.18% and 77.02% at 10, 75 and 200 μM, respectively). OH-Tyr only affected the CD36 levels in the presence of native LDL (28.43% and 54.02% at 10 and 75%), although this might be due to cytotoxic effects. This experiment was also carried out for macrophages (see Table 2). A dose-dependent decrease in CD36 was observed in SQ-treated macrophages cultured with either native LDL (64.58%, 20.18% and 48.43% at 10, 75 and 200 μM, respectively) or moderate oxLDL (89.06%, 73.92% and 56.41% at 10, 75 and 200 μM, respectively). A slight decrease was observed after adding Tyr (96.02%, 99.29% and 92.94% at 10, 75 and 200 μM, respectively) and OH-Tyr (80.97% and 88.64% at 75 and 200 μM, respectively) together with moderate oxLDL. Finally, SQ per se produced a strong dose-dependent reduction of CD36 in the absence of native or oxLDL (85.85%, 62.58% and 41.31% at 10, 75 and 200 μM, respectively).

3.2 Inhibition of monocyte/macrophage differentiation

The expression of CD45 and CD14 showed that none of the compounds tested inhibited the activation of monocytes into macrophages in the presence of oxLDL. Nonetheless, only SQ was capable of preventing such differentiation when native LDL was co-administered (data not shown).

Table 1. Expression of CD36 (%) in monocytes in presence of oxLDL and squalene, tyrosol and hydroxytyrosol

Treatment	Tyrosol				Hydroxytyrosol				Squalene			
	0 (μM)	10 (μM)	75 (μM)	200 (μM)	0 (μM)	10 (μM)	75 (μM)	200 (μM)	0 (μM)	10 (μM)	75 (μM)	200 (μM)
Antioxidant + native LDL	100	180.56	204.73	172.03	100	28.43	54.02	ND	100	73.93	44.07	54.73
Antioxidant + mild oxLDL	100	141.50	113.20	152.83	100	181.13	133.01	169.81	100	96.22	181.13	96.22
Antioxidant + moderate oxLDL	100	83.10	137.83	93.24	100	117.56	89.18	127.70	100	75.00	89.18	77.02

oxLDL, oxidised LDL; ND, not detected.

3.3 Anti-proliferative and cytotoxic activity of minor compounds

Figure 1A depicts the cytotoxic effects of Tyr, OH-Tyr and SQ on human U937 monocyte-like histiocytic lymphoma cells. These results show that OH-Tyr promotes a dose-dependent diminution of cell viability (85%, 70% and 61% at 10, 75 and 200 μM) ($p < 0.05$). No significant cytotoxic effects were seen for Tyr or SQ. There was a noticeable increase in viability (120%) at 75 μM SQ. With regard to cell proliferation, Fig. 1B illustrates an increase at 10 μM for SQ (124%) and Tyr (118%). OH-Tyr produced an intense antiproliferative repercussion at relatively high concentrations (above 10 μM) (55% and 16% at 75 and 200 μM) ($p < 0.05$), while SQ and Tyr affected proliferation at even higher concentrations (above 75 μM) (115% and 91% at 75 and 200 μM, respectively) and (128% and 106% at 75 and 200 μM, respectively) ($p < 0.05$), respectively.

3.4 Cytotoxic activity of minor compounds on macrophages

Human monocytes were differentiated into macrophages with PMA and the outcome was assessed by measuring the expression of CD14 and CD45, using flow cytometry. Our experiments revealed the successful expression of these markers in 99% of the cells (data not shown). Cell viability after adding Tyr, OH-Tyr or SQ is shown in Fig. 1C. Interestingly, Tyr was the most cytotoxic substance when it was added to macrophages at 10 and 200 μM (84% and 74%, respectively) ($p < 0.01$). Neither OH-Tyr nor SQ importantly affected viability even at the highest concentrations.

3.5 Apoptosis and necrosis in U937 cells caused by minor compounds

Viable, apoptotic and necrotic monocytic cells incubated together with Tyr, OH-Tyr, or SQ are shown in Fig. 2(A, B and C). Flow cytometry confirmed a dramatic dose-dependent diminution of viable cells (more than 50% at 200 μM compared to the control group) after adding OH-Tyr (64% and 47% at 75 and 200 μM, respectively) ($p < 0.05$). A concomitant increase in apoptotic and necrotic cells was seen at 75 (18%) and 200 μM (14%) ($p < 0.05$) (Fig. 2A). There were no such differences for SQ or Tyr (Figs. 2B and C).

4 Discussion

It is widely accepted that dietary interventions can modulate gene expression, not only in animal models but also in humans. In this sense, and in terms of cardiovascular disease, the traditional Mediterranean diet supplemented with virgin OO, or OO alone (due to its molecular components such as polyphenols, MUFA and minor compounds such as SQ) can influence the expression of key genes involved in atherosclerosis-related events such as vascular inflammation and foam cell formation [10,11,17,29]. In this paper, we report new knowledge about the role of three minor compounds of OO in the expression of the CD36 receptor in the presence/absence of oxLDL. We also address the different

Table 2. Expression of CD36 (%) in macrophages in presence of oxLDL and squalene, tyrosol and hydroxytyrosol

Treatment	Tyrosol				Hydroxytyrosol				Squalene			
	0 (μM)	10 (μM)	75 (μM)	200 (μM)	0 (μM)	10 (μM)	75 (μM)	200 (μM)	0 (μM)	10 (μM)	75 (μM)	200 (μM)
Antioxidant alone	100	97.36	62.46	81.87	100	119.01	88.40	111.42	100	85.85	62.58	41.31
Antioxidant + native LDL	100	117.16	115.20	103.63	100	101.89	92.18	98.61	100	64.58	20.18	48.43
Antioxidant + mild oxLDL	100	92.61	83.77	109.90	100	106.31	175.61	112.72	100	41.75	89.79	103.50
Antioxidant + moderate oxLDL	100	96.02	99.29	92.94	100	101.41	80.97	88.64	100	89.06	73.92	56.41

oxLDL: oxidised LDL.

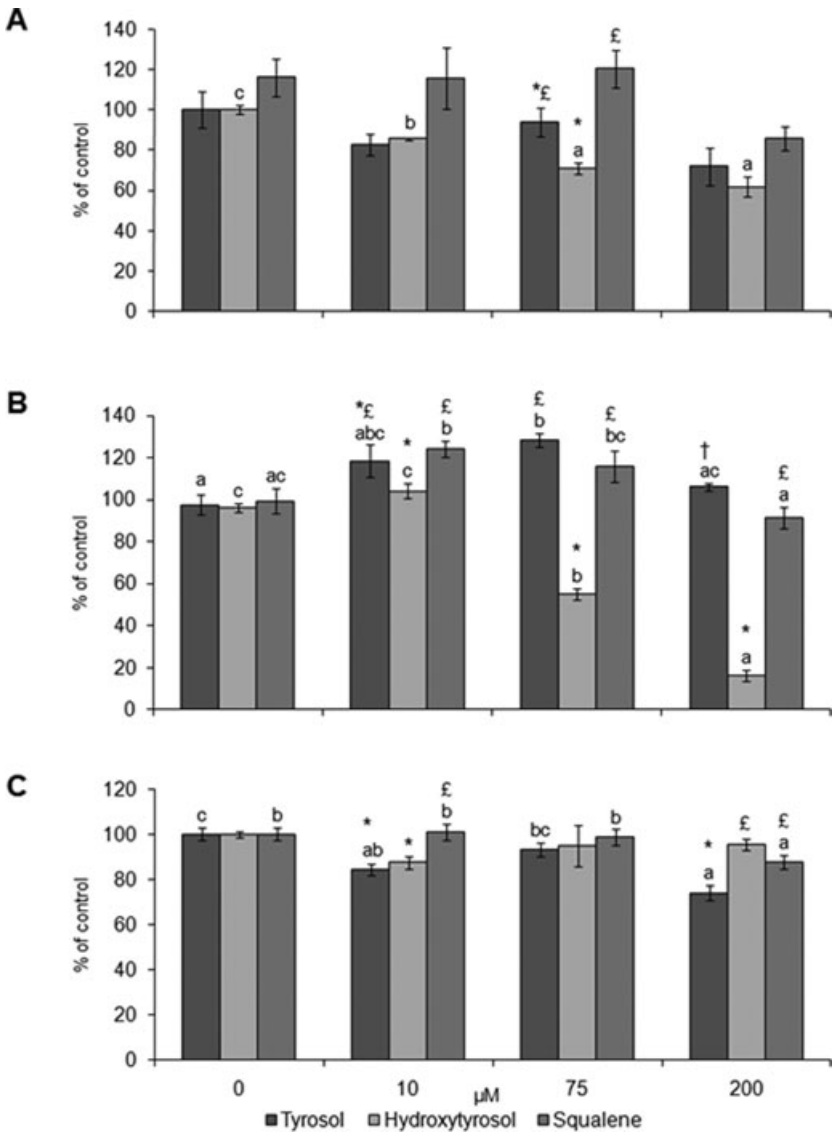

Figure 1. Effects on proliferation and cytotoxic activity of squalene, tyrosol and hydroxytyrosol in U937 monocyte-like cells and macrophages. Values are expressed as means ± SEM ($n = 6$). Bars with different letters significantly differ among concentrations for each compound. Different symbols indicate statistical significance among the compounds for each concentration. (A) Cytotoxicity in monocytes (Bars: $p < 0.05$; symbols: $p < 0.001$); (B) Proliferation in monocytes (Bars: $p < 0.05$; symbols: $p < 0.05$); (C) Cytotoxicity in macrophages (Bars: $p < 0.01$; symbols: $p < 0.01$). OH-Tyr, hydroxytyrosol; Tyr, tyrosol; SQ, squalene.

effects of these molecules on monocytes and macrophages in terms of cell viability, proliferation and cytotoxicity.

SQ is an isoprenoid compound with a low index of peroxidability and a beta-carotene-like structure, which acts as a metabolic precursor of cholesterol and other sterols. Dietary intake and the endogenous synthesis of cholesterol are the two main sources of serum SQ in humans [20]. There are very few reports about this molecule and atherosclerosis, although recently Guillén et al. [30] showed in ApoE-knockout male mice that SQ feeding reduced the atherosclerotic lesion area independently of plasma lipids and the activation of circulating monocytes. To date, little has been reported about SQ and its modulating effects on the expression of CD36 receptor. We find that SQ reduces the CD36 expression without provoking cytotoxicity, either in monocytes or macrophages, but how SQ exerts this inhibitor effect on the receptor remains unclear. This isoprenoid strongly inhibits the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase through negative feedback [31]. This inhibitory property could be partly responsible for decreasing the expression of CD36 by inactivating PPARγ (peroxisome proliferators-activated receptor gamma), a feature that is also presented by pitavastatin. This statin is a HMG-CoA reductase inhibitor that reduces the expression of CD36 at mRNA and cell surface levels [32] by diminishing PPARγ activity in macrophages [33]. However, this possibility could be discarded because SQ did cause a diminution of CD36 without affecting monocyte/macrophage differentiation. This is a proatherogenic step which is also promoted by PPARγ [34]. Therefore, further studies are needed to clarify which CD36-related signalling pathways are affected by SQ but not by Tyr and OH-Tyr.

The phenolic fraction of virgin OO is composed of different molecules, including Tyr and OH-Tyr [11]. These

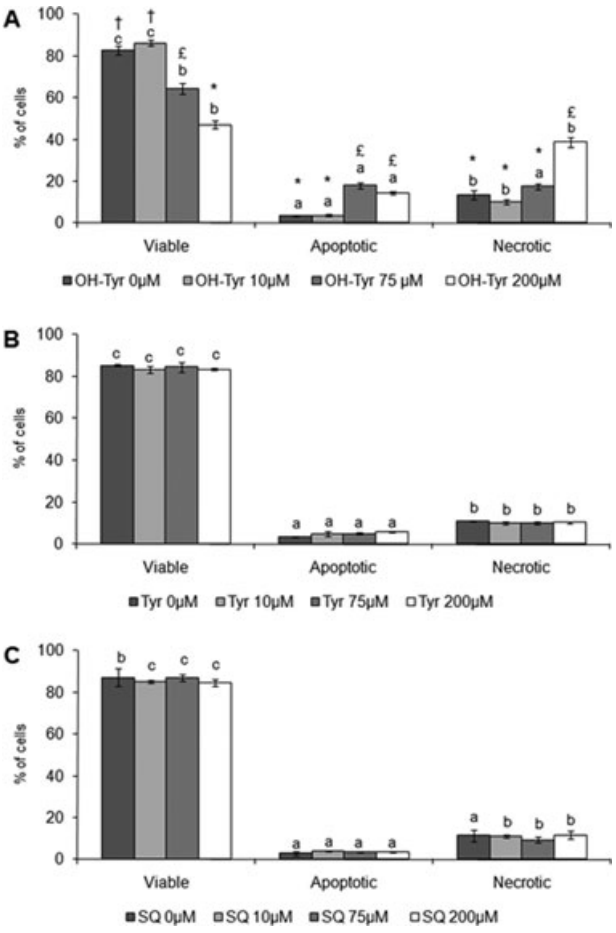

Figure 2. Effect of different concentrations of tyrosol (A), hydroxytyrosol (B) and squalene (C) on cell viability, apoptosis and necrosis. Values are expressed as means ± SEM ($n = 4$). Bars with different letters significantly differ among viable, apoptotic and necrotic cells for each concentration ($p < 0.05$). Different symbols indicate statistical significance among concentrations for each compound ($p < 0.01$). OH-Tyr, hydroxytyrosol; Tyr, tyrosol; SQ, squalene.

phenolic compounds produce important effects at cardiovascular level, with antioxidant, anti-inflammatory and antithrombotic properties; moreover, they prevent lipid peroxidation, induce favourable changes in the lipid profile and improve the endothelial function [10]. Very little is known about the role of Tyr and OH-Tyr on CD36 expression or on monocyte/macrophage differentiation. In a previous report, Tyr was shown to prevent monocyte/macrophage activation in the RAW 264.7 cell line by inhibiting pro-inflammatory effectors (iNOS, COX-2, NF-κB, STAT-1α, IRF-1) [35]. Our discordant results can be explained because the latter authors employed higher concentrations of Tyr (1, 2 and 4 mM), different cells and different inductors of differentiation (gliadin and IFN-γ). With regard to OH-Tyr, in vivo studies do not clarify the relationship between OH-Tyr and atherosclerosis. A study made in hyperlipidemic rabbits showed that the antioxidant status improved in OH-Tyr-supplemented groups, while the size of atherosclerotic lesions decreased, in comparison with control animals [36]. Nevertheless, the administration of OH-Tyr in low-cholesterol diets in ApoE-deficient mice increased the atherosclerotic lesions associated with the degree of monocyte activation and remodelling of plasma lipoproteins [37]. Few studies have been made of OH-Tyr and CD36 expression, and our findings do not reveal any effect of these compounds, either in CD36 expression or in monocyte/macrophage differentiation. Nevertheless, our data do supply more evidence about the phenotype-dependent cytotoxicity of OH-Tyr, which is correlated with apoptotic events [11]. These phenotype-dependent cytotoxic and antiproliferative roles attributed to OH-Tyr were first reported by Ragione et al. [38], who observed cytotoxic and antiproliferative events in human HL60 cells and peripheral lymphocytes, while two colon cell lines (HT-29 and CaCo2) were unaffected by OH-Tyr. Our study highlights the powerful inhibition of proliferation and viability in U937 monocytic cells by the augmentation of apoptotic and necrotic cells at concentrations higher than 10 μM. These data are in accordance with previous reports [39]. However, OHTyr did not affect cell growth in macrophages. This cell-dependent dual role of OHTyr could result in diminished arrival of monocytes at the arterial intima. Moreover, this molecule has demonstrated anti-inflammatory properties [11] and is capable of inhibiting pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells [40]. Overall, current and past knowledge seems to indicate that OH-Tyr could prevent atherogenic processes by decreasing monocyte migration toward the arterial intima, as well as by inhibiting pro-inflammatory gene expression.

Future studies are needed to clarify whether these compounds accomplish their effects inside or outside cells. We did not measure the uptake of these antioxidants by cells, but, based on previous studies, a cellular uptake is expected. For example, SQ has to be endocytosed in order to act as a HMG-CoA reductase inhibitor by means of negative feedback [31], while phenolic compounds exert antioxidant and modulator effects on gene expression [11] previous cellular uptake as was described by Di Benedetto et al. [41] with HPLC in cells. These authors reported that OH-Tyr and Tyr probably produce effects by intracellular accumulation.

In conclusion, we show that SQ derived from virgin OO can reduce CD36 surface receptor expression in monocytes and macrophages in the presence of oxLDL. In contrast, this isoprenoid molecule does have antiproliferative or cytotoxic effects in monocytes and macrophages; neither does it restrict monocyte/macrophage differentiation. These data suggest that SQ does not prevent monocyte migration and activation into macrophages, but that it would inhibit oxLDL uptake by macrophages, by reducing the expression of CD36. The inverse mechanism is presented by OH-Tyr. The present

report, thus, provides new data about the role of minor compounds derived from virgin OO with respect to the prevention of atherosclerosis.

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