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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 leafs. 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.

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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

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

2. Virgin Olive Oil bioactives

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Nutrigenomics of VOO.

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

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

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

Molecular and cellular effects of oleic acid (C18:1, n-9) have been extensively characterized as this MUFA is relatively abundant in food and is at the same time a product of the cellular 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 Δ9 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 cardio-prevention 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

of the cellular redox at the interface between the master regulators of transcriptional and metabolic pathways involved in the stress adaption response, such as the Nrf2 transcription factor, and the pentose phosphate pathway (reviewed in [76]). Altogether these effects are crucial to preserve the endocrine and metabolic functions of this organ also including its capability to control the compensatory and regenerative processes associated with the differentiation of adipose stem cells [75]. Obesity can lead to develop pathologic phenotypes in which chronic inflammation can impair these functions of the adipose tissue thus increasing the risk of cardiometabolic events and that of other age-related disorders [77]. Olive oil bioactives are among the dietary phytochemicals that have been described to influence the control of "inflammagenes" [77; 78]. In a recent comparative study among OO polyphenols, Richard et al. [79] described HT as the most effective inhibitor of inflammatory pathways that stimulate the production of NO, the eicosanoid PGE2, and cytokines such as IL-1α, IL-1β, IL-6, IL-12, TNF-α, and the chemokines CXCL10/IP-10, CCL2/MPC-1. Corresponding effects of inhibition were observed in the gene expression of the inducible nitric oxide synthase (iNOS), IL-1α, CXCL10/IP-10, MIP-1β, matrix metalloproteinase-9, and prostaglandin E2 synthase. The inhibition of COX-2 and iNOS genes, responsible for the transcriptional control of TNF-α, was confirmed in other studies in human monocyte-machrophages [80; 81; 82] and in vivo in a model of inflammatory response in rats in which the inhibitory effects of HT on COX-1 and -2 have been reported to have the same potential of the non-steroidal anti-inflammatory drugs ibuprofen and celecoxib [83]. Also the control of NF-κB transcription factor is proposed to play a major role in the anti-inflammatory gene response to HT [84]. Moreover, at nutritionally relevant concentrations, HT was shown to have additive effects with other VOO components such as OA, in preventing gene defects associated with the metabolic defect of inflamed adipocytes, a major event in metabolic syndrome and cardiovascular risk [85]. HT and OA synergize in preventing the downregulative effect of TNF-α on gene expression and secretion of adiponectin, a cardioprotective hormone of the adipose tissue. This effect was caused by this inflammatory cytokine through JNK (stress kinase)-mediated suppression of PPARγ activity.

The activity of HT on stress-activated kinases, such as JNK, and transcription factors, such as NF-κB and PPARγ, also suggests effects of this biophenol on the regulation of cell cycle and apoptotic pathways, which have sure relevance in the chemo-prevention and possibly hormetic role of this compound (Section 4) as well as on anticancer properties described below in Sections 6 and 7. Similarly to oleuropein and other related polyphenols, HT possesses cytotoxic activity and depending on the experimental model it has been described to

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

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

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

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

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

Metabolomics, proteomics and epigenetics of VOO bioactives

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

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

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

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

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

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

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

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

4. Toward a hormetic role of EVOO phenolic compounds

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

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

demonstrated that VOOP metabolites (obtained by chemical synthesis) did not possess any significant antioxidant activity when used at physiological concentration. In particular, the glucuronides of Tyr, OH-Tyr and HVA were not able to inhibit Cu-catalyzed LDL oxidation, while the parent molecule OH-Tyr had strong antioxidant effect. Tuck et al. [109] observed contrasting results, but they used VOOPs metabolites purified from human urines; in particular, they observed that Homovannillic Acid and OH-Tyr-3-O-Glucuronide had a high radical scavenging activity (DPPH test), while the sulfate conjugate of OH-Tyr did not possess any radical scavenging activity. Finally, Deiana demonstrated that low concentration of 3 different OH-Tyr glucuronide (obtained by chemical synthesis) were able to slightly protect renal cells against H_2O_2 induced membrane oxidative damage, but they did not exert any significant protection against H_2O_2 induced cellular injury [110].

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Recently, it has been shown that olive oil phenols were able to modulate both the gene and miRNA expression profiles evaluated by the microarray technique in mice treated from age 10 to 16 months with an EVOO naturally rich in phenols (H-EVOO) and these changes were associated with reduced age-related decline of motor coordination and contextual memory [97]. At the end of the treatment, most of the genes modulated by aging resulted to be down-regulated and restricted to the cerebral cortex. Compared to L-EVOO (the same oil deprived of phenols), the treatment with H-EVOO was instead associated with a significant up-regulation of genes associated with synaptic plasticity and with motor and cognitive behavior, such as Notch1, BMPs, NGFR, GLP1R and CRTC3. The agrin pathway was also significantly modulated.

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

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

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

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

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

beginning of the intervention. These changes can be related to a complex modulation of gene and miRNA expression patterns.

Finally, it is worth mentioning the recent interest in evaluating the effects of exogenous plant-derived miRNAs [140]. Exogenous miRNAs from animal and plant dietary sources can be transported to human blood and tissues through exosomes [141; 142] and the possibility of cross-kingdom gene regulation has been raised. mi-RNAs have been detected with next generation sequencing in olives [143], but up to date the bioavailability of these miRNAs ingested with olives or VOO remains unexplored [144]. Future work is needed to clarify whether part of the beneficial effects of olive oil can also be attributed to such olive-derived miRNAs.

6. Chemoprevention effects of olive oil polyphenols: molecular and cellular mechanisms.

In the last few years, VOOPs have received growing attention because of their healthy properties including the chemopreventive activity. Several processes are essential for cancer development: DNA damage, sustained proliferation and insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, tissue invasion, metastatization and inflammation [145]. Some VOOPs are capable to affect many if not all these processes thus interfering with the carcinogenic process. This chemopreventive activity of VOOPs is the result of specific gene regulation effects some of which are now identified and are described here in this section.

Oxidative DNA damage plays a central role in both the stages of cancer initiation and promotion/progression. The protection effect of different VOOPs against the H2O2-induced DNA damage has been investigated in HL60 human lymphoblasts and in peripheral blood mononuclear cells (PBMC). HT (3,4-DHPEA) significantly reduced the extent of DNA damage at concentrations as low as 1 μM, as evaluated by the single cell gel electrophoresis (SCGE or Comet assay) [146]. Other compounds structurally related to HT showed the same effect on the H2O2-induced DNA damage, but the potency of the different compounds changed. In particular, tyrosol (p-HPEA), a phenol compound lacking of the ortho-hydroxyl group on the phenol ring, had less effect than 3,4-DHPEA [146]. These VOOPs were also able to prevent the oxidative DNA damage induced in human lymphocytes by the co-culture with monocyte-macrophages stimulated with PMA, an ex vivo model that mimics the pathophysiology of oxidative stress of an inflammatory lesion [147]. In this experimental system, tyrosol was more effective than HT in preventing oxidative DNA damage [146].

Similar results were obtained in Jurkat cells exposed to continuously generated H2O2 and treated with phenolic extracts from OO and olive mill waste water as well as with isolated compounds (HT and caffeic acid) [148]. Additional studies have demonstrated that pretreatment of HeLa cells with VOO phenolic extract also prevents DNA damage induced by H2O2 [149]. The effect of VOO was tested also in vivo. Quiles et al. tested the effects of feeding male Wistar rats with diets containing different sources of fat, such as VOO and sunflower oil (SO) [150]. Lower levels of DNA double-strand breaks in peripheral blood lymphocytes were found in young animals fed on VOO, which reached around one half of the damage found in SO treated animals. The same measurements were carried out in aged rats showing that the age-related increase of DNA double-strand breaks was significantly lower in rats fed a diet containing VOO [150]. Another study from the same group showed the higher efficacy of VOO compared to SO in decrease deletions of the mitochondrial genes ND1 and ND4 of rat liver [151]. These results strongly suggest that VOOPs may prevent one of the main steps in the initiation phase of carcinogenesis, i.e. the oxidative lesion of DNA, a process that is responsible for oncogene activation [152].

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

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

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

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

Redox-dependent properties of anticancer VOOPs

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Vitamin E was discovered in 1992 by Evans and Bishop as a factor essential for rat fertility [189]. Nowadays, this term is used to identify a family of eight tocochromanols produced in

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

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

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

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

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

As far as the radical species produced during H-donation reactions of tocopherols concern, tocopheryl radicals and their quinone derivatives, such as tocopheryl quinone (TQ), show different degree of stability and cytotoxicity. Desmethyl quinone derivatives generate much greater toxicity than α-tocopheryl quinone [40; 199]. Such a potentially harmful activity is based on peculiar signaling and gene modulation effects that may help to explain the beneficial roles so far reported for the desmethyl forms of vitamin E [200; 201]. The same type of considerations has been made on the quinone analogues of VOO phenolics [202]. The mitochondrial-dependent pro-apoptotic activity of α-tocotrienol in HER2-neu positive breast cancer cells and the corresponding in vivo anticancer activity are a specific example of these properties [92; 203]. Molecular players of this signaling include among the others, phospholipase A2, PKC isoforms and their corresponding phosphatases, pro-survival and stress-related MAPKs, and the regulatory components of cell cycle (reviewed in [37; 204]). The lower chemoprevention potential of α-TOH suggests a physiological regulatory activity in lipid/phosphorylative signalling of cells which is another potential reason for its preferential selection and bioavailability to tissues. The physiological role of α -TOH in cellular signaling and functional control of biological processes distinct from other forms of vitamin E can also be deduced from the gene response of immune cells. In fact, in vivo a high intake of α-TOH sustains the gene response of stimulated T-cells thus suggesting immune-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 menas 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 μM. Plasma α-TOH is approximately 10 to 20-fold less abundant than α-TOH; δ-TOH shows even lower levels, usually below 0.5 μM.

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

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

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

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

Emerging aspects of vitamin E biology and function

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

excess of this molecule together with the non-alpha forms to hepatic catabolism and excretion [37]. In the last years, the metabolites produced during the hepatic catabolism of vitamin E have been identified to regulate gene expression and signaling pathways with biological effects that are specific or superior than those produced by the vitamin precursor [215; 216; 217; 218; 219]. The metabolism of vitamin E is initiated by the enzymatic activity of a tocopherol ω-oxidase that produces the long-chain metabolite (LCM) 13'-hydroxy-6-hydroxychromanol (13'-OH) (Figure 2) [220; 221]. This early metabolite found at low nanomolar levels in human serum [191; 217] is further oxidized by the ω-oxidase activity of alcohol or aldehyde dehydrogenase enzymes to the corresponding 13'-COOH derivative that is the substrate of the β-oxidation like shortage of the side chain to form the final carboxyethylchroman metabolite found in plasma and then of urine and bile as main end-products of vitamin E catabolism in animal organisms [195; 222; 223]. Convincing evidence was obtained on the identification of a form of the cytochrome P450 family of enzymes as the main tocopherol ω-oxidase of liver cells, namely the CYP4F2 isoenzyme [221; 224; 225]. This isoenzyme may represent a point of convergence between the metabolism of vitamin E and that of middle and long-chain fatty acids with roles in the inflammatory signalling of cells [226]. These include the PUFA arachidonic acid and the eicosanoid derivatives prostaglandin A1 and E1, some lipotoxins, the 5- and 12-hydroxyeicosatetraenoic acid (HETE) forms, and particularly leukotriene B4, a potent mediator of inflammation. Indeed CYP4F2 gene encodes for the leukotriene B4 ω-oxidase 1 enzyme protein and, together with CYP4A11, it is the main 20-HETE-synthesizing enzymes in humans, a bioactivation process of arachidonic acid substrate with prominent role in the control of renal processess that regulate ion absorption, blood flow, vascularization, and thus blood pressure of rodents and possibly humans (reviewed in [227]). According with this hypothesis of a functional convergence between the metabolism of vitamin E and that of inflammatory lipids, the supplementation of mononuclear leukocytes with vitamin E has been described to control inflammatory pathways that include NF□B transcription factor and the enzymes iNOS and COX-2, as well as lipoxygenase isoenzymes (reviewed in [37; 228] and references therein). Among the products of this metabolism, LCMs of α-TOH and γ-TOH, appear to produce regulatory effects on these inflammatory processes that are more potent than those promoted by their vitamin precursor forms. Effects on these metabolites [229] and to a lower extent of CEHC metabolites [230; 231] were also reported on genes responsible of cell cycle regulation. More recently LCMs have been found to act in a feed-forward mechanism with the dietary fatty acids oleate and

linoleate to control the expression of CYP4F2 gene in human hepatocytes, a regulatory response that is under the transcriptional influence of PPARγ nuclear receptor [215].

Other bioactive metabolites of α-TOH include α-TOH phosphate, the enzymatic product of an unknown cellular kinase that has been identified in tissues and biological fluids [204].

The effect of EVOO rich in α-TOH on the circulating level of LCMs in healthy volunteers and fatty liver patients is under investigation (Piroddi et al., unpublished data).

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

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

On this ground, VOO-derived nutraceuticals or functional ingredients for food fortification or formulation of nutritional supplements,have been developed with proposed use in chemoprevention of chronic inflammation and age-related diseases, particularly cancer and CVD. These applications however need more extensive evaluation as far as nutritional and health effects concern, and such an important evaluation should be implemented at the individual level. The perspective of using the available molecular data to predict and even assess the gene response to VOO bioactive in protocols of personalized nutrition and medicine is very attractive and worth of further investigation. Omics technology is going to make this perspective real in the near future.

Legends to figures.

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	Cmax (μM) free form	Cmax (μM) conjugated form	ref
HT in H2O	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]

Table 2. Main plasma antioxidants

	μM
Uric Acid	200-400
Ascorbic Acid	50-100
α-tocopherol	20-40
Protein thiols	400-500
Low MW thiols	0.1-20

From: [129]

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 H2O (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-129-5p	mmu-miR-369-5p* 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.

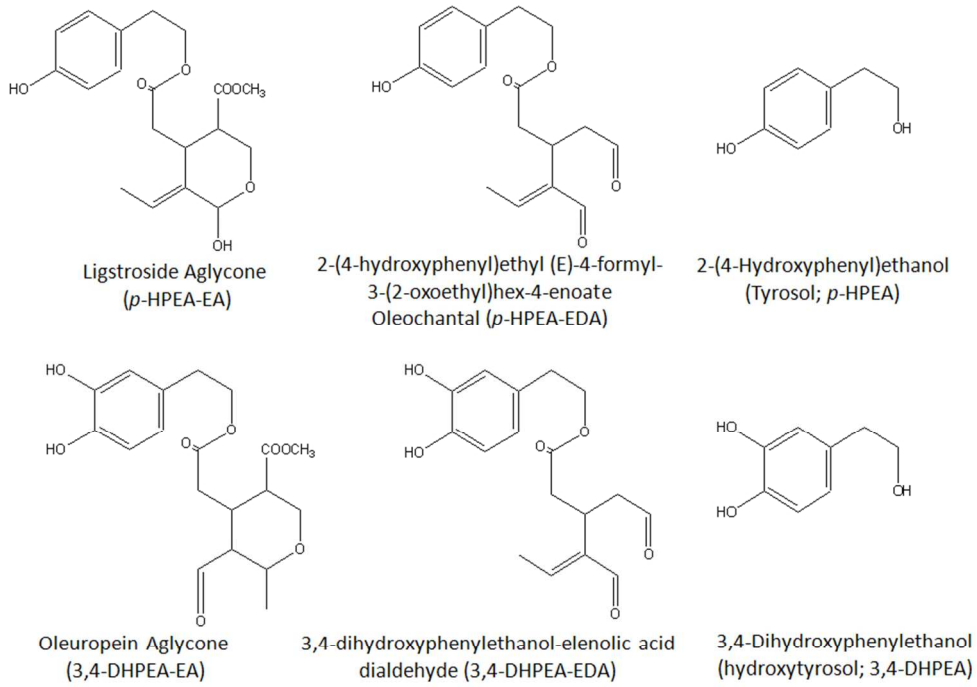
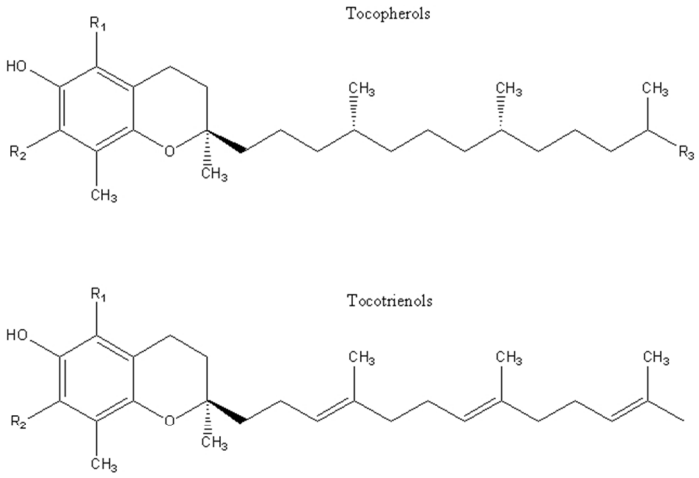


Figure 1

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

251x188mm (96 x 96 DPI)



Vitamin E isomers and metabolites	R_1	R_2	R_3*
α	CH_3	CH_3	OH
β	CH_3	H	OH
γ	H	CH_3	OOH
δ	H	H	OOH

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

Figure 2.

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

244x179mm (96 x 96 DPI)