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Nutrigenomics of extra-virgin olive oil: a review.

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Abstract

Nutrigenomics data on the functional components of olive oil are still sparse, but the available literature on this subject is increasing. Olive oil is the main source of fat and a key health-promoting component of the Mediterranean diet with proposed positive effects on genes involved in the pathobiology of most prevalent age- and lifestyle-related human conditions such as cancer cardiovascular disease and neurodegeneration. Other effects on the regulation of health-promoting genes have been identified for bioactive components of olives and olive leaves. Omics technologies are offering unique opportunities to identify nutritional and health biomarkers associated with these gene responses and to use them with a personalized and even predictive approach, which is a main breakthrough in modern medicine and nutrition. Gene regulation properties of the functional components of olive oil, such as oleic acid, biophenols and vitamin E, point to a role for these molecules as natural homeostatic and even hormetic factors for an application as cytoprotection and early prevention agents in conditions of premature and pathologic aging. Even therapeutic applications can be foreseen in conditions of chronic inflammation, and particularly in cancer, which will be discussed in detail in this review paper as major clinical target of olive oil and its functional components.

Word count: 192

Keywords:

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extravirgin olive oil;

polyphenols;

vitamin E;

nutrigenomics.

1. Introduction

Olive Oil (OO) in its production variants of virgin and extra virgin OO (VOO and EVOO, respectively), is universally recognized as a symbol, and the major source of fat, of the Mediterranean diet [1]. In all the traditional forms of this diet found in the Mediterranean basin, VOO, the OO obtained directly from olives and solely by mechanical means, is proposed as main health-promoting component with effects that include a reduced risk of morbidity and mortality for cancer, neurodegenerative diseases such as Parkinson's and Alzheimer's Disease, metabolic syndrome and cardio-cerebro-vascular events [2; 3; 4; 5; 6].

Gene modulation by VOO combined with the other components of Mediterranean diet have been investigated to provide a mechanistic rationale to such a positive clinical outcome (recently reviewed in [7; 8]). Solid evidence was obtained on converging effects of VOO and Mediterranean diet on the homeostatic control of genes having a role in immune-inflammatory pathways, vessel protection and blood pressure control, metabolic regulation and detoxification of reactive species. The actual molecular players of these nutrigenomic effects have been tentatively identified in animal models and humans [7; 9; 10] and the available evidence is strong enough to consider VOO a natural functional food. Besides having a high content of monounsaturated fatty acids (MUFAs), VOO contains a number of "bioactives", such as biophenols (Figure 1) and vitamin E (Figure 2), the latter being the main fat-soluble vitamin of this oil (Section 8), the pattern of which shows huge variability in olives and VOO products available for human consumption [11].

The impact of the accumulated evidence on this edible oil on health and nutrition policies of different regions has been huge. Recommendations of World Health Organization for a healthy diet include VOO as a source of unsaturated fats that should be preferred to more saturated ones, found for instance in fatty meat and dairy products [12], which is in agreement with previous health claims on oleic acid of the European Food Safety Authority (EFSA) [13] and Food and Drug Administration (FDA) [14] (Section 2). Further recognition of the importance of functional components in VOO has been provided by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), concerning polyphenols, and vitamin E [15; 16].

Nutrigenomics encompassing high-throughput omics technologies, such as transcriptomics, proteomics, metabolomics, interactomics and fluxomics, and their implementation with the latest bioinformatics tools, is now available to characterize the molecular markers of these claims. In fact, a growing body of evidence is accumulating on the identification of genes and

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3 1 metabolic responses that link the functional properties of VOO bioactives with the nutritional
4 2 and health-promoting activity of the bioactive molecules found in this edible oil. These
5 3 aspects are discussed in this review paper, which is aimed at providing an updated description
6 4 of the most recently identified gene-bioactive functional interactions produced during the
7 5 consumption of VOO.
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9 7

10 8 11 9 12 10 13 11 14 12 15 13 16 14 17 15 18 16 19 17 20 18 21 19 22 20 23 21 24 22 25 23 26 24 27 25 28 26 29 27 30 28 31 29 32 30 33 31 34 32 35 33 36 34 37 35 38 36 39 37 40 38 41 39 42 40 43 41 44 42 45 43 46 44 47 45 48 46 49 47 50 48 51 49 52 50 53 51 54 52 55 53 56 54 57 55 58 56 59 57 60 58

9 The nutritional and healthy values as well as the sensory and biological properties of VOO
10 have been ascribed to the presence of bioactive components such as Monounsaturated and
11 Polyunsaturated Fatty Acids (MUFAs and PUFAs), squalene, phytosterols, triterpenic acids
12 and dialcohols, pigments, tocopherols and polyphenols.

13 Initially, the health-promoting effects of VOO have been attributed to its high MUFAs
14 amount. Among them, the oleic acid (18:1 ω -9), representing 49% to 83% of the total FA in
15 VOO, is supposed to be the most important one from a healthy point of view [17]. In fact, it
16 exerts high efficiency in the modulation of gastrointestinal and metabolic functions and of
17 extrinsic cardiovascular risk factors. The ameliorative effect of oleic acid in olive oil is
18 thought to occur via modifications to plasma lipid and lipoprotein patterns and levels. Cell
19 membrane composition and fluidity, inhibition of coagulation, improvement in glucose
20 homeostasis and blood pressure, and attenuation of inflammation and oxidative states in
21 fasting conditions, have also been described to be affected. Other important functions
22 associated to the oleic acid consumption include the a better control of the secretory activity
23 of pancreas and liver (bile secretion) and an improved protection of the gastric mucosa by a
24 reduced secretion of hydrochloric acid that helps constraining the risk of gastric-duodenal
25 ulcers [18]. However, the most convincing evidence in medicine on the health-promoting
26 activity of this MUFA was obtained in cardiovascular prevention trials. This evidence has
27 resulted in the formulation of the health claims introduced above and now appearing on olive
28 oil labels. More in detail, the FDA in 2004 issued “the benefits on the risk of coronary heart
29 disease of eating about two tablespoons (i.e. 23g) of VOO daily, due to the MUFAs (oleic
30 acid) in olive oil” [14]. The European Authority EFSA has moved in the same direction with
31 the following sentence in a recent opinion: “Replacing saturated fats in the diet with
32 unsaturated fats contributes to the maintenance of normal blood cholesterol levels. Oleic acid
33 is an unsaturated fat” [13].

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3 1 On the other hand, the PUFA linoleic acid (18:2 ω -6) and linolenic acid (18:3 ω -3),
4 2 respectively, as essential FA, are indispensable components of the cell structure and are also
5 3 fundamental for the development of the brain and retina, especially during the growth [19]. In
6 4 humans they are biosynthetic precursors of other long chain unsaturated fatty acids, namely
7 5 arachidonic acid (20:4 n-6) and eicosapentaenoic acid (20:5 n-3), which are involved in
8 6 eicosanoid metabolism, thereby regulating important functions of inflammatory leukocytes,
9 7 platelets and vascular cells. Furthermore, most of the nutrition guidelines agree in considering
10 8 the VOO ratios PUFA/SFA and ω -3/ ω -6 as the best occurring in natural fats. Some
11 9 parameters, such as the area of production, altitude, climate, fruit variety, and stage of
12 10 maturity of the fruit can greatly affect the FA composition of virgin olive oil. It is generally
13 11 accepted that cooler areas produce oil with higher monounsaturated content than warmer
14 12 climates [20].

15 13 Current epidemiological and experimental studies strongly support the fact that the beneficial
16 14 effects of VOO are also due to its minor bioactive components. Among them, the squalene,
17 15 besides its well known anticancer properties, shows several biological activities, with the
18 16 antioxidant one being similar to that of trans retinol [21; 22]. The squalene content in olive oil
19 17 is especially high (up to 0.7% (7 mg/g)) when compared to other oils and human dietary fats.
20 18 Moreover, squalene plays a key role as intermediate metabolite in cholesterol metabolism. In
21 19 vivo and in vitro studies have shown that it regulates the absorption, synthesis, esterification
22 20 and elimination of cholesterol [23] by stimulating acyl-coenzyme A. At the same time it
23 21 reduces cholesterol, thereby increasing the efficiency of statins and reduces the UV-induced
24 22 DNA damage thus preventing human skin photo-aging [24]. Squalene is also an important
25 23 intermediate in the biosynthetic pathway of sterols in both plants and animals [25]. The
26 24 phytosterols represent a major fraction of unsaponifiables molecules in VOO (ranging from
27 25 80 to 260 mg/100g of VOO), mainly represented by β -sitosterol (\geq 93.0% of total sterols). In
28 26 vivo phytosterols, and particularly β -sitosterol, are effective in reducing the concentrations of
29 27 total and LDL cholesterol, and in stimulating the apoptotic signaling of prostate cancer cells;
30 28 moreover, these sterols are used in the natural treatment of benign prostatic hyperplasia [26;
31 29 27]. The beneficial effect of phytosterols is obtained only with a daily intake of at least 0.8 g
32 30 of plant sterols/stanols, according to the Claim of EFSA named "The sterols / stanols ratio
33 31 contributes to the maintenance of normal levels of blood cholesterol" [16].

34 32 The triterpenes are bioactive molecules found in olive skin and in the leaves of olive trees.
35 33 Hydroxyl pentacyclic triterpene acids (HPTA) (oleanolic and maslinic acid) and dialcohols
36 34 (uvaol and erythrodiol) are responsible for several biological activities attributed to VOO,

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3 1 such as anti-inflammatory, hepatoprotective, anticancer, antiviral, anti-HIV, anti-microbial,
4 2 antifungal, anti-diabetic, gastroprotective and anti-hyperlipemia [28; 29; 30; 31]. Recent
5 3 findings demonstrated also different neuroprotective effects exerted by maslinic and oleanolic
6 4 acid [32; 33; 34]. However, their concentration in VOO is very weak, ranging between 17 and
7 5 344 mg/Kg for oleanolic acid, 19 and 250 mg/Kg for maslinic acid and traces of ursolic, while
8 6 it is significantly higher in crude olive pomace oil. According to some authors, the main
9 7 factors causing variability in the HPTA concentration are the oil free acidity olive variety,
10 8 olive ripeness, and oil extraction system [35].

11 9 Furthermore, VOO contains a considerable amount of pigments (chlorophylls and
12 10 carotenoids). Consistent clinical evidence has been obtained on the antioxidant activity of
13 11 carotenoids as well as on other molecular effects that were associated with the prevention or
14 12 amelioration of serious human ailments [24; 36]; these include cancer and cardiovascular
15 13 disease, and skin and eye disorders. In the latter, carotenoids enhance the optical density of
16 14 macular pigments and protects against the formation of age-related cataracts.. Their health-
17 15 promoting properties are mostly due to carotenes (e.g. β -carotene) and xanthophylls (e.g.
18 16 lutein). In particular, carotenes (precursors of vitamin A) are proposed to quench the singlet
19 17 oxygen, a reactive intermediate of the molecular oxygen formed during the process of light-
20 18 induced oxidation (photo oxidation) of the biomolecules. Lutein shows higher efficiency in
21 19 protecting cellular membranes against lipid peroxidation and in preventing oxidative damage
22 20 to the retina [24].

23 21 Tocopherols (vitamin E) are presented in VOO essentially as α -tocopherol ($\geq 90\%$ of
24 22 tocopherols in EVOO) i.e. the main form of this vitamin E also found in human tissues [37]
25 23 (Section 8). This is one of the most important lipophilic antioxidants found in nature [38; 39]
26 24 and its role in preventing lipid peroxidation of cellular membranes and lipoproteins [40] has
27 25 been recognized in the recent health claim released from EFSA: "Vitamin E helps to protect
28 26 cells from oxidative stress" [16]. Moreover, both the redox-dependent and -independent
29 27 properties of this vitamin have been demonstrated to influence the expression of homeostatic
30 28 genes that protect tissues from oxidative and inflammatory processes associated with aging,
31 29 degenerative diseases and cancer [37]. Noteworthy, the levels of α -tocopherol in different
32 30 VOO products show marked variability depending on pedoclimatic factors and agronomic
33 31 practices, such as the area of origin, the cultivar and the stage of fruit ripening [20; 41]. The
34 32 data obtained assessing 430 samples of EVOO have showed a range of variability between 23
35 33 and 751 mg/kg [17].

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3 1 Besides the compounds described above, it is well known that biophenols are the most
4 2 represented bioactive molecules of VOO. There are more than 100 different biophenols
5 3 reported in olive products (fruit, oil, leaves, and waste) [42]. The composition in terms of
6 4 phenolic compounds is different in olive fruit, oil, leaves, and waste. Additionally agronomic
7 5 (varieties, ripeness, and agro-climatic aspects) and technological factors (variables of
8 6 mechanical extraction process and storage conditions) have a significant impact on the
9 7 biophenol composition of olives, VOOs and by-products [43].

10 8 The main biophenols occurring in olives include the phenyl alcohols hydroxytyrosol (HT),
11 9 and tyrosol, and the secoiridoids oleuropein and ligstroside showed in Figure 1. Other forms
12 10 include verbascoside, lignans, and flavonoids (rutin and glycosides of luteolin and apigenin).
13 11 In VOO the main classes of phenols are phenolic acids, phenolic alcohols, flavonoids,
14 12 secoiridoids (as aglycon derivatives) and lignans. Secoiridoids are characterized by the
15 13 presence in their molecules of elenolic acid (EA) or its derivative forms (Figure 1); they occur
16 14 only in plants belonging to the family of Oleaceae, which includes *Olea europaea* L., thus the
17 15 only natural food sources are table olives and VOO. The most abundant secoiridoids in VOO
18 16 are the dialdehydic form of decarboxymethyl-EA linked to HT or tyrosol (3,4-DHPEA-EDA
19 17 and p-HPEA-EDA, respectively), an isomer of oleuropein aglycone (3,4-DHPEA-EA), and
20 18 the ligstroside aglycone (p-HPEA-EA) (Figure 1), found and characterized for the first time
21 19 by Montedoro et al. [44]. These substances are aglycone derivatives of secoiridoid glucosides
22 20 contained in the olive fruit, originating during the oil mechanical extraction process, by the
23 21 hydrolysis of oleuropein, demethyloleuropein, and ligstroside through the activity of
24 22 endogenous β -glucosidases [45]. The composition in those compounds may be extremely
25 23 variable due to the combination of several factors including agronomical, technological and
26 24 storage factors. Most of the variables involved in such modifications, in fact, have been
27 25 widely investigated during the last 35 years. As an example about the variability in the
28 26 concentration in VOO, in 210 oil samples obtained in industrial plants, average values of the
29 27 prevalent phenolic alcohols, phenolic acids and secoiridoids was in total 352.4 mg/Kg (133.5
30 28 and 950.5 mg/Kg were the lower and the upper quintile, respectively) [45].

31 29 Being responsible for the bitter and pungent sensory attributes of VOO, the secoiridoid
32 30 derivatives in VOO represent a rare case of the healthy value of a food product which is
33 31 directly perceptible by means of sensory stimuli. All the scientific evidences regarding the
34 32 role of those compounds in the prevention of several diseases have contributed in increasing
35 33 consumer awareness about the positive correlation between VOO bitterness and pungency
36 34 and its good quality [46].

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3 1 VOO phenolics may exert beneficial effects as a consequence of their antioxidant,
4 2 antimicrobial and anti-inflammatory activities. Observational and epidemiological studies
5 3 demonstrated the efficacy of the VOO's phenolic compounds on the prevention of chronic
6 4 and inflammatory diseases such as cardio-cerebro-vascular disease and cancer, which is
7 5 consistent with the results of clinical trials that have confirmed the importance of phenolic
8 6 compounds cardiovascular risk protection afforded by VOO (recently reviewed in [45; 47; 48;
9 7 49]). In 2011 the EFSA agency released a health claim [15; 16] concerning the recognition of
10 8 the effectiveness of the ingestion of VOO phenols (HT and its derivatives, 5 mg/day per 20 g
11 9 of VOO) in protecting LDL from oxidation. This is the sole example between the available
12 10 health claims that identified in the phenotype of LDL cholesterol oxidation an underlying
13 11 event in cardiovascular risk to be targeted with a specific dose of a natural functional
14 12 ingredient of food.

15 13 Besides these effects on LDL protection, other molecular mechanisms by which the phenolic
16 14 fraction of VOO could protect human tissues from the pathogenic cues of chronic and
17 15 degenerative diseases have been tentatively identified. These mechanisms presented in detail
18 16 in the next sections, are proposed to include effects on other aspects of lipid metabolism such
19 17 as on HDL levels, the anti-atherogenic fraction of cholesterol [50], as well as on oxidative
20 18 stress and inflammatory parameters (Section 3 and 4), platelet function [51] and activity of
21 19 the fibrinolytic factors PAI-1 and FVII [52], and on endothelial parameters such as the release
22 20 and pro-oxidant effects of the vasodilating gas NO [53], blood cell adhesiveness [54] and
23 21 angiogenic activity that is an important target in chemoprevention and therapy of different
24 22 cancers (Section 7) together with the control of DNA damage as an early event in
25 23 carcinogenesis [48], and with specific effects on signal transduction and gene regulation
26 24 pathways that control proliferation, invasiveness and apoptotic death of cancer cells (Section
27 25 6).

28 26 The possibility that biophenols of VOO and from other food items may reach key molecular
29 27 targets of human cells and tissues to produce such an impressive series of effects, depends on
30 28 the metabolism and bioavailability features of the active forms of these compounds. Data
31 29 regarding the metabolism of VOO phenolics in humans are very limited, and contrasting
32 30 results have been obtained regarding the amounts and forms in which they are present in
33 31 plasma and excreted in urine (reviewed in [55]). These aspects further discussed below
34 32 (Section 4) have been assessed in some human trials that conclusively demonstrated how
35 33 VOO phenolics are resistant to the acidic conditions of the stomach and thus are readily
36 34 absorbed in humans (55–60 % for HT and oleuropein aglycone) [56]. However, blood

1 kinetics were found to vary among the different VOO biophenols. Oleuropein is rapidly
2 absorbed after oral administration with a maximum plasma concentration occurring 2 h after
3 administration [57]. Tyrosol and HT are the products of the metabolic transformation of this
4 and other VOO phenolics such as ligstroside aglycone, which are absorbed in a dose-
5 dependent manner in humans [57]. HT appears in plasma minutes after oral administration,
6 with maximal concentrations observed in 5–10 min and then the renal clearance produces a
7 rapid drop of circulating levels within the first hour [58].

8 Notwithstanding, the free form of HT, that is commonly investigated in cellular tests, is
9 almost completely undetectable in plasma and urine, being > 95% in the conjugated form,
10 mainly condensed with glucuronyl residues, while methylconjugates are a minor form of the
11 molecule excreted in human urine [57; 59]. The same is for oleuropein [60; 61]. Therefore,
12 HT should reach tissues mainly as conjugated form.

13 If the biological activity of HT or other biophenols could be attributed to endogenous
14 metabolites is still matter of investigation and preliminary data on antioxidant properties of
15 HT have provided conflicting results in literature (reviewed in [62]). Other options may
16 include the role of cellular esterase enzymes in the local metabolism and bioactivation of
17 derivatized forms that may have great relevance in the pathophysiology of GI tract, mainly in
18 gut and hepatic tissue. These aspects are worth of further and more accurate investigation also
19 considering the limits imposed by the metabolism of VOO bioactives in animal models and
20 particularly in rodents when compared to humans (reviewed in [55]). Genomics and
21 molecular data on the absorption and biotransformation of VOO biophenols in humans are
22 also elusive and this has fuelled speculation and a biased interpretation of mechanisms that
23 lay behind the biological effects of these molecules.

24 Olive Oil vitamin E (Section 8), similarly to dietary fatty acids, is readily absorbed and
25 delivered to liver and then to systemic lipid pools, through the pre-hepatic and post-hepatic
26 lipoprotein metabolism.

27 28 **3. Nutrigenomics of VOO.**

29
30 In the last few years, the impressive growth of omics technologies has offered the opportunity
31 to deepen the knowledge on the molecular and metabolic effects of VOO and its functional
32 components. High-throughput transcriptomic and metabolomic profiling of VOO
33 administered alone or in combination with Mediterranean diet have been implemented in
34 animal models and humans in the frame of healthy or pathological conditions. Nutrigenomics

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3 1 of biophenols has also been investigated separately from other components of VOO.
4 2 Transcriptomic fingerprints from a series of these studies have been recently compared in the
5 3 elegant review paper of Konstantinidou et al. [7] revealing key molecular targets of VOO in
6 4 the area of prevention and management of CVD and other inflammatory and age-related
7 5 disorders. Among the gene transcripts investigated in peripheral blood cells, few of these
8 6 were identified as common molecular signs of the intervention with VOO phenolic
9 7 compounds or with the Mediterranean diet combined to VOO. The following pathways
10 8 associated with coronary artery disease [63] appear to harbour most of the genes identified in
11 9 transcriptomic studies on VOO: oxidoreductase activity (JUN), hydroxymethylglutaryl-CoA
12 10 reductase activity (HMB-CoA), adipocytokine receptor signaling pathway (ADIPOQ,
13 11 GLUT4, NFkB, TNF- α), VEGF signaling pathway (COX2), hematopoietic cell lineage
14 12 (CD14), and cytokine–cytokine receptor interaction (CCL5, LEP, IL6, IL8R, IL7R, IL1B,
15 13 TNF- α , IFN γ).

16 14 Most significant changes toward a protective mode were observed in atherosclerosis,
17 15 inflammation, and oxidative stress-related genes such as MCP, IL7R, IFN γ , TNF α and the β -
18 16 adrenergic receptor B2. Monocyte chemoattractant protein-1 (MCP1), also known as CCL2,
19 17 chemokine C-Cmotif ligand 2, is modulated by the VOO polyphenols within and out of the
20 18 context of the Mediterranean diet, while TNF- α gene was a point of transcriptional
21 19 convergence between the Mediterranean diet and VOO intake. MCP1 is a crucial chemokine
22 20 responsible for the recruitment of monocytes to inflammatory lesions in the vasculature and
23 21 its decreased expression is a convincing evidence of the anti-inflammatory effect of VOO. In
24 22 association with other inflammatory mediators, it plays a fundamental role in monocyte
25 23 chemotaxis to sites of injury and infection; the levels of this protein have been shown to
26 24 increase in conditions of chronic inflammation and accelerated aging, such as rheumatoid
27 25 arthritis or lupus [64] and chronic kidney disease [65]. TNF- α , one of the earliest
28 26 inflammatory cytokines generated during monocyte activation, was downregulated together
29 27 with interferon gamma (IFN γ) and interleukin-7 receptor (IL7R) in some intervention studies
30 28 with diets rich in VOO polyphenols [66; 67]. TNF- α sustains the production of late
31 29 inflammatory cytokines, such as IL-6 [68]. These control the activation of cascades of genes
32 30 associated with the synthesis of acute phase proteins, endothelial cell activation, metabolism
33 31 and stress response of tissues.

34 32 Molecular and cellular effects of oleic acid (C18:1, n-9) have been extensively characterized
35 33 as this MUFA is relatively abundant in food and is at the same time a product of the cellular
36 34 biosynthesis of FA, a process activated in response to dietary carbohydrates [69]. ChRBP

transcription factor is essential to control the series of genes that implement the biosynthetic steps of this metabolism, i.e. the “de novo lipogenesis” (DNL), starting from the molecular precursor in this polymerization process, e.g. acetyl-CoA. A critical step in oleic acid biosynthesis downstream of palmitic acid (C16:0) formation, the major product of DNL, and elongation to stearic acid (C18:0), is the $\Delta 9$ desaturation which is catalyzed by the enzyme protein Stearoyl-Coenzyme A desaturase [70]. This gene may represent a major player of the lipotoxicity that an increased DNL may generate in cellular systems through the stressogenic activity of palmitic acid, a pro-oxidant and pro-apoptotic agent with proposed pathogenic roles in non-alcoholic steatohepatitis [71]. The capability to synthesize oleic acid at the cellular level is thus indicative of an efficient lipid metabolism that promotes the FA catabolism through the β -oxidation pathway. Accordingly, both gene transcription and lipidomic data demonstrate that oleic acid exerts much less toxicity than SFA, such as palmitic and stearic acid, when assessed in murine and human hepatocytes [70] and in β -cells, the insulin-secreting cellular component of the endocrine pancreas [71; 72]. The same findings have been reported in other cellular models outside of the gastrointestinal tract such as cardiomyocytes [73]. Moreover, in these studies oleic acid and other unsaturated species have been convincingly demonstrated to be protective against the lipotoxicity of palmitic acid.

HT, one of the most active biophenols of VOO, has been extensively investigated in a number of in vitro and in vivo studies focused on anti-cancer, anti-inflammatory and cardioprevention effects (reviewed in [62; 74]). Biochemistry, pharmacokinetics, and toxicology data obtained on HT as minor component of VOO or pure molecule of natural or synthetic origin, point to a use of this biophenol as a potential drug for the chemoprevention of highly prevalent chronic and inflammatory diseases. In a recent study by Giordano et al. [9], mice fed with a diet rich in HT (0.03 % w/w) showed a significant modification of a series of glutathione-related genes in the adipose organ, one of the target organs in cardiometabolic prevention [75]. Among the responding genes the microsomal and cytosolic glutathione S-transferase, selenium and non-selenium glutathione-peroxidases, forms 1 and 7, respectively, and γ -glutamyltransferase 5 were found; this in vivo transcriptional effect of the adipose tissue could produce functional interactions with other components of the detoxification and antioxidant protection system such as superoxide dismutase 1, 2 and 3, and some members of the cytochrome P450 family of genes, namely CYP1A1, 1A2 and 2E1. HT was also observed to influence the redox status of the tripeptide GSH in cultured adipocytes, i.e. the GSH/GSSG ratio, which points to a role of this functional component of VOO in the homeostatic control

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3 1 of the cellular redox at the interface between the master regulators of transcriptional and
4 2 metabolic pathways involved in the stress adaption response, such as the Nrf2 transcription
5 3 factor, and the pentose phosphate pathway (reviewed in [76]). Altogether these effects are
6 4 crucial to preserve the endocrine and metabolic functions of this organ also including its
7 5 capability to control the compensatory and regenerative processes associated with the
8 6 differentiation of adipose stem cells [75]. Obesity can lead to develop pathologic phenotypes
9 7 in which chronic inflammation can impair these functions of the adipose tissue thus
10 8 increasing the risk of cardiometabolic events and that of other age-related disorders [77].
11 9 Olive oil bioactives are among the dietary phytochemicals that have been described to
12 10 influence the control of “inflammagens” [77; 78]. In a recent comparative study among OO
13 11 polyphenols, Richard et al. [79] described HT as the most effective inhibitor of inflammatory
14 12 pathways that stimulate the production of NO, the eicosanoid PGE₂, and cytokines such as
15 13 IL-1 α , IL-1 β , IL-6, IL-12, TNF- α , and the chemokines CXCL10/IP-10, CCL2/MPC-1.
16 14 Corresponding effects of inhibition were observed in the gene expression of the inducible
17 15 nitric oxide synthase (iNOS), IL-1 α , CXCL10/IP-10, MIP-1 β , matrix metalloproteinase-9,
18 16 and prostaglandin E₂ synthase. The inhibition of COX-2 and iNOS genes, responsible for the
19 17 transcriptional control of TNF- α , was confirmed in other studies in human monocyte-
20 18 macrophages [80; 81; 82] and in vivo in a model of inflammatory response in rats in which
21 19 the inhibitory effects of HT on COX-1 and -2 have been reported to have the same potential
22 20 of the non-steroidal anti-inflammatory drugs ibuprofen and celecoxib [83]. Also the control of
23 21 NF- κ B transcription factor is proposed to play a major role in the anti-inflammatory gene
24 22 response to HT [84]. Moreover, at nutritionally relevant concentrations, HT was shown to
25 23 have additive effects with other VOO components such as OA, in preventing gene defects
26 24 associated with the metabolic defect of inflamed adipocytes, a major event in metabolic
27 25 syndrome and cardiovascular risk [85]. HT and OA synergize in preventing the
28 26 downregulative effect of TNF- α on gene expression and secretion of adiponectin, a
29 27 cardioprotective hormone of the adipose tissue. This effect was caused by this inflammatory
30 28 cytokine through JNK (stress kinase)-mediated suppression of PPAR γ activity.
31 29 The activity of HT on stress-activated kinases, such as JNK, and transcription factors, such as
32 30 NF- κ B and PPAR γ , also suggests effects of this biophenol on the regulation of cell cycle and
33 31 apoptotic pathways, which have sure relevance in the chemo-prevention and possibly
34 32 hormetic role of this compound (Section 4) as well as on anticancer properties described
35 33 below in Sections 6 and 7. Similarly to oleuropein and other related polyphenols, HT
36 34 possesses cytotoxic activity and depending on the experimental model it has been described to

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3 1 inhibit either initiation or promotion/progression phases of carcinogenesis. In fact, this
4 2 molecule can prevent the DNA damage induced by different genotoxic molecules of pre-
5 3 tumoral models and, at the same time, it was found to arrest cell cycle thus inhibiting
6 4 proliferation and inducing apoptosis in different tumors cell lines (Section 6).

7 5 Signaling and gene regulation effects of HT and oleuropein have been consistently
8 6 demonstrated to depend on redox-dependent properties of these molecules that include the
9 7 stimulation of hydrogen peroxide production both at the extracellular and intracellular level
10 8 [86; 87]. However, redox-independent processes could also have a role in the pro-apoptotic
11 9 activity of HT documented on different tumour cells [86].

12 10 According with a redox-dependent mechanism of action within the
13 11 cytoprotection/chemoprevention function of HT, the treatment of cells with this biophenol
14 12 was found to activate a series of antioxidant and detoxification genes, including heme
15 13 oxygenase-1 (15-fold upregulation), glutaredoxin (1.65) and glutathione peroxidase (1.53)
16 14 [88]. These are typical Nrf2 transcription factor-dependent genes that collectively produce the
17 15 adaption response of cells to oxidative stress and, more in general, to noxious stimuli deriving
18 16 from the exposure to electrophiles and lipophilic xenobiotics [76; 89] also including natural
19 17 and food-derived biophenols [90]. Nrf2 operates the transcriptional control of these genes in
20 18 concert with other elements. In the case of the in vitro anti-cancer effects of HT, changes in
21 19 the expression of the transcription factors STAT3, STAT6, SMAD7 and ETS-1 as well as of
22 20 the telomerase subunit TERT have been described. Trans-regulation effects between these
23 21 regulatory elements may occur by means of functional interactions with components of the
24 22 redox sensing and signaling platform of the cell. Glutathione S-transferase P was recently
25 23 observed to represent a protein hub for a redox-sensitive protein interaction network that
26 24 coordinates the transcriptional activity of Nrf2 and STAT3 with the signaling of stress
27 25 kinases, detoxification and redox-regulating enzymes and cell cycle checkpoints [76; 89].
28 26 Such a regulatory network is one of the cellular interactomes with important role in the
29 27 crosstalk between complex cellular responses at the interface between inflammatory pathways
30 28 and oxidative stress [76] that surely deserve further investigation as far as hormetic effects of
31 29 HT and other VOO bioactives may have on human tissues (Section 4).

32 30 Extensive in vitro investigation on anticancer activity (reviewed in [55; 62]) clearly
33 31 demonstrates that HT affects the regulation of genes associated with the arrest of cell cycle
34 32 during G0/G1 or G2/M transitions, which results in cell senescence and activation of the
35 33 canonical (mitochondrial-dependent) pathway of apoptotic cell death. The same signaling and
36 34 cell cycle regulation activity is reported for another functional biocomponent of VOO, e.g.

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3 1 vitamin E, even if the desmethyl and tocotrienol forms of this vitamin seem to possess higher
4 2 activity (reviewed in [91; 92; 93]) when compared with the main form present in VOO,
5 3 namely α -tocopherol (Section 8).
6 4
7 5

6 *Metabolomics, proteomics and epigenetics of VOO bioactives*

7 6 Efforts have been made to identify metabolomics fingerprints of VOO administration to
8 7 humans, alone or in the context of the beneficial effects that the Mediterranean diet is
9 8 proposed to have on human health. The effect of the Mediterranean diet supplemented with
10 9 either EVOO (MD + EVOO) in nondiabetic subjects on the ¹H-NMR urinary metabolome
11 10 was recently investigated in one of the PREDIMED intervention trials with a follow-up of 1
12 11 and 3 years [94]. Potential metabolome biomarker discriminating MD + EVOO from
13 12 baseline and from a low-fat diet (LFD) were concerning the metabolism of carbohydrates (3-
14 13 hydroxybutyrate, citrate, and cis-aconitate), creatine, creatinine, amino acids (proline, N-
15 14 acetylglutamine, glycine, branched-chain amino acids, and derived metabolites), lipids (oleic
16 15 and suberic acids), and microbial cometabolites (phenylacetylglutamine and p-cresol).
17 16 Hippurate, trimethylamine-N-oxide, histidine and derivatives (methylhistidines, carnosine, and
18 17 anserine), and xanthosine were predominant after the administration of the LFD.

19 18 Metabolomics investigations were recently extended to plasma metabolites that may provide
20 19 biomarker and mechanistic cues of the influence of EVOO and MD on cardiovascular risk
21 20 (recently reviewed in [95]). Although still very speculative in nature, such efforts led to
22 21 tentatively identify specific candidates of a targeted investigation that include branched-chain
23 22 and aromatic amino acids, the glutamine-to-glutamate ratio, some short- to medium-chain
24 23 acylcarnitines, gut flora metabolites (choline, betaine, and trimethylamine N-oxide), urea
25 24 cycle metabolites (citrulline and ornithine) and specific lipid subclasses. These candidates
26 25 together with a large number of untargeted metabolites are now under investigation to further
27 26 examine the effects of VOO and other food interventions within the MD dietary pattern on
28 27 CVD risk.

29 28 Next-generation omics are now available in several laboratories, which should help to expand
30 29 and even increase the scientific level of this research. This technology and its extensive
31 30 application in the next years are expected to boost to development of protocols for the
32 31 personalized evaluation of nutritional and health outcomes of dietary patterns and functional
33 32 ingredients with a predictive power that was unimaginable before. These omics approaches
34 33 include the investigation of epigenetic drift (epigenetic modifications as they occur as a direct
35 34 function with age) of blood cells, and its ancillary elements, including platelets, secreted

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3 1 microvesicles (MVs), and microRNA (miRNA) [96]. These are reflective of various diseases
4 2 as well as of lifestyle changes, making them extremely sensitive biomarkers of human health
5 3 and nutrition interventions. Animal studies have ascertained these aspects in the case of VOO
6 4 biophenols. Recently, the effects of these molecules on miRNA and gene modulation have
7 5 been investigated in the mouse brain [97] and findings are compatible with positive regulatory
8 6 effects on neuronal function and synaptic plasticity, leading to improve cognitive, motor and
9 7 emotional behaviour in aged animals treated with these bioactives (this theme is further
10 8 discussed in Section 5).

11 9 Dietary FA also influence miRNA expression of adult rats and to a certain extent of the
12 10 offspring of mothers that consumed different dietary sources of fat including VOO and these
13 11 changes appear to have effects on lipid composition and metabolism of the liver [98]. These
14 12 results point to a major impact of VOO components on epigenetic players that warrant further
15 13 investigation and translation into human clinical trials.

16 14 The impact of VOO phenolics on proteomic profiles of urine and blood was also investigated.
17 15 A recent randomized controlled trial confirmed the positive effect of VOO on a series of
18 16 urinary proteomic biomarkers associated with the cardiovascular risk in healthy volunteers,
19 17 but this effect was independent from the polyphenol content of the administered VOO [99]. In
20 18 the same study, the urinary proteome was not associated with other types of risk such as that
21 19 for chronic kidney disease or diabetes.

22 20 The effects of VOO phenolic compounds on a targeted blood proteome of
23 21 hypercholesterolemic patients, that of HDL apoproteins, was investigated in a recent double-
24 22 blind randomized controlled trial [100]. The comparison with VOO enriched in its phenolics
25 23 or complemented with thyme polyphenols showed minor differences in the quantitative
26 24 changes produced by the reference treatment, i.e. that with VOO (25 ml/day for 3 weeks) thus
27 25 suggesting minor effects on HDL proteome of VOO biophenols. Of the 127 HDL-associated
28 26 proteins identified in the proteomic profiling of these patients, 15 were differently expressed
29 27 after the three VOO interventions compared to baseline, with some quantitative changes
30 28 specific to each treatment. These changes suggest a cardioprotective impact on the HDL
31 29 proteome with the up-regulation of proteins related to cholesterol homeostasis, protection
32 30 against lipoprotein oxidation and blood coagulation while down-regulated proteins are
33 31 implicated in acute-phase response, lipid transport, and immune response. Pathway analysis
34 32 revealed the involvement of these changes in the control of LXR/RXR nuclear receptor, acute
35 33 phase response, and atherosclerosis.

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3 1 The effect of VOO on blood and urinary proteome biomarkers is obviously the result of
4 2 complex effects on tissues and particularly on the liver proteome. Unluckily, these aspects
5 3 have not been investigated in human liver, but in a recent animal study a proteomic approach
6 4 was used to characterize molecular targets of the prevention effects of VOO on fibrotic liver
7 5 damage during chronic exposure to the free radical generating agent CCL4 [101]. In this
8 6 experimental model of inflammation and oxidative stress, the administration of EVOO
9 7 improved histology and molecular markers of lipid peroxidation and fibrosis, with effects on
10 8 the liver expression of proteins associated with antioxidant protection, cellular detoxification,
11 9 and the intermediary metabolism. In detail, the proteins that increased in association with the
12 10 liver protection effect of VOO were: thioredoxin domain-containing protein 12,
13 11 peroxiredoxin-1, thiosulphate sulphurtransferase, calcium-binding protein 1, Annexin A2 and
14 12 heat shock cognate 71 kDa protein. Conversely, COQ9, cAMP-dependent protein kinase type
15 13 I-alpha regulatory subunit, phenylalanine hydroxylase and glycerate kinase were decreased.
16 14 These results point to a major effect of VOO on the liver proteome and possibly on other
17 15 proteomes, such as those of blood plasma and other biological fluids, that may have both
18 16 casual and causal association with metabolic risk factors of age- and lifestyle-dependent
19 17 diseases of the liver.
20 18

21 19 **4. Toward a hormetic role of EVOO phenolic compounds**

22 20 Many biological effects of VOO phenolics (VOOPs) introduced above have been ascribed to
23 21 their antioxidant activity that has been largely demonstrated in vitro [102] and seems to be
24 22 retained in vivo [103; 104]. However, a crucial element in determining VOOPs antioxidant
25 23 activity in vivo is represented by their bioavailability from diet (i.e. to achieve a biological
26 24 effect in a specific tissue or organ, VOOPs should be present in the active form in that tissue
27 25 or organ).

28 26 Several supplementation studies in human and animal models demonstrated that VOOPs are
29 27 rapidly absorbed and undergo to first-pass intestinal/hepatic metabolism [57; 105; 106; 107].
30 28 This process leads to the formation of sulphate and glucuronide conjugates to such an extent
31 29 that the free forms are almost undetectable in body fluids (around 98% of Tyr and HT in
32 30 plasma and urine are present in conjugated forms [108]. The conjugation and the resulting
33 31 loss of the OH groups decreases the radical scavenging activity of Ps, completely modifying
34 32 the antioxidant nature of the parent molecules. Actually, the few studies considering the
35 33 antioxidant activity of VOOPs metabolites demonstrated a general drop in their radical
36 34 scavenging activity. Using a LDL oxidation test and DPPH assay, Khymenets[66]

1 demonstrated that VOOP metabolites (obtained by chemical synthesis) did not possess any
2 significant antioxidant activity when used at physiological concentration. In particular, the
3 glucuronides of Tyr, OH-Tyr and HVA were not able to inhibit Cu-catalyzed LDL oxidation,
4 while the parent molecule OH-Tyr had strong antioxidant effect. Tuck et al. [109] observed
5 contrasting results, but they used VOOPs metabolites purified from human urines; in
6 particular, they observed that Homovannillic Acid and OH-Tyr-3-O-Glucuronide had a high
7 radical scavenging activity (DPPH test), while the sulfate conjugate of OH-Tyr did not
8 possess any radical scavenging activity. Finally, Deiana demonstrated that low concentration
9 of 3 different OH-Tyr glucuronide (obtained by chemical synthesis) were able to slightly
10 protect renal cells against H₂O₂ induced membrane oxidative damage, but they did not exert
11 any significant protection against H₂O₂ induced cellular injury [110].

12 Moreover, due to their low bioavailability, the conjugated forms are present in plasma at very
13 low concentration. In fact, the maximum concentration reached in human plasma by HT
14 metabolites after a single dose supplementation does not exceed 5 µM (Table 1), and similar
15 results have been obtained also after longer supplementation [111]. This concentration
16 appears quite low especially if we compare it to those of the main plasma antioxidants (Table
17 2), with which they should compete.

18 Inside tissues and cells the concentration of PCs is even lower than that present in plasma, and
19 this is quite critical as molecular components of cells and tissues should be key targets of the
20 antioxidant action. Unfortunately, it is very difficult to evaluate the presence of PCs in human
21 tissues and, to the best of our knowledge, there are no human studies on VOOP accumulation
22 in tissues and also animal studies are very limited. Serra et al. [112] quantified VOOP
23 metabolites in different tissues of rats supplemented with a high dose of VOOPs (3 g/kg of
24 phenolic extract from olive cake containing 100 mg PC/g extract). They observed that VOOPs
25 were distributed through the blood stream almost everywhere in the body, however PCs were
26 present especially in their conjugated forms and the tissue concentration was quite low
27 (nmol/g tissue). In a recent paper, tissue uptake of HT was studied in rats after the
28 supplementation of a refined oil containing HT in a dose compatible with human dietary
29 intake (1 mg HT/Kg). The concentration of HT metabolites recovered in rat tissues was even
30 lower (pmol/g tissue) than that previously observed [113]. Finally, in the study of Rublio et
31 al. [114] VOOP metabolites were detected in rat red blood cell (RBC) after an oral
32 administration of a VOO phenolic extract (1.5 g extract/Kg by intragastric gavage). The
33 maximum RBC concentration reached by HT and its metabolites was about 200 nM.
34 Considering the high efficiency of our cellular antioxidant defence system, providing

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3 1 cytosolic GSH at 1-10 mM concentration, such small amount of VOOP metabolites in cells
4 2 should not play a direct influence on the cellular redox.

5 3 To sum up, the bioavailability of these compounds is quite low and the attained
6 4 concentrations (in plasma and tissue) after ingestion appear to be too low to compete with and
7 5 impact on the endogenous antioxidant system and the redox homeostasis of tissues. Therefore,
8 6 a direct antioxidant action in vivo seems quite improbable. However, several supplementation
9 7 studies demonstrated that VOO or VOOP consumption induces an increase antioxidant
10 8 capacity of human plasma [104; 115; 116; 117] and prevents oxidative damage [103].

11 9 To explain this apparent contradiction several hypotheses have been made. First of all, it has
12 10 been proposed that the conjugated metabolites may act as storage forms and that parent
13 11 molecules could be freed from their glucuronide and/or sulphate conjugates in tissues. A
14 12 recent animal study suggested that the de-conjugation process could really occur in vivo; in
15 13 this study, the content of OH-Tyr and OH-Tyr metabolites was measured in rat RBCs before
16 14 and after an oral administration of olive extract. The results show a decrease in the level of
17 15 conjugated forms and a parallel increase of the parent molecule concentrations in RBCs
18 16 suggesting that the OH-Tyr conjugated forms could be hydrolysed intracellularly [114].

19 17 Incorporation of VOOPs into lipoproteins could represent another mechanism through which
20 18 they could prevent the oxidative damage. Several human studies demonstrate that VOO
21 19 consumption increase the ex vivo LDL resistance against oxidation (Table 3). On this basis,
22 20 the EFSA released a claim concerning the benefits of olive oil polyphenols (5 mg/d) for the
23 21 protection of LDL from oxidation [15]. But, if at least 10 μ M HT concentration is needed to
24 22 lower LDL levels, as demonstrated in in vitro studies [106], how the low attained plasma
25 23 concentrations of this PC can explain such results? The answer could be found in the
26 24 formation of molecular interactions between VOOPs and the LDL particle; these interactions
27 25 can determine a much higher local concentration that will be able to protect LDL from
28 26 oxidation. Unfortunately, just few studies have assessed physical and chemical aspects of
29 27 VOOP incorporation into LDL (Table 3), and further results are needed to explore the
30 28 possibility that this incorporation represents a prerequisite for the inhibition of LDL
31 29 oxidation. Because, VOOPs-LDL interaction appears to be transient (time-coinciding with
32 30 the plasma absorption peak) and strongly affected by dialysis procedure (90 % of the HT
33 31 present in LDL disappears within the first 10 minutes of dialysis) [118], the selection of the
32 32 right time point and analytical method used for LDL preparation should be considered as key
33 33 aspects in future human studies.

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3 1 Finally, also it can be hypothesized that the systemic antioxidant effect (increase of
4 2 antioxidant capacity and prevention of oxidative damage) of VOOPs could be the result of an
5 3 indirect effect, which may lead to improve the endogenous antioxidant defence essentially
6 4 through transcriptional effects on phase II drug metabolizing genes [90]. By its own nature,
7 5 antioxidants (PCs among them) are prone to oxidation and their oxidation products if not
8 6 redox cycled can be toxic and then operate as hormetic compounds, activating adaptive
9 7 cellular stress response pathways [119]. Therefore, the oxidized forms of VOOP could induce
10 8 a temporary oxidative stress and cause the activation of signalling pathway that in turn trigger
11 9 the up-regulation of endogenous antioxidant and detoxification enzymes, which may lead to
12 10 higher level of protection against the long-term damaging effects of oxidative stress.

13 11 As introduced above (Section 3), one of the most important pathways of cellular hormesis is
14 12 the Nrf2/antioxidant response element (ARE) system. Nrf2 regulates the expression of phase
15 13 II detoxification and antioxidant genes in response to harmful stimuli and food bioactives
16 14 [90]. Emerging evidence indicates that VOOPs can activate the Nrf2 pathway both in cellular
17 15 models [120; 121; 122] and in vivo [123]. Finally, several human studies demonstrated that
18 16 the consumption of VOO or VOOPs activates this endogenous antioxidant response system
19 17 increasing enzyme activities such as plasma GPX [124; 125] and erythrocyte CAT [126], and
20 18 the levels of GSH [127] and its redox control [116] in blood plasma.

21 19 Then we can speculate that at the low doses reached in vivo VOOPs can activate
22 20 cytoprotective pathways, not acting directly as free radical scavenger, but as a sort of Nrf2-
23 21 targeted “early warning signal” (positive hormetic effect).
24 22

25 23 **5. Functional components of olive oil as anti-aging agents: modulation of the miRNome.**

26 24

27 25 Epidemiological studies indicate that olive oil consumption is associated with reduced
28 26 mortality and improved cognitive function in elderly subjects at high cardiovascular risk [128;
29 27 129]. Clinical trials have shown that the assumption of these compounds, either in VOO or as
30 28 extracts, induced a decrease in a series of inflammation and oxidative stress parameters in the
31 29 blood [130]. As many of these parameters are associated with cardiovascular risk, and
32 30 considering that cardiovascular pathologies are the first death cause in the over-65 population,
33 31 it can be hypothesized that the protection from this particular risk is one of the main
34 32 mechanisms through which VOO phenols can reduce both mortality and some age-associated
35 33 pathologies.

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3 1 Preclinical animal studies have confirmed the protective effects of olive oil phenols on
4 2 cognitive function, both in normal and in accelerated aging rodent models [131], showing also
5 3 positive effects on motor function and emotional behaviors related to anxiety [132; 133]. HT,
6 4 tyrosol, their sulfate metabolites and oleuropein have been found in the brain after
7 5 administration of VOO extracts to laboratory animals, although in smaller amount as
8 6 compared to other organs [112]. Thus, it is possible that these behavioral actions are also due
9 7 to a direct interaction with the brain tissue.

10 8 Moreover, both in vitro and in vivo evidences indicate that VOO phenolics have the ability to
11 9 modulate cellular pathways that are relevant to the aging process, such as those related to cell
12 10 protection and survival, energy metabolism, and the inflammation process [134].

13 11 While part of these actions can be due to direct interaction with proteins, they can also be
14 12 ascribed to the modulation of gene expression. DNA microarray-based studies have shown
15 13 that a large number of genes is modulated in the aging heart and brain of mice, and that
16 14 dietary interventions have the ability to counteract these changes [135]. Very few works have
17 15 addressed the modulation of gene expression by VOO components during aging. Bayram et
18 16 al. [123] have reported an induction of genes related to antioxidant defenses and longevity in
19 17 SAMP8 mice, a widely used model of accelerated senescence, treated for 4.5 months with a
20 18 diet rich in VOO phenols (10% extra virgin olive oil, EVOO).

21 19 The role of miRNAs, the small non-coding RNAs, in modulating gene expression at the post-
22 20 transcriptional level has gained increasing importance during the last decade, and it has been
23 21 shown that miRNA regulation might also play an important role in the shaping of age-related
24 22 mRNA changes [136]. Furthermore, a dietary intervention, namely calorie restriction, known
25 23 to exert anti-aging actions, has been shown to induce the down-regulation of miR-30e and
26 24 miR-34a, up-regulated in the aging brain [137].

27 25 Recently, it has been shown that olive oil phenols were able to modulate both the gene and
28 26 miRNA expression profiles evaluated by the microarray technique in mice treated from age
29 27 10 to 16 months with an EVOO naturally rich in phenols (H-EVOO) and these changes were
30 28 associated with reduced age-related decline of motor coordination and contextual memory
31 29 [97]. At the end of the treatment, most of the genes modulated by aging resulted to be down-
32 30 regulated and restricted to the cerebral cortex. Compared to L-EVOO (the same oil deprived
33 31 of phenols), the treatment with H-EVOO was instead associated with a significant up-
34 32 regulation of genes associated with synaptic plasticity and with motor and cognitive behavior,
35 33 such as Notch1, BMPs, NGFR, GLP1R and CRT3. The agrin pathway was also
36 34 significantly modulated.

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3 1 Opposite to genes, miRNAs in the cerebral cortex were mostly up-regulated by aging, and the
4 2 treatment with olive oil phenols modified the miRNA profile in aging mice so that it resulted
5 3 very similar to the young mice profile. In particular, 63 miRNAs were significantly down-
6 4 regulated by the H-EVOO treatment [97].

7 5 Further, some of the H-EVOO-modulated miRNAs were found to target genes associated
8 6 with synaptic plasticity and neuronal function protection, whose expression was also modified
9 7 by the treatment. A computational analysis on miRNAs modified according to the changes of
10 8 their respective target genes allowed to identify a further restricted list of 14 age-modulated
11 9 miRNAs, and a partially overlapped list of 6 treatment-modulated miRNAs, shown in Table 4
12 10 (Giovannelli, unpublished data). Among the age-modulated miRNAs, those with the top
13 11 scores were miR-681, -709, -706, all reduced in aging, and -30a-5p, which was up-regulated
14 12 in older mice. Of the 6 treatment-modulated miRNAs, all down-regulated in the H-EVOO
15 13 group, 5 were also up-regulated in aging: miR-30a-5p, -434-5p, -369-5p, -451 and -126-3p.
16 14 At the top ranking score was miR-30a-5p, predicted to control a large numbers of genes
17 15 involved in several pathways, among which axon guidance, ubiquitin-mediated proteolysis,
18 16 regulation of actin cytoskeleton and long-term potentiation. MiR-126, a well-studied miRNA
19 17 in vascular biology, has been found increased in colon-derived myofibroblasts cells upon in
20 18 vitro treatment with wine-derived polyphenols, and demonstrated to be associated to reduced
21 19 expression of inflammatory genes [138]. No association with aging or polyphenols has been
22 20 previously described for miR-434, -369 and -451.

23 21 Among the miRNAs consistently reported to be associated with the aging process in the brain,
24 22 mir-30, mir-34 and mir-124 have been reported to be up-regulated in aged animals, and these
25 23 changes were associated to learning dysfunctions in animal models and in Alzheimer's
26 24 disease [139]. As these and other miRNAs can be down-regulated by dietary interventions,
27 25 the modulation of the miRNome, and of specific miRNAs, by natural compounds might
28 26 represent a novel mechanism of action for nutraceutical compounds, and become part of a
29 27 neuroprotective strategy in the prevention of age-related pathologies.

30 28 Recently, much interest has focused on circulating miRNAs, as potential biomarkers that can
31 29 be measured in plasma by simple PCR techniques. In a future perspective, specific miRNAs
32 30 might serve as markers in intervention trials in elderly humans, to evaluate the extent of age-
33 31 associated dysfunctions and the efficacy of anti-aging treatments.

34 32 In conclusion, olive oil phenols are able to counteract age-induced alterations in brain
35 33 function, and the treatment has proved effective also in animals that were fully adult at the

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3 1 beginning of the intervention. These changes can be related to a complex modulation of gene
4 and miRNA expression patterns.
5 2

6 3 Finally, it is worth mentioning the recent interest in evaluating the effects of exogenous plant-
7 derived miRNAs [140]. Exogenous miRNAs from animal and plant dietary sources can be
8 transported to human blood and tissues through exosomes [141; 142] and the possibility of
9 cross-kingdom gene regulation has been raised. mi-RNAs have been detected with next
10 generation sequencing in olives [143], but up to date the bioavailability of these miRNAs
11 ingested with olives or VOO remains unexplored [144]. Future work is needed to clarify
12 whether part of the beneficial effects of olive oil can also be attributed to such olive-derived
13 miRNAs.
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19 13 20 14 21 15 **6. Chemoprevention effects of olive oil polyphenols: molecular and cellular mechanisms.** 22 16 23 17

24 18 In the last few years, VOOPs have received growing attention because of their healthy
25 properties including the chemopreventive activity. Several processes are essential for cancer
26 development: DNA damage, sustained proliferation and insensitivity to antigrowth signals,
27 evasion of apoptosis, sustained angiogenesis, tissue invasion, metastatization and
28 inflammation [145]. Some VOOPs are capable to affect many if not all these processes thus
29 interfering with the carcinogenic process. This chemopreventive activity of VOOPs is the
30 result of specific gene regulation effects some of which are now identified and are described
31 here in this section.
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36 23 Oxidative DNA damage plays a central role in both the stages of cancer initiation and
37 promotion/progression. The protection effect of different VOOPs against the H₂O₂-induced
38 DNA damage has been investigated in HL60 human lymphoblasts and in peripheral blood
39 mononuclear cells (PBMC). HT (3,4-DHPEA) significantly reduced the extent of DNA
40 damage at concentrations as low as 1 μ M, as evaluated by the single cell gel electrophoresis
41 (SCGE or Comet assay) [146]. Other compounds structurally related to HT showed the same
42 effect on the H₂O₂-induced DNA damage, but the potency of the different compounds
43 changed. In particular, tyrosol (p-HPEA), a phenol compound lacking of the ortho-hydroxyl
44 group on the phenol ring, had less effect than 3,4-DHPEA [146]. These VOOPs were also
45 able to prevent the oxidative DNA damage induced in human lymphocytes by the co-culture
46 with monocyte-macrophages stimulated with PMA, an ex vivo model that mimics the
47 pathophysiology of oxidative stress of an inflammatory lesion [147]. In this experimental
48 system, tyrosol was more effective than HT in preventing oxidative DNA damage [146].
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3 1 Similar results were obtained in Jurkat cells exposed to continuously generated H₂O₂ and
4 2 treated with phenolic extracts from OO and olive mill waste water as well as with isolated
5 3 compounds (HT and caffeic acid) [148]. Additional studies have demonstrated that
6 4 pretreatment of HeLa cells with VOO phenolic extract also prevents DNA damage induced by
7 5 H₂O₂ [149]. The effect of VOO was tested also in vivo. Quiles et al. tested the effects of
8 6 feeding male Wistar rats with diets containing different sources of fat, such as VOO and
9 7 sunflower oil (SO) [150]. Lower levels of DNA double-strand breaks in peripheral blood
10 8 lymphocytes were found in young animals fed on VOO, which reached around one half of the
11 9 damage found in SO treated animals. The same measurements were carried out in aged rats
12 10 showing that the age-related increase of DNA double-strand breaks was significantly lower in
13 11 rats fed a diet containing VOO [150]. Another study from the same group showed the higher
14 12 efficacy of VOO compared to SO in decrease deletions of the mitochondrial genes ND1 and
15 13 ND4 of rat liver [151]. These results strongly suggest that VOOPs may prevent one of the
16 14 main steps in the initiation phase of carcinogenesis, i.e. the oxidative lesion of DNA, a
17 15 process that is responsible for oncogene activation [152].

18 16 Regarding the effects of VOOPs on cancer progression it was found that 3,4-DHPEA reduces
19 17 the proliferation of different human tumour cell lines derived from colon (HCT116, SW480),
20 18 prostate (PC3 and LnCap), breast (MDA and MCF-7) [86] and leukemia (HL60) [153].
21 19 Among the solid tumour cells those derived from breast were the most sensitive to 3,4-
22 20 DHPEA treatment followed by colon and prostate cells. The greatest inhibition of
23 21 proliferation rate was found in HL60 cells (IC₅₀ = 75µM) and this response was associated
24 22 with an increased apoptotic cell death. On the other hand, p-HPEA failed to inhibit cell
25 23 growth and to induce apoptosis in the same experimental conditions [153]. Two secoiridoid
26 24 derivatives of 3,4-DHPEA and pHPEA linked to the dialdehydic form of elenoic acid (EDA)
27 25 and relatively abundant in olive oil were also investigated. 3,4-DHPEA-EDA and pHPEA-
28 26 EDA were able to inhibit the proliferation of HL60 cells and pHPEA-EDA was more potent
29 27 than 3,4-DHPEA-EDA to suppress cell growth and to induce apoptosis in these cells [154].
30 28 Worth of note, the secoiridoid derivatives of 3,4-DHPEA and pHPEA were more efficient
31 29 than phenolics in producing these cellular responses of leukemia cells. In particular, the
32 30 apparent IC₅₀ calculated on cell proliferation for pHPEA-EDA was 10µM while pHPEA
33 31 was ineffective also at 100µM. When pHPEA and 3,4-DHPEA were combined with the
34 32 dialdehydic form of elenoic acid, the physicochemical and structural properties of the
35 33 resulting compounds undergo to major modifications [154]. The molecular and mechanistic
36 34 aspects of the cellular response to 3,4-DHPEA have been investigated in HL60 cells. The

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3 1 expression of some cyclins, kinases cycline dependent (CDK) and inhibitors of kinases
4 2 cycline dependent (CDKi), are under the influence of this molecule, e.g. 3,4-DHPEA or HT
5 3 [155], and demonstrate changes in cell cycle checkpoints that are consistent with a block of
6 4 the cycle in G0/G1 phase. Most relevant changes observed at the proteomic and
7 5 transcriptomic level were an increased expression of cyclin D3 and of the CDKi p21 and p27.
8 6 At the same time, CDK6 decreased and the cyclins E, B1, and A remained unchanged
9 7 together with p15 levels. These findings are suggestive of the inhibitory activity that 3,4-
10 8 DHPEA may exert on Rb phosphorylation, a key pathway in the control of genes responsible
11 9 for DNA synthesis and G1-S transition [156].

12 10 3,4-DHPEA-targeted checkpoints of cell cycle, such as p21 and p27, also provide regulation
13 11 effects on the cellular differentiation process [157]. Accordingly, 3,4-DHPEA similarly to the
14 12 positive controls DMSO and ATRA, induced the granulocytic differentiation of HL60 cells
15 13 [155].

16 14 The same or even more potent activity of 3,4-DHPEA on these anti-cancer effects associated
17 15 with a modified regulation of cell cycle were also observed for other VOO bioactives, such as
18 16 pinoresinol. This is one of the simplest lignans present in olives and VOO, which was found
19 17 to exert a potent anti-proliferative activity both in HL60 cells (IC50% 8 μ M) and in the
20 18 multidrug resistant variant HL60R (IC50% 32 μ M). Again, this effect was associated with a
21 19 block of cell cycle in G0/G1 phase, up-regulation of the CDK inhibitor p21 and induction of
22 20 cellular differentiation [158].

23 22 *Redox-dependent properties of anticancer VOOPs*

24 23 Underlying molecular events in the anticancer function of main VOOPs, such as tyrosol and
25 24 HT, have been reported to include effects on the redox homeostasis of cancer cells (see
26 25 Section 4). Mechanisms of the redox sensing for these molecules may include a direct
27 26 interaction with cellular proteins and lipids at the level of plasmalemma or even
28 27 intracellularly for the VOOP forms with cellular bioavailability. Both the redox-dependent and
29 28 independent interactions with the cellular components are markedly influenced by the
30 29 structural and physico-chemical features of VOOP compounds. For instance, different
31 30 reaction rates with radical probes have been reported for phenolics that show very close
32 31 structural similarity [102] and lipophilic properties of biophenols influences the possibility to
33 32 modify the physical properties of the lipid bilayer or even permeate cellular membranes to a
34 33 relevant extent thus reaching intracellular interactors/sensors. The latter is for instance the
35 34 case of oleocanthal [159], an anti-inflammatory fat-soluble biophenol first identified in VOO

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3 1 by the group of Montedoro and Servili [44]. As far as, the redox properties of 3,4-DHPEA are
4 2 concerned, H₂O₂ was generated and released in the culture medium of cancer cells that
5 3 responded to the anti-proliferative and pro-apoptotic activity of this molecule (16). Steady-
6 4 state levels of H₂O₂ were influenced by the presence of serum and pyruvate in the culture
7 5 medium, and from the ability of cells to remove this oxidant agent. Worth of note, other
8 6 VOOPs structurally related to HT, such as p-HPEA, do not stimulate the production of H₂O₂
9 7 in the culture medium and the apoptotic death of HL60 cells [160].

10 8 Recently, the attention of different groups has been focused on the effects of 3,4-DHPEA on
11 9 different inflammatory pathways either in association with cancerogenesis or other chronic
12 10 diseases such as arthritis, pathologic obesity and atherosclerosis (reviewed in [161; 162]). In
13 11 this regard, it was demonstrated that 3,4-DHPEA reduced the production of superoxide anions
14 12 (O₂⁻), prostaglandin E₂ (PGE₂), and the expression of cyclooxygenase-2 (COX-2) in
15 13 freshly-isolated human monocytes [80]. Similar effects were previously reported in murine
16 14 and human macrophage cell lines [79; 163; 164].

17 15 The decreased generation of O₂⁻ suggests that 3,4-DHPEA can inhibit the activity of the
18 16 enzyme NADPH-oxidase, a membrane protein complex expressed in several cellular systems
19 17 involved in the production of reactive oxygen species at the inflammatory site [165]. This
20 18 effect on O₂⁻ production was also observed with other OOPs with an efficacy that changed
21 19 according with the following order of magnitude: pHPEA-EDA ≈ 3,4-DHPEA > 3,4-
22 20 DHPEA-EDA > p-HPEA [80]. In LPS-stimulated human monocytes, 3,4-DHPEA
23 21 significantly decreased PGE₂ production and release in the culture medium. PGE₂ is an
24 22 inflammatory mediator involved in angiogenesis, proliferation, invasion and apoptosis [166;
25 23 167]. This 3,4-DHPEA dependent decrease of PGE₂ secretion resulted from the
26 24 downregulation of COX-2 gene [80]. In the same experimental system, 3,4-DHPEA
27 25 stimulated the production of TNF- α , one of the earliest pro-inflammatory cytokine with key
28 26 role in immune cell activation and cancer mechanisms [168; 169]. The addition of exogenous
29 27 PGE₂ to the cell culture medium hampered this effect, pointing to a PGE₂-mediated control
30 28 of 3,4-DHPEA on TNF- α gene expression and cytokine secretion. Pharmacologic activation
31 29 of adenylate cyclase by Forskolin and the consequent increment of intracellular cAMP,
32 30 reduced the LPS-stimulated secretion of TNF- α in 3,4-DHPEA-treated monocytes [82].
33 31 Therefore, in close functional similarity with non steroidal anti-inflammatory drugs
34 32 (NSAIDs), such as celecoxib and rofecoxib (19), 3,4-DHPEA behaves as a natural anti-
35 33 inflammatory agent decreasing COX-2 activity and PGE₂ production, and increasing the
36 34 secretion of TNF- α in LPS-activated human monocytes.

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3 1 The in vivo anticancer activity of oleuropein, the precursor of olive oil secoiridoids, has been
4 2 also investigated and convincing pre-clinical in vitro findings have been obtained in several
5 3 models of hormone-sensitive cancers (reviewed in [170]). In animal models oleuropein has
6 4 been described to prevent the colitis-associated molecular changes reducing the risk of
7 5 developing colorectal cancer [171]. Recent studies in a MCF-7 breast carcinoma xenograft
8 6 generated in ovariectomised nude mice, demonstrated that an oleuropein-enriched diet
9 7 compared to the baseline control diet decreases the tumour growth rate and significantly
10 8 prevents the formation of both peripulmonary and parenchymal lung metastases [172]. This
11 9 earliest in vivo evidence on the chemopreventive activity of this VOO compound in breast
12 10 cancer is worth of further clinical investigation.

11 12 **7. Olive oil polyphenols and their effect on tumor vascularization and progression**

13
14 14 Angiogenesis is the formation of new blood vessels from the pre-existing vasculature [173;
15 15 174]. It is a physiologic process regulated through the fine balance between stimulatory
16 16 factors, such as Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor
17 17 (bFGF), Placental Growth Factor (PlGF), inhibitory factors as Tissue Inhibitor of
18 18 MetalloProteinase (TIMP), Angiopietin-2 (Ang-2), Interferon- α (IFN- α), Thrombospondin
19 19 family of genes (TSP) and other molecular players with accessory pro-angiogenic properties,
20 20 such as endothelial Nitric Oxide Synthase (eNOS) and Cyclooxygenase-2 (COX-2). This
21 21 process could shift to a pathological condition when feedback mechanisms are deficient or
22 22 completely lost, a condition promoted by the tumor microenvironment, that is the cellular
23 23 environment surrounding the tumor, including blood vessels, immune cells, fibroblasts and
24 24 signaling molecules [175; 176]. Tumor and microenvironment are closely related and interact
25 25 constantly by the releasing of extracellular signals that promote tumor angiogenesis and pro-
26 26 tumoral stimuli. Indeed, tumor cells can produce chemokines and cytokines that stimulate
27 27 angiogenesis in terms of growth, progression, and dissemination; these events are grouped
28 28 together under the definition of angiogenic switch, a process that allows the tumor
29 29 transformation from a microscopic lesion with a low malignant potential to a high aggressive
30 30 mass favouring malignancy and metastatization.

31 31 The newly-formed tumor vasculature is structurally and functionally abnormal and it differs
32 32 from the normal vasculature by means of several phenotypic aspects. Blood vessels are leaky,
33 33 tortuous, dilated and show a disorganized pattern of interconnection that witnesses the loss of
34 34 the vascular architecture. The endothelial cells show an aberrant morphology, pericytes (cells

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3 1 that provide support for the endothelial cells and are able to regulate blood flow) are loosely
4 2 attached or absent, and the basement membrane is often abnormal. These structural
5 3 abnormalities contribute to spatial and temporal heterogeneity in tumor blood flow.

6 4 Under physiological situations, such as in wound healing, angiogenesis is closely related with
7 5 the mobilization of inflammatory cells that is a transient process. However, in certain
8 6 pathological conditions, such as in cancer, a continuous recruitment of inflammatory cells is
9 7 observed, which are also a source of ROS. This tight connection between the inflammation-
10 8 dependent generation of ROS and angiogenesis is believed to play a leading role during
11 9 tumorigenesis, from angiogenic switch to metastatization [177].

12 10 Considering the relevant role of angiogenesis in tumor progression, several studies have
13 11 focused on the prevention of the angiogenic switch, e.g. angioprevention [173], which is the
14 12 inhibition of angiogenesis using chemopreventive drugs targeted to key players of this
15 13 process. Accordingly, angiopreventive compounds interrupt autocrine or paracrine stimuli
16 14 generated by the cancer cells or the tumor microenvironment that in turn influence specific
17 15 endothelial pathways, such as inhibition of VEGF-pathway, c-Met (Hepatocyte Growth
18 16 Factor Receptor) axis and COX-2 signaling.

19 17 For instance, Terzuoli [178] demonstrated that DPE (2-(3,4-dihydroxyphenyl)-ethanol), a
20 18 polyphenol present in virgin olive oil, exerts its effects on colon cancer cell growth and
21 19 angiogenesis through the inhibition of Hypoxia Inducible Factor-1alpha (HIF-1 α) pathway.
22 20 HIF-1 α stimulates the migration of mature endothelial cells increasing vascularization of
23 21 hypoxic areas in the tumor microenvironment and this response stimulates the activation of
24 22 microsomal prostaglandin-E synthase-1 and VEGF pathways [179]. In consideration of the
25 23 close relationship between angiogenesis and inflammation in the tumor microenvironment.
26 24 Scoditti et al. [180] investigated the protective role of oleuropein and HT in endothelial
27 25 models after the induction of an inflammatory state with the pro-angiogenic factor phorbol
28 26 12-myristate 13-acetate-PMA. The Authors demonstrated that these polyphenols suppress
29 27 inflammatory angiogenesis attenuating COX-2 expression and the production and release of
30 28 Matrix MetalloProteinase-9 (MMP-9).

31 29 HT, oleic acid and taxifolin have also been studied for their anti-angiogenic potential in
32 30 endothelial cells [181]. Lamy and colleagues demonstrated that these polyphenols present in
33 31 olive oil inhibit angiogenesis downregulating the phosphorylation of VEGF receptor-2 and the
34 32 downstream pathways [182].

35 33 The auto-phosphorylation of different tyrosine residues is linked to a specific effect on
36 34 endothelial cells, for examples, Tyr951 is essential for endothelial cell migration, whereas

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3 1 Tyr1059 is required for VEGF-induced MAPK pathway activation that leads to cell
4 2 proliferation. Secoiridoid derivatives, in particular (-)-oleocanthal, have been assessed for
5 3 their capability to promote chemopreventive effects. In two different studies (-)-oleocanthal
6 4 was demonstrated to exert inhibitory effects towards c-Met 8;9 [183; 184] ; c-Met is a proto-
7 5 oncogene encoding the heterodimeric tyrosine kinase receptor of Hepatocyte Growth Factor
8 6 (HGF) that has been associated with several cellular mechanism, such as cell proliferation,
9 7 epithelial-to-mesenchymal transition (EMT), invasion and metastasis. Furthermore, the
10 8 HGF/c-Met axis represents an additional VEGF-independent mechanism of tumor
11 9 angiogenesis, and its inhibition correlates with repression of angiogenesis.

12 10 Certain anti-angiogenic agents transiently "normalize" the abnormal structure and function of
13 11 tumor vasculature to make them more efficient for oxygen and drug delivery [185]. Drugs
14 12 that are capable to induce vascular normalization can modulate hypoxia increasing the
15 13 efficacy of conventional therapies [185]. Palmieri et al. [186] studied the anoxic environment
16 14 surrounding tumor cells and the correlation of antioxidant activity by olive phenols. They
17 15 demonstrated that polyphenols decreased the anoxia-associated levels of iNOS, COX-2, TNF-
18 16 α , MMP-2/-9 and restored that of the TIMP-1 leading to inhibition of vascular injury and
19 17 correlated hemodynamic alteration (vascular normalization hypothesis [187]).

20 18 All these studies are focused on the chemoprevention properties of VOOPs as anti-angiogenic
21 19 agents that delay or impede the neovascularization by the tumor cells and the tumor
22 20 microenvironment. Early work by some of us demonstrated the anti-angiogenic potential of a
23 21 phenol-rich olive mill wastewater purified extract, an aqueous byproduct of VOO production
24 22 process. We found that this phenol extract inhibits the angiogenic process, lowering the index
25 23 of proliferation, migration/invasion and network formation of endothelial cells both in vitro
26 24 and in vivo [188]. These results further demonstrate the chemoprevention potential of these
27 25 natural functional agents that deserve further exploration in preclinical and clinical protocols
28 26 of therapy for tumor relapse and progression also to face the problem of the severe collateral
29 27 effects that the available anti-angiogenesis drugs are known to produce.

30 **8. Signaling and gene response of vitamin E: a focus on the main fat-soluble vitamin in** 31 **olives and olive oil.**

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33 33 Vitamin E was discovered in 1922 by Evans and Bishop as a factor essential for rat fertility
34 34 [189]. Nowadays, this term is used to identify a family of eight tocopherols produced in

1 photosynthetic organisms [190], 4 tocopherols (TOH) and 4 tocotrienols (T3) (Figure 2).
2 These molecules share as common structural component a chroman group with a hydroxyl
3 moiety in position 6 and a 13-carbon atom side chain in position 2. This chain has phytyl-
4 derived or geranylgeranyl-derived (or isoprenoid) structure in the subfamily of tocopherols
5 and tocotrienols, respectively. In each of these two subfamilies the four vitamers identified
6 with Greek letters, from alpha to delta, have different methylation patterns of the chroman
7 ring with alpha as the fully methylated (trimethylated) and delta as the desmethyl
8 (monomethylated) form. Both the degree of methylation and the presence of unsaturations in
9 the side chain confer specific biological, physico-chemical and analytical characteristics to the
10 different forms of vitamin E [191].

11 Tocopherols are considerably more widespread in the plant kingdom than tocotrienols [192]
12 and are found in leaves, seeds, roots, tuber, fruits, stems, hypocotyls and cotyledons of higher
13 plants with very heterogeneous concentrations and relative composition of the vitamers [190;
14 192]. In general, α -tocopherol is the main form of tocopherol in photosynthetic tissues. Seeds
15 generally accumulate ten to twenty times more tocopherols than leaves and in some cases,
16 such as Arabidopsis, soy or sunflower seeds, the desmethyl forms of tocopherol largely
17 predominate over the content of α -tocopherol. The presence of tocotrienols in photosynthetic
18 tissues is relatively rare but various tocotrienols can be present in significant amounts in
19 monocot seeds [193].

20 As introduced in Section 2, α -TOH accounts for more than 90% of the vitamin E found in
21 EVOO with key role in the antioxidant protection of other lipid components of this edible oil
22 [20; 41]. Absolute levels of this vitamin however vary substantially in different oil products;
23 in a recent comparative analysis on 430 samples of EVOO showed levels of this vitamin
24 between 23 and 751 mg/kg [17].

25 α -TOH also represents the main form of vitamin E of the human organism (reviewed in [194;
26 195]) and this could be the consequence of a series of physicochemical and biological
27 properties, which are unique among this family of fat-soluble molecules. Convincing
28 evidence was obtained on the fact that α -TOH provides optimal structural interactions with
29 membrane phospholipids. A differential distribution with respect to cholesterol and ceramides
30 has been described to occur on cellular membranes and this may help to regulate fluidity and
31 the functional properties of the different areas of the plasmalemma [34; 196]. Reduction
32 potential and lipid-lipid interaction properties of α -tocopherol are also distinctive of a role for
33 this molecule as the main non-enzymatic antioxidant of cellular membranes. The dynamic
34 interaction of α -TOH with the allylic moieties of unsaturated phospholipids strategically

1 influences the antioxidant protection and the physical stability of the membrane. α -TOH is by
2 far the most important hydrogen donating species of biological membranes and in general of
3 macromolecular complexes such as the lipoprotein particles [40] and other lipid structures
4 even in plant organisms [190]. This is a key and early event in the radical chain-breaking
5 activity that non-enzymatic fat-soluble antioxidants embedded in the lipid structure may
6 promote. A further requisite for chain breakers is to generate a corresponding radical with
7 higher degree of stability with respect to the lipid hydroperoxyl radical formed during free
8 radical damage. This non-enzymatic step is a one-electron reaction that in the end promotes
9 the formation of a lipid hydroperoxide for a subsequent two-electron step of enzymatic
10 reduction that restores the lipid structure of PUFA at the site of free radical attack. In the cell
11 membrane this late step is catalysed by the peroxidase activity of enzyme proteins such as the
12 selenium-dependent glutathione peroxidase 4 [197]; other glutathione- or thioredoxin-
13 dependent enzymatic reactions take place in the different compartments of cells and tissues to
14 implement this antioxidant protection of lipid environments, the 1-Cys Peroxiredoxin 6
15 reaction is reported as one of these antioxidant enzymes (reviewed in [198]).

16 As far as the radical species produced during H-donation reactions of tocopherols concern,
17 tocopheryl radicals and their quinone derivatives, such as tocopheryl quinone (TQ), show
18 different degree of stability and cytotoxicity. Desmethyl quinone derivatives generate much
19 greater toxicity than α -tocopheryl quinone [40; 199]. Such a potentially harmful activity is
20 based on peculiar signaling and gene modulation effects that may help to explain the
21 beneficial roles so far reported for the desmethyl forms of vitamin E [200; 201]. The same
22 type of considerations has been made on the quinone analogues of VOO phenolics [202]. The
23 mitochondrial-dependent pro-apoptotic activity of α -tocotrienol in HER2-neu positive breast
24 cancer cells and the corresponding in vivo anticancer activity are a specific example of these
25 properties [92; 203]. Molecular players of this signaling include among the others,
26 phospholipase A2, PKC isoforms and their corresponding phosphatases, pro-survival and
27 stress-related MAPKs, and the regulatory components of cell cycle (reviewed in [37; 204]).

28 The lower chemoprevention potential of α -TOH suggests a physiological regulatory activity
29 in lipid/phosphorylative signalling of cells which is another potential reason for its
30 preferential selection and bioavailability to tissues. The physiological role of α -TOH in
31 cellular signaling and functional control of biological processes distinct from other forms of
32 vitamin E can also be deduced from the gene response of immune cells. In fact, in vivo a high
33 intake of α -TOH sustains the gene response of stimulated T-cells thus suggesting immune-
34 modulation effects useful to promote host defence and the inflammatory response of tissues

[205], a response that was also observed in other experimental models and in humans [206]. On the contrary, γ -TOH assessed under the same experimental conditions, repressed T-cell inflammatory genes with higher efficacy than α -TOH, pointing to an anti-inflammatory role for this desmethyl form of vitamin E [205].

Emerging evidence demonstrates that α -TOH may exert some of its roles by means of the produce some of its biological roles useful to

Therefore, available data are sufficient to propose roles for α -TOH as key cytoprotection factor with homeostatic effects that spread virtually to all the cellular components and biological fluids [207]. Such an important series of functions justifies the definition for this molecule as an “essential factor” also representing a valid reason for the hepatic metabolism to preferentially retain α -TOH and distribute it with the lipoprotein particles to blood and then to peripheral tissues (reviewed in [194; 195]). In fact, its levels in plasma are by far the highest among the fat-soluble molecules reported to possess antioxidant activity with levels that vary depending on gene characteristics and dietary habits from 20 to 60 μ M. Plasma α -TOH is approximately 10 to 20-fold less abundant than α -TOH; δ -TOH shows even lower levels, usually below 0.5 μ M.

The selective liver uptake and distribution of α -TOH is under the control of the α -Tocopherol Transfer Protein (α -TTP), a cytosolic protein whose gene defect causes a severe and even fatal form of spinocerebellar ataxia associated with vitamin E deficiency, also known with the term of AVED (OMIM #277460; [208]) that can be successfully treated by vitamin E therapy [209; 210].

Nutritional considerations

Regardless of the variability among VOO products, the intake of α -TOH with this oil within a Mediterranean diet food pattern is of nutritional relevance and this can become even more relevant if the quality of VOO is the highest in terms of vitamin E levels. The amount of tocopherols in other edible oils not common in the Mediterranean diet is between 0.31 and 39.2 mg/100 g for α -TOH, 0.92 and 64.9 for γ -TOH, 2.8 and 20.38 for δ -TOH [211]. These composition data support a much higher intake of γ -TOH than α -TOH in regions that consume soy and corn oil as main source of fat in their diets, such as US and Australia [194]. High intakes of tocotrienols are found in Asiatic regions cause of the sustained consumption of palm oil or other characteristic oil products such as annatto oil [91].

Non-alpha forms and tocotrienols have been reported to promote beneficial effects such as cancer prevention and cholesterol lowering activity [91; 194], but so far the most convincing

1 literature on nutritional and health-promoting effects of vitamin E was produced on α -TOH
2 [40]. Along with antioxidant and gene regulation properties reported above for this vitamer, it
3 is reported to have higher biological activity in different tests used to comparatively assess the
4 nutritional properties of the forms of this vitamin such as the rat foetal resorption, H_2O_2 -
5 induced red blood cell haemolysis and prevention of muscular degeneration in animal models
6 of vitamin E deficiency. Accordingly, nutritional recommendations for non-alpha forms of
7 vitamin E are expressed in terms of α -TOH equivalents (one equivalent is defined by the
8 biological activity of 1 mg of RRR- α -TOH in the resorption-gestation test, i.e. β -TOH should
9 be multiplied by 0.5, γ -TOH by 0.1, and α -T3 by 0.3 [212])

10 These aspects altogether support the definition of α -TOH as the actual molecule that provides
11 vitamin E activity to humans and that one on which nutritional recommendations should
12 focus. Although the debate on nutritional requirements and optimal intake of this vitamin is
13 still open [37], the European Food Safety Authority (EFSA) panel on Dietetic Products,
14 Nutrition, and Allergies recently assessed the intakes in healthy populations with no apparent
15 deficiency for α -tocopherol in the EU and used these data to define the Adequate Intakes
16 (AIs) for this vitamin [213]. These were set at 13 mg/day for men and 11 mg/day for women,
17 and at 6 and 9 mg/day for children aged 1 to 3 years and 3 to 10 years, respectively. A value
18 of 5 mg/day was proposed for infants aged 7–11 months and no evidence for an increased
19 intake was reported for pregnant or lactating women. However, recent studies suggest that
20 vitamin E requirements could increase from 15 to 25 mg/d or more, depending on the intake
21 of polyunsaturated fatty acids (PUFA); consequently, additional needs of vitamin E could be
22 considered at least for some individuals [214] and other adjustments on recommended intakes
23 are needed in case of some diseases (reviewed in [37]).

24 From these data it is obvious that a significant proportion of these AI values can be met with
25 few tablespoons of EVOO rich in vitamin E. Therefore, a further reason for nutritionists to
26 recommend EVOO as a health-promoting food is that this can represent a significant dietary
27 source of α -TOH.

28 29 *Emerging aspects of vitamin E biology and function*

30 If on one hand the hepatic metabolism of vitamin E provides a preferential route for α -TOH
31 to be selected and delivered to tissues, on the other hand it may represent a site of biological
32 activation for this molecule as it is for other fat-soluble vitamins such as vitamin A and D
33 (reviewed in [37]). As discussed above, α -TTP and possibly other binding proteins of liver
34 cells select the quota of α -TOH to be retained in the human organism thus directing the

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3 1 excess of this molecule together with the non-alpha forms to hepatic catabolism and excretion
4 2 [37]. In the last years, the metabolites produced during the hepatic catabolism of vitamin E
5 3 have been identified to regulate gene expression and signaling pathways with biological
6 4 effects that are specific or superior than those produced by the vitamin precursor [215; 216;
7 5 217; 218; 219]. The metabolism of vitamin E is initiated by the enzymatic activity of a
8 6 tocopherol ω -oxidase that produces the long-chain metabolite (LCM) 13'-hydroxy-6-
9 7 hydroxychromanol (13'-OH) (Figure 2) [220; 221]. This early metabolite found at low
10 8 nanomolar levels in human serum [191; 217] is further oxidized by the ω -oxidase activity of
11 9 alcohol or aldehyde dehydrogenase enzymes to the corresponding 13'-COOH derivative that
12 10 is the substrate of the β -oxidation like shortage of the side chain to form the final
13 11 carboxyethylchroman metabolite found in plasma and then of urine and bile as main end-
14 12 products of vitamin E catabolism in animal organisms [195; 222; 223]. Convincing evidence
15 13 was obtained on the identification of a form of the cytochrome P450 family of enzymes as the
16 14 main tocopherol ω -oxidase of liver cells, namely the CYP4F2 isoenzyme [221; 224; 225].
17 15 This isoenzyme may represent a point of convergence between the metabolism of vitamin E
18 16 and that of middle and long-chain fatty acids with roles in the inflammatory signalling of cells
19 17 [226]. These include the PUFA arachidonic acid and the eicosanoid derivatives prostaglandin
20 18 A1 and E1, some lipotoxins, the 5- and 12-hydroxyeicosatetraenoic acid (HETE) forms, and
21 19 particularly leukotriene B4, a potent mediator of inflammation. Indeed CYP4F2 gene encodes
22 20 for the leukotriene B4 ω -oxidase 1 enzyme protein and, together with CYP4A11, it is the
23 21 main 20-HETE-synthesizing enzymes in humans, a bioactivation process of arachidonic acid
24 22 substrate with prominent role in the control of renal processes that regulate ion absorption,
25 23 blood flow, vascularization, and thus blood pressure of rodents and possibly humans
26 24 (reviewed in [227]). According with this hypothesis of a functional convergence between the
27 25 metabolism of vitamin E and that of inflammatory lipids, the supplementation of mononuclear
28 26 leukocytes with vitamin E has been described to control inflammatory pathways that include
29 27 NF- κ B transcription factor and the enzymes iNOS and COX-2, as well as lipoxygenase
30 28 isoenzymes (reviewed in [37; 228] and references therein). Among the products of this
31 29 metabolism, LCMs of α -TOH and γ -TOH, appear to produce regulatory effects on these
32 30 inflammatory processes that are more potent than those promoted by their vitamin precursor
33 31 forms. Effects on these metabolites [229] and to a lower extent of CEHC metabolites [230;
34 32 231] were also reported on genes responsible of cell cycle regulation. More recently LCMs
35 33 have been found to act in a feed-forward mechanism with the dietary fatty acids oleate and
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1 linoleate to control the expression of CYP4F2 gene in human hepatocytes, a regulatory
2 response that is under the transcriptional influence of PPAR γ nuclear receptor [215].
3 Other bioactive metabolites of α -TOH include α -TOH phosphate, the enzymatic product of
4 an unknown cellular kinase that has been identified in tissues and biological fluids [204].
5 The effect of EVOO rich in α -TOH on the circulating level of LCMs in healthy volunteers
6 and fatty liver patients is under investigation (Piroddi et al., unpublished data).
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9. Conclusions and perspectives

Olives and OO are a source of bioactive phytonutrients the additive and synergistic effects of which are reported to exert unique and powerful health-promoting effects in antagonism with risk factors for inflammatory and age-dependent conditions such as cardiovascular disease, tumorigenesis and cancer progression, and the cognitive decline of neurodegenerative diseases. Some of these effects have been confirmed in randomized clinical trial and are now the subject of nutritional and health claims endorsed by the institutional authorities in Europe and US. From a nutritional perspective, VOO is a precious source of the MUFA oleic acid and with the average intake of the traditional Mediterranean diet, products with sustained content of α -tocopherol can provide a significant part of, if not all, the adequate intake for this vitamin. These components of VOO are claimed to help regulating the metabolism and antioxidant protection of lipoprotein particles and cellular membranes, thereby leading to a better control of inflammatory and endothelial risk factors for cardiovascular and cerebrovascular disease such as insulin resistance and an increased generation and progression rate of atherosclerotic lesions. Under the form of EVOO, it provides a mixture of biophenols with biological roles that further expand these effects. Antioxidant, anti-inflammatory and anti-cancer have been among the most investigated ones in vitro, in animal models and even in humans.

Health-promoting and nutritional properties of VOO and its functional molecules are now supported by a first set of data coming from nutrigenomics investigations that evaluated this oil alone or within the Mediterranean diet food pattern. These include few but significant gene transcription, metabolomics and proteomics studies carried out in the last few years. The resulting bunch of data tentatively defined a molecular signature for the positive effects of VOO bioactives; biological targets and mechanisms of action are now better understood and available for experimental validation and even clinical application; molecular and cellular targets identified in the nutrigenomics of VOO biophenols are suggestive of specific effects on disease mechanisms. These include receptors, signaling kinases and transcription factors associated with cellular stress and inflammation, lipoprotein damage and endothelial function and more in general with pathways responsible for cell cycle regulation and metabolism that include mitochondrial function and signaling, ER stress, DNA damage, and the response to growth factors, cytokines and hormones (mainly associated with insulin resistance). Efforts have been made to characterize the molecular effects of the individual components and particularly of phenolic compounds such as tyrosol, HT, oleuropein and ligstroside

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3 1 derivatives, hydrocarbons (as squalene), sterols (as β -sitosterol) carotenoids and
4 2 phospholipids. Olive oil phenolics are the most characterized species and available omics data
5 3 clearly confirm the potential for the bioavailable form of these molecules as homeostatic
6 4 factors of cells of the GI tract, particularly of liver and pancreas, as well as of inflammatory
7 5 and vascular cells at the systemic level. Gene expression data are consistent with a role as
8 6 anti-inflammatory and immunomodulating molecules, and recent evidence clearly
9 7 demonstrates for some of these molecules a potent activity as hormetic agents with effects
10 8 on antioxidant and detoxification genes and thus on the control of the cellular redox. Specific
11 9 responses to VOOPs such as HT, oleuropein and oleocanthal have been observed on cell
12 10 cycle regulation and anticancer genes.

13 11 On this ground, VOO-derived nutraceuticals or functional ingredients for food fortification or
14 12 formulation of nutritional supplements, have been developed with proposed use in
15 13 chemoprevention of chronic inflammation and age-related diseases, particularly cancer and
16 14 CVD. These applications however need more extensive evaluation as far as nutritional and
17 15 health effects concern, and such an important evaluation should be implemented at the
18 16 individual level. The perspective of using the available molecular data to predict and even
19 17 assess the gene response to VOO bioactive in protocols of personalized nutrition and
20 18 medicine is very attractive and worth of further investigation. Omics technology is going to
21 19 make this perspective real in the near future.
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3 **1** **Legends to figures.**
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5 **2**
6 **3** **Figure 1. Structures of main secoiridoid derivatives and phenyl alcohols of VOO. From**
7 **[102].**
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9 **5** **Figure 2. Structures of tocopherols, tocotrienols and their earlier hepatic long-chain**
10 **6** **metabolites.**
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Figure 1. Structures of main secoiridoid derivatives and phenyl alcohols of VOO. From [102].

Figure 2. Structures of tocopherols, tocotrienols and their earlier hepatic long-chain metabolites.

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Conflict of interest statement

The authors have no conflict of interest to declare.

Table 1. Maximum human plasmatic concentration of free and conjugated HT after single dose supplementation

Supplement	Dose	Number of subject	C _{max} (μM) free form	C _{max} (μM) conjugated form	ref
HT in H ₂ O	2.5 mg HT/kg	10	1.1±0.2		[115]
EVOO VOO	30 ml (26.5 mg PC) 30 ml (7.9 mg PC)	13		0.53±0.30 0.86±0.24	[125]
LPC EVOO MPC EVOO HPC EVOO	30 ml (5.7 mg PC) 30 ml (11.5 mg PC) 30 ml (17.2 mg PC)	12		1.81±0.98 5.21±3.19 6.33±2.50	[126]
Olive leaf extract	9.7 mg HT 14.5 mg HT	9		0.96±1.00 0.97±0.64	[127]
Olive leaf extract	250 mg extract	8		2.18±0.65	[128]

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3 1 Table 2. Main plasma antioxidants
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	μM
Uric Acid	200-400
Ascorbic Acid	50-100
α -tocopherol	20-40
Protein thiols	400-500
Low MW thiols	0.1-20

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For Peer Review

Table 3. Human Intervention Studies with OOPs: effects on LDL Oxidation

Supplement	Type of study	Control	n subject	Dose of VOO PC	Effect	LDL PC incorporation	ref
EVOO	Chronic 3 weeks	Sunflower oil	10	na	Mild (not signific) effect on ex vivo LDL oxidation	na	[130]
EVOO (50g/day)	Chronic 4 weeks	Refined oil (no PCs)	14	na	No difference between treatment	No	[112]
Fortified olive oil (47 g)	Acute 0-2 hrs	Placebo	12	31 mg	No difference vs control	na	[131]
EVOO (69 g/day)	Chronic 3 weeks	Phenol-poor EVOO	49	21 mg vs 3 mg	No difference between treatment Fasting blood	na	[132]
EVOO	Chronic 4 weeks	Olive Oil	10 hyperlipidemic		Reduction of ex vivo LDL oxidation	na	[133]
HPC OO (25 ml/day)	Chronic 4 days	LPC OO MPC OO	12	11 mg vs 3 vs 0.2	Reduced oxLDL in plasma (dose dependent effect)	na	[121]
VOO (25 ml/day)	Chronic 3 weeks	Refined oil (no PC) Common oil (medium PC)	30	4 mg	Reduction of ex vivo LDL oxidation Reduced oxLDL in plasma (dose dependent effect)	na	[134]
HPC OO (25 ml/day)	Chronic 3 weeks	LPC OO MPC OO	182	8 mg vs 4 vs 0.1	Reduced oxLDL in plasma (dose dependent effect)	na	[135]
HPC OO (40 ml)	Acute 0-6 hrs	LPC OO MPC OO	12	13 mg vs 6 vs 0.1	Reduced oxLDL in plasma vs LPC OO	Yes	[48]
VOO (25 ml/day)	Chronic 3 weeks	Refined oil (no PC) Common oil (medium PC)	33	21 mg vs 0	Reduction of ex vivo LDL oxidation Reduced oxLDL in plasma (dose dependent effect)	Yes	[136]
HT in H ₂ O (2.5mg/kg)	Acute 0-2 hrs	Time 0	10	2.5mg/kg	No difference respect time 0	Yes but measured 10/20 min after supplementation	[115]
VOO (25 ml/day)	Chronic 3 weeks	Refined oil (no PC)	36	16 mg vs 0	Reduced oxLDL in plasma	Yes as conjugated	[137]
HPC OO (25 ml/day)	Chronic 3 weeks	LPC OO	18	8 mg vs 0	Reduced oxLDL in plasma	na	[65]
HPC OO (25 ml/day)	Chronic 3 weeks	LPC OO	25	8 mg vs 0	Decrease LDL concentration Decrease of number of small LDL Reduction of ex vivo LDL oxidation	na	[138]

na: not analyzed/available

Table 4. miRNAs modified by aging and/or treatment with olive oil phenols in concordance with the changes of the respective target genes.

Aging-modified miRNAs	Treatment-modified miRNAs
mmu-miR-681	mmu-miR-30a-5p*
mmu-miR-709	mmu-miR-484
mmu-miR-706	mmu-miR-434-5p*
mmu-miR-30a-5p*	mmu-miR-369-5p*
mmu-miR-129-5p	mmu-miR-451*
mmu-miR-434-3p	
mmu-miR-380-3p	
mmu-miR-30a-3p	
mmu-miR-434-5p*	
mmu-miR-433-3p	
mmu-miR-451*	
mmu-miR-720	
mmu-miR-126-3p*	
mmu-miR-369-5p*	

C57Bl mice were treated from age 10 to 16 months with an extra-virgin olive oil naturally rich in phenols (n=9), or with an extra-virgin olive oil deprived of phenols (n=9, control group), and miRNA expression was evaluated in the cerebral cortex through microarray. Age-related changes in miRNAs expression were obtained by comparing the control group of aged mice with a group of young animals (age 4 months, n=6). Treatment effects were evaluated comparing the two groups of aged mice. * miRNA present in both the lists.

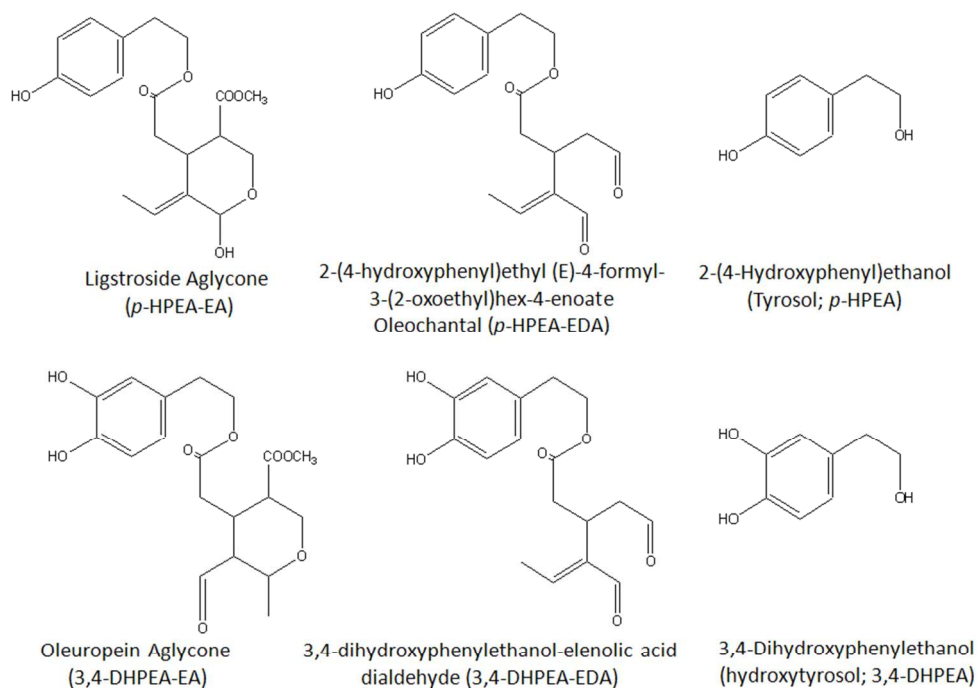


Figure 1

Figure 1. Structures of main secoiridoid derivatives and phenyl alcohols of VOO. From [102].

251x188mm (96 x 96 DPI)

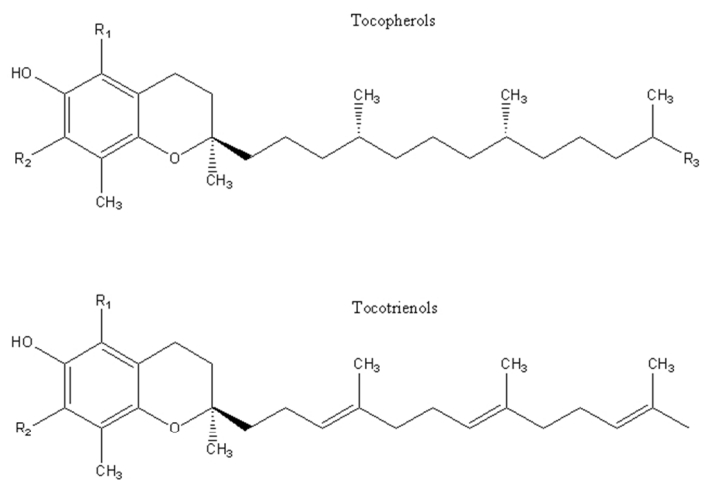


Figure 2.

Vitamin E isomers and metabolites	R ₁	R ₂	R ₃ [*]
α	CH ₃	CH ₃] OH OOH
β	CH ₃	H	
γ	H	CH ₃	
δ	H	H	

* Long-chain metabolites:
13'-Hydroxychromanol
13'-Carboxychromanol

Figure 2. Structures of tocopherols, tocotrienols and their earlier hepatic long-chain metabolites.

244x179mm (96 x 96 DPI)