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Minor Components of Olive Oil: Evidence to Date of Health Benefits in Humans

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Olive oil is a functional food, which in addition to a high level of monounsaturated fatty acids also contains multiple minor components with biological properties. A large number of studies, mainly experimental, have been carried out on some of these components. However, the precepts of evidence-based medicine require adequate scientific evidence (level I or II) to be provided before nutritional recommendations for the general public can be formulated. In this review, we summarize the state of the art of the body of knowledge and discuss the extent to which there exists evidence for the health benefits of the minor components of olive oil.

Key words: LDL oxidation, minor components, olive oil, oxidative damage, phenolic compounds

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INTRODUCTION

Olive oil is a functional food, which in addition to having a high level of monounsaturated fatty acids (MUFA), also contains multiple minor components with biological properties. So far, most of the cardioprotective effects of olive oil in the context of the Mediterranean diet have been attributed to its high MUFA content. It is important, however, to emphasize that oleic acid is also one of the predominant fatty acids in widely consumed animal foods in Western diets, such as poultry and pork.[1] Meat intake was positively related to the level of oleic acid in plasma phospholipids in a female population in Malmö, Sweden.[2] In this population, plasma levels of oleic acid were higher than those of a female population in Granada, Spain, but there were no differences in levels of polyunsaturated fatty acids (PUFA).[2] It is thus plausible that a high oleic acid intake is not the primary agent responsible for the healthful properties of olive oil.

The content of the minor components of an olive oil varies depending on the cultivar, climate, ripeness of the olives at harvesting, and the processing system employed. Different processing methods produce virgin, ordinary, or pomace olive oil.[3] Virgin olive oil is produced by direct pressing or centrifugation of the olives. Virgin olive oils with an acidity greater than 3.0 degrees are submitted to a refining process in which some components, mainly phenolic compounds and to a lesser degree squalene, are lost.[4] By mixing virgin and refined olive oil, an ordinary olive oil (UE 1991) is produced and marketed. After virgin olive oil production, the rest of the olive drupe and seed is processed and submitted to a refining process, resulting in pomace olive oil, to which a certain quantity of virgin olive oil is added before marketing.

The minor components of virgin olive oil are classified into two types: the unsaponificable fraction, defined as the fraction extracted with solvents after the saponification of the oil, and the soluble fraction, which

includes the phenolic compounds. A large number of studies, mainly experimental models, have been performed on certain minor components of the olive oil. However, the precepts of evidence-based medicine require high-level scientific evidence to be provided before nutritional recommendations for the general public can be formulated. The scientific evidence required is provided by randomized, controlled, double-blind clinical trials (level I evidence), and to some extent by large cohort studies (level II evidence). Basic research, despite its usefulness in permitting a mechanistic approach to be adopted, does not provide evidence for nutritional recommendations. Of course, the level of evidence of a particular study depends not only on its design, but also on its quality (external and internal validity, homogeneity of the sample, and statistical power). Finally, evidence is built by the agreement of the results of several similar studies.[5,6] In this review, we summarize the state of the art of the body of knowledge and the extent to which we possess evidence of the health benefits of olive oil minor components.

UNSAPONIFIABLE MINOR COMPONENTS

The unsaponifiable fraction of virgin olive oil is rich in minor components that have antioxidant and anti-inflammatory properties. Incubation of endothelial cells with triacylglycerol-rich proteins enriched with the unsaponifiable minor components of olive oil reduces the release of proinflammatory and prothrombotic factors from the cells.[7] Components of the unsaponifiable fraction of olive oil in order of their increasing polarity are: hydrocarbons, tocopherols, fatty alcohols, triterpenic alcohols, 4-methylsterols, sterols, other terpenic compounds, and polar pigments (chlorophylls and pheophytins). The major component of the unsaponifiable fraction is the hydrocarbon squalene, a polyunsaturated triterpene formed by the condensation of six units of isoprene.

Squalene is a precursor in the biosynthesis of cholesterol and all of the steroid hormones. Compared with other vegetable oils, squalene appears in elevated proportions in olive oil (around 400 mg/kg).[4] It is an inhibitor of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase and increases the activity of the acyl coenzyme A cholesterol acyltransferase. It has been suggested that the former activity, by reducing farnesyl pyrophosphate availability for "prenylation" of the ras oncogene, is responsible for the tumor-inhibitory activity of squalene observed in animal models.[8,9] In experimental studies, squalene has also acted as a free radical scavenger by reducing lipid peroxidation in the retina.[10] Due to olive oil consumption, the intake of squalene in Mediterranean countries is 10 times higher than that in northern European countries or the United States.[9] As a working hypothesis, it has been suggested that the high squalene content of olive oil is one of the protective factors that might explain the low incidence of certain cancers in Mediterranean populations.[8] However, no attempts have been made to establish a direct relationship between squalene consumption and the incidence of cancers.

The levels of tocopherol (100–250 mg/kg of olive oil, mainly as α-tocopherol) and carotenoids (0.5–10 mg/kg) present in "real-life" daily consumption of olive oil are far below what are regarded as effective in clinical studies.[11] Their ingestion through olive oil, however, does contribute to the total pool of vitamins and antioxidants in the body. The biological implications of fatty alcohols and methyl sterols are not known at present. Sterols are bile acid sequestrants and acyl coenzyme A cholesterol acyltransferase inhibitors. Olive oil is rich in sterols. Pomace olive oil has a higher sterol content (up to 2600 mg/kg) than virgin olive oil (up to 1600 mg/kg). The consumption of sterol-rich food leads to lower levels of plasma cholesterol,[12] and has been shown to reduce the bioavailability of α-tocopherol and β-carotene in normocholesterolemic individuals.[13] On the other hand, in some[14] cross-sectional studies, but not in all,[15] high concentrations of plasma sterol were associated with a personal or family history of coronary heart disease. Thus, there are contradictory data, which will have to be resolved, on the protective role of plant sterols on coronary heart disease.

The four most abundant simple triterpenes in olive oil are oleanolic and maslinic acids and erythrodiol and uvaol alcohols. Because triterpenes are concentrated mainly in the skin of fruits, their content in pomace olive oil is about 10 times higher than in other types of olive oils.[16] In experimental studies and animal models, olive oil triterpenes have displayed antiinflammatory,[17] antioxidant,[18] cardiotonic, antidysrhythmic, and vasodilatory activity.[19,20] Further studies are required to determine their beneficial effect in humans following olive oil consumption.

SOLUBLE MINOR COMPONENTS

Phenolic compounds from olive oils have been the subject of great interest in recent years. The major phenolic compounds in olive oil are: 1) simple phenols (e.g, hydroxytyrosol, tyrosol, vanillic acid); 2) secoiridoids (e.g., oleuropein glucoside), SIDs, which are the dialdehydic form of oleuropein (SID-1) and ligstroside (SID-2) lacking a carboxymethyl group, and the aglycone form of oleuropein glucoside (SID-3) and ligstroside (SID-4); and 3) polyphenols, which are lignans (e.g., (+)-pinoresinol and (+)-1-acetoxypinoresinol) and fla-

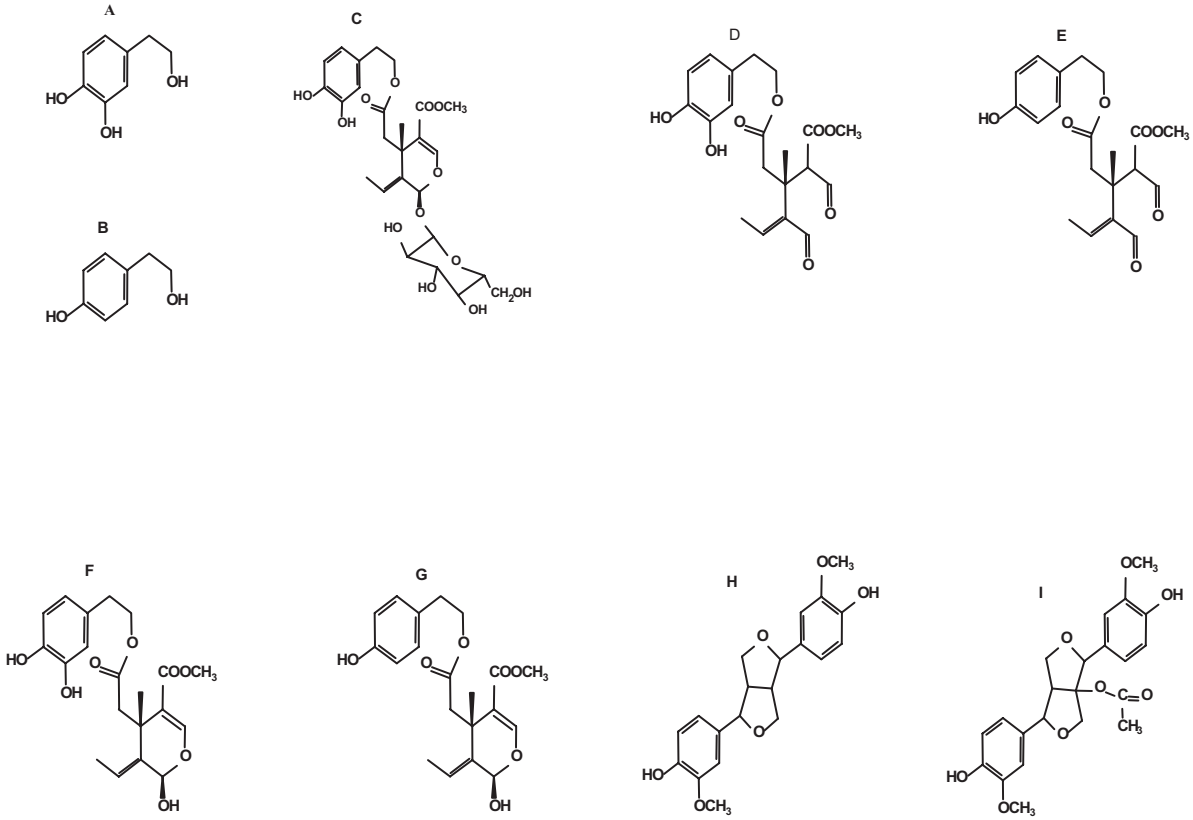

Figure 1. Structures of the major phenolic compounds identified in olives and olive oil. A, Hydroxytyrosol; B, tyrosol; C, oleuropein glucoside; D, SID-1; E, SID-2; F, SID-3; G, SID-4; H, (+)-pinoresinol; I, (+)-1-acetoxypinoresinol.

vonols (Figure 1). Tyrosol, hydroxytyrosol, and their secoiridoid derivatives make up around 90% of the total phenolic content of virgin olive oil.[21] About 80% or more of the phenolic compounds of olive oil are lost in the refining process. Their content is thus higher in virgin olive oil (around 230 mg/kg, common range 130–350 mg/kg) than in other types of olive oil.[4]

In experimental studies, olive oil phenols have been shown to: 1) have antioxidant effects, greater than those of vitamin E, on lipids and DNA oxidation[3, 22-25]; 2) prevent endothelial dysfunction by decreasing the expression of cell adhesion molecules,[26] increasing nitric oxide (NO) production and inducible NO synthesis[27] and quenching vascular endothelium intracellular free radicals[28]; 3) inhibit platelet-induced aggregation[29]; and 4) enhance the mRNA transcription of the antioxidant enzyme glutathione peroxidase (GSH-Px). Controversial results, however, have been obtained on this last issue depending on the tissue in which the gene expression was evaluated.[23,30] Recently, an ibuprofen-like activity has been described for oleocanthal, a ligstroside aglycone present in olive oil.[31] Other potential activities of olive oil phenolic compounds include chemopreventive activity.[25] In animal models, olive oil phenolics retained their antioxidant properties in vivo[32] and delayed the progression of atherosclerosis.[33]

BIOAVAILABILITY AND DISPOSAL OF OLIVE OIL PHENOLIC COMPOUNDS IN HUMANS

It has been suggested that non-absorbable phenolic compounds may display local antioxidant activities in the gastrointestinal tract.[34] This idea is supported by the capacity of isolated phenolic compounds to scavenge both the free radicals generated by the fecal matrix[25] and those induced in epithelial cells of the intestine.[35] However, one of the prerequisites for assessing the physiological significance of olive oil phenolic compounds in human beings is the ability to determine their bioavailability. Tyrosol and hydroxytyrosol are absorbed by humans in a dose-dependent manner with the phenolic content of the olive oil administered.[36] Even from moderate doses (25 mL/d), which are lower than the traditional daily dietary intake in Mediterranean countries,[37,38] around 98% of these phenolics are present in plasma and urine in conjugated forms, mainly glucuronoconjugates, suggesting the existence of an extensive first-pass intestinal/hepatic metabolism of the ingested primary forms.[39] The biological activity of olive oil phenolics must therefore derive from their metabolites. In fact, the 3-O-glucuronide of hydroxytyrosol shows stronger activity as a radical scavenger than hydroxytyrosol itself.[40] Sources of hydroxytyrosol from olive oil

are its free form (about 10% of the dose[41]), its glucoside,[42] and oleuropein. Oleuropein is absorbed, metabolized in the body, and recovered in urine, mainly in the form of hydroxytyrosol.[43]

A major unresolved drawback in the evaluation of the disposition of hydroxytyrosol is the fact that after strict dietary control, as well as after hours of fasting, it is not possible to minimize hydroxytyrosol concentration in biological fluids. One explanation could be that hydroxytyrosol is also known as DOPET (3,4- dihydroxyphenylethanol), a well-known metabolite of dopamine. In fact, homovanillic acid, one of the main metabolites of dopamine, has also been reported as a major metabolite of hydroxytyrosol.[44] Urinary concentrations of tyrosol are dependent on the administered tyrosol dose, whereas urinary concentrations of hydroxytyrosol tend to accumulate. The previously discussed interrelationship between hydroxytyrosol and dopamine may be a confounding factor in the interpretation of analytical results. For this reason, tyrosol may well be a better biomarker of sustained doses of virgin olive oil consumption for clinical studies.[45]

Dietary phenolic compounds can bind low-density lipoproteins (LDL).[46] The susceptibility of LDL to oxidation depends not only on its fatty content, but also on its LDL antioxidant content (e.g., vitamin E and phenolic compounds).[47] Phenolic compounds that can bind LDL are likely to perform their peroxyl-scavenging activity in the arterial intima, where full LDL oxidation occurs in microdomains sequestered from the richness of antioxidants present in plasma.[48] Tyrosol has been shown to bind human LDL ex vivo (Figure 2). When isolated LDL or plasma were incubated with virgin olive oil phenolic extracts, an increase of the phenolic compounds previously bound to LDL was observed. This increase was directly related to an increase of the LDL resistance to oxidation.[49] Consumption of olive oil rich in phenolic compounds for 1 week led to an increase in the total phenolic content of LDL in human subjects.[50] The fact that phenolic compounds from olive oil can protect the phenolic content of human LDL reinforces their role as antioxidants in vivo.

ANTIOXIDANT EFFECT OF OLIVE OIL PHENOLIC COMPOUNDS IN HUMANS

Postprandial lipemia is recognized as a risk factor for atherosclerosis development because it is associated with oxidative changes.[51] Several studies have examined the antioxidant effect of phenolic compounds from olive oil after a single dose in humans. In some studies, no changes, either in the ex vivo susceptibility of LDL to oxidation[52-54] or in the in vivo measurements of LDL and DNA oxidation,[55] were observed in the postprandial state after the ingestion of olive oils providing from 0 to 100 mg of phenolic compounds. In these studies, the ingestion of 50 mL of virgin olive oil enhanced total plasma antioxidant capacity,[54] while the ingestion of 25 mL of low-phenolic-compound olive oil reduced the activity of GSH-Px.[55] Visioli et al.[56] described a decrease in F_2 isoprostanes after a 50-mL dose of olive oils enriched with tyrosol and hydroxytyrosol that provided from 24 to 98 mg of phenolic compounds. After sustained consumption (25 mL/d for 4 days), a decrease in

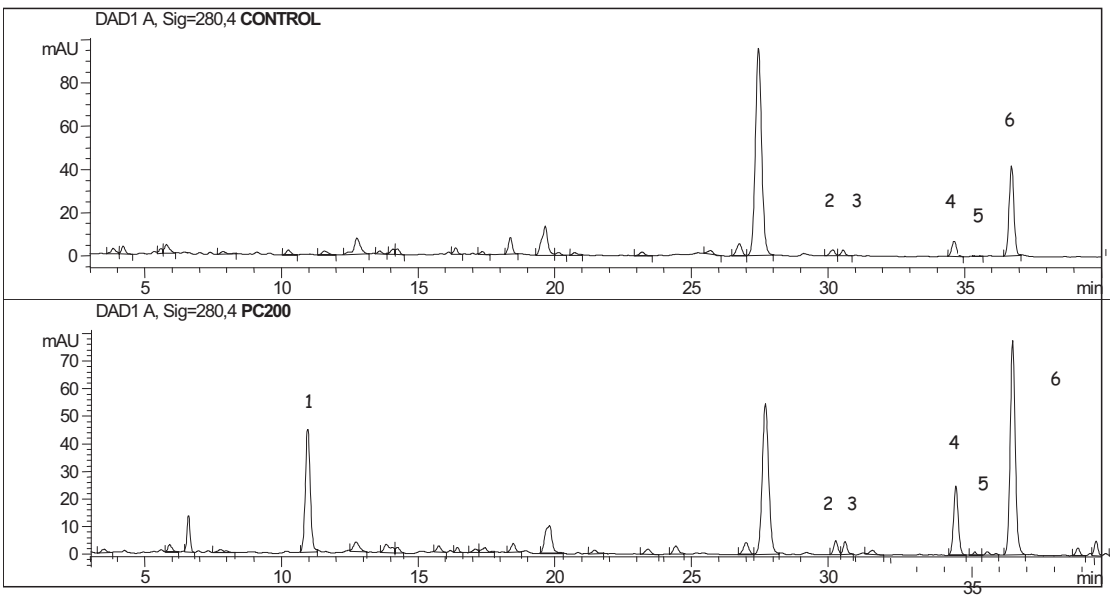

Figure 2. Phenolic compounds in low-density lipoprotein (LDL) after plasma incubation with virgin olive oil phenolics at concentrations of 0 mg/L (Control) and 200 mg/L (PC 200). Peak numbers: 1, tyrosol; 3, flavonoid derivative; 2, 4, 5, and 6, phenolics with flavonoid-like spectra.

postprandial levels of circulating oxidized LDL and DNA oxidation was reported after the ingestion of a single 25-mL dose of olive oil providing 10.4 mg of phenolic compounds. This effect was not observed after the same dose of olive oil with a lower phenolic content.[38] The results of postprandial studies are difficult to evaluate and compare because some studies do not mention whether postprandial lipemia and/or hyperglycemia occur,[52,56] while in other studies neither hyperlipemia nor hyperglycemia occur in the postprandial state after olive oil ingestion.[38,54-56]

Several randomized, crossover, controlled human studies have been performed, and these are potentially capable of providing the first level of evidence on the in vivo antioxidant effect of sustained doses of phenolic compounds from olive oil (Table 1). In four studies of healthy volunteers, there was no evidence that the consumption of phenols in the amounts provided by dietary olive oil accounted for benefits on the ex vivo susceptibility of LDL to oxidation.[53,54,57,58] In two of those studies, in vivo biomarkers such as plasma malondialdehyde, lipid hydroperoxides, and protein carbonyls were also evaluated without any effect being identified that could be attributed to the phenolic content of the olive oil.[57,58]

In recent years, there have been two similar studies in healthy volunteers.[37,38] The protective effects of olive oil phenols on in vivo circulating oxidized LDL, malondialdehyde in urine, DNA oxidation, plasma GSH-Px (Table 1), and HDL cholesterol levels (Table 3) were found in male subjects. No changes in F2-isoprostanes were observed. Subjects were subjected to a strict very low-antioxidant diet in washout and intervention periods or to a controlled diet in order to avoid high antioxidant consumption. Low-phenolic olive oil was used for cooking purposes during intervention periods and for raw and cooking purposes during washout periods. This permitted the homogenization of both the main fat ingestion of participants and the LDL fatty acid content.[37,38]

There have been several randomized, crossover studies of patients in whom an enhanced oxidative stress status was reported.[59-61] Protective effects on the resistance of LDL to oxidation were found in one study of peripheral vascular disease patients.[62] In mildly hyperlipidemic patients, an increase in total antioxidant capacity directly related to the phenolics from the olive oil consumed but without changes in plasma F_2-isoprostanes has been recently reported.[63] In another recent study, protective effects related to the phenolic content of the olive oil on circulating oxidized LDL and lipid peroxides in coronary heart disease patients were found (Table 2).[64]

There are extensive differences among these studies (Tables 1 and 2). These include differences in the experimental design, control of diet, sample population, age of the participants (from 18 $\pm$ 0.2 years[54] to 57 $\pm$ 20 years[37]), measurement or not of markers of the compliance of the intervention, and in the sensitivity and specificity of the oxidative stress biomarkers evaluated. It appears, however, in spite of the differences among these studies, that the protective effects on oxidative stress of olive oil phenolics in humans are more likely to be displayed under oxidative stress conditions; that is, in males rather than females, in elderly people, in males subjected to a very strict antioxidant diet, and in hyperlipidemic, peripheral vascular disease, or coronary heart disease patients.

A review of the effects of intervention with antioxidants and nutrients in relation to oxidative DNA damage and repair concluded that only studies involving male subjects showed consistent antioxidant effects in terms of reduced levels of oxidized pyrimidines.[65] This can be explained by the fact that the balance of pro-oxidant and antioxidant reactions is well regulated in the body. For this reason, an intervention with an antioxidant-rich compound without any oxidative stress involved may exert only a marginal effect that could not be detected with the current state-of-the-art of the oxidative biomarkers. Moreover, as a general rule, the markers most sensitive to olive oil phenolic ingestion were those directly associated with LDL lipoprotein rather than "whole-body" measurements (i.e., circulating oxidized LDL versus F_2-isoprostanes). The fact that ingesting olive oil phenolics promotes an increase in LDL phenolic content, as mentioned above, could account for this. In addition to olive oil phenolic antioxidant activity, the combined protective effect of both the phenolic and the MUFA content, with which LDL is enriched after virgin olive oil ingestion,[51] must not be ignored.

ANTITHROMBOTIC AND ANTIHYPERTENSIVE EFFECT OF OLIVE OIL PHENOLIC COMPOUNDS

Few human studies have been performed to assess the in vivo antithrombotic potential of olive oil phenolic compounds (Table 3). The administration of pure hydroxytyrosol to human volunteers lowered thromboxane B_2 (TXB_2) production in a time-dependent manner.[66] Two recently published studies in individuals with enhanced oxidative stress support the in vivo antithrombotic activity of olive oil phenolic compounds in humans. The administration of virgin olive oil providing 6.6 mg/d of hydroxytyrosol for 7 weeks to mildly hyperlipemic individuals decreased serum TXB_2 production compared with refined olive oil administration.[63] In diabetic patients, a 46% decrease in serum TXB_2 production was observed after 4 days of consumption of olive mill waste that provided 12.5 mg/d (25 mg/d on day 1) of hydroxytyrosol.[67]

Table 1. Antioxidant Effect of Olive Oil Phenolic Compounds in Randomized, Crossover, Controlled Studies in Healthy Volunteers

Subjects (n) (sex)	Intervention	Intervention Period	Washout Period	Baseline Adjustment	Compliance Biomarkers	Oxidative Markers	Effects	Reference
10 (men)	Virgin olive oil vs oleic acid-rich sunflower oil[a]	3 weeks	1 week with usual diet	No	No	Ex vivo LDL resistance to oxidation	Decrease of Dienes	Nicolaiew et al.[53] (1998)
14 (10 women and 4 men)	Virgin vs refined olive oil (50 g/d)	4 weeks[b]	4 weeks[c]	No	No	Ex vivo LDL resistance to oxidation	None	Bonanome et al.[54] (2000)
46 (31 women and 15 men)	High-phenol vs low-phenol olive oil (69 g/d) (sauces and baked products)	3 weeks	2 weeks without olives and olive oil	No	No	Ex vivo LDL resistance to oxidation MDA, FRAP Lipid peroxides Protein carbonyl	None (all markers)	Vissers et al.[57] (2001)
25 (14 women and 11 men)	High-phenol vs low-phenol olive oil (70 g/d, raw)	3 weeks	2 weeks without olives and olive oil	No	No	Ex vivo LDL resistance to oxidation MDA, FRAP Lipid peroxides Protein carbonyl	None (all markers)	Moschandreas et al.[58] (2002)
30 (men)	Virgin vs common vs refined olive oil (25 mL/d, raw)	3 weeks with refined olive oil for cooking	2 weeks with refined olive oil for raw and cooking purposes	Yes	Yes	Ex vivo LDL resistance to oxidation Circulating oxidized LDL	Increase with olive oil phenol content Decrease with olive oil phenol content	Marrugat et al.[37] (2004)
						Antibodies against oxidized LDL	None	
12 (men) refined olive	High vs refined olive oil; medium vs low phenol olive oil (25 mL/d, raw)	4 days with oil for cooking, and very low-antioxidant diet	10 days with for raw and cooking purposes; very low-antioxidant diet	Yes	Yes	Circulating oxidized LDL MDA in urine 8-oxo-dG in urine and lymphocytes	Decrease with olive oil phenol content (all markers)	Weinbrenner et al.[38] (2004)
						F_2-isoprostanes	None	
						Glutathione peroxidase	Increase with olive oil phenol content	

[a]Added to meals, quantity not defined. Only percentage of MUFA (21%) in diet available. [b]Characteristics of the washout period not defined. MDA, malondialdehyde; FRAP, Ferric reducing ability of plasma; 8-xo-dG, 8-oxo-deoxyguanosine in urine.

Table 2. Antioxidant Effect of Olive Oil Phenolic Compounds in Randomized, Crossover, Controlled Studies in Patients

Subjects (n, sex)	Intervention	Intervention Period	Washout Period	Baseline Adjustment	Compliance Markers	Oxidative Markers	Effects	Reference
Peripheral vascular disease patients (24 men)	Virgin vs refined for all purposes	3 months usual diet	3 months	No	No	Lipid peroxides in LDL Macrophage plasma oxidized LDL uptake	Decrease with olive oil phenol content (all markers)	Ramírez-Tortosa et al.[62] (1999)
Hyperlipemic Patients (22) (12 men and 10 women)	Virgin vs refined (raw) (40 mL/day)	7 weeks usual diet	4 weeks with usual diet	Yes	No	Plasma total antioxidant capacity	Increase with olive oil phenol content	Visioli et al.[63] (2005)
Coronary heart disease patients (40 men)	Virgin vs Refined (raw) (50 mL/day) cooking purposes,	3 weeks with refined olive oil for cooking	2 weeks with refined olive oil for raw and	Yes	Yes	Circulating oxidized LDL Lipid peroxides (all markers) GSH-Px	Decrease with olive oil phenol content Increase with olive oil phenol content	Fitó et al.[64] (2005)

Olive oil consumption is associated with low blood pressure and has been shown to reduce the need for antihypertensive treatment in hypertensive patients.[68,69] Only two studies (Table 2) on the antihypertensive effect of olive oil minor components in humans have been identified. Ruiz-Gutiérrez et al.[70] compared the effects of two similar MUFA-rich diets (virgin olive oil and high-oleic sunflower oil) in hypertensive women. These authors reported that only the virgin olive oil-rich-diet induced a significant reduction in both systolic and diastolic blood pressure. This suggests a role for the minor components of olive oil on blood pressure levels. Fitó et al.[64] recently reported a decrease in systolic blood pressure after a virgin olive oil versus refined olive oil intervention in hypertensive, stable coronary heart disease patients, especially in those with systolic blood pressures above 140 mmHg at the beginning of the study.[64]

FUTURE DIRECTIONS

Minor components of olive oil show properties that can account for benefits in human health. For some components of the unsaponificable fraction, there is a lack of human studies that might provide evidence of the benefits resulting from olive oil consumption. Despite their cholesterol-lowering properties, the protective role of sterols on the development of coronary heart disease remains to be elucidated. Further clinical studies with individuals who are prone to oxidative stress or large sample-size studies in healthy individuals are required to determine the conditions under which ingestion of phenolic compounds from olive oil can provide the greatest benefits. The evidence provided by in vivo human studies of the antithrombotic and antihypertensive properties of the phenolic compounds in olive oil is promising, and further randomized, controlled trials are required to strengthen the evidence. Possible benefits on the lipid cardiovascular risk profile also deserve further attention.

An overview of the flaws and highlights of the studies performed to date permits us to underline some key characteristics of nutritional intervention studies on the healthy effects of the minor components of olive oil:

1. There must be satisfactory dietary control of washout and intervention periods, primarily of the type of fat ingested for raw and cooking purposes. The type of fat ingested influences the oxidative damage to lipids.[71] Differences among participants in the fat ingested for raw and cooking purposes during washout periods, and for cooking purposes during intervention periods, can be an important confounder in the assessment of the effects of the phenolic compounds of olive oil.
2. Adjustment of the endpoint values of the biomarkers

Table 3. Healthy Effects of Olive Oil Phenolic Compounds in Randomized, Cross-Over, Controlled Studies

Subjects (n, sex)	Intervention	Intervention Period	Washout Period	Baseline Adjustment	Compliance Biomarkers	Biomarker	Effects	Reference
Hypertensive (16 women)	Virgin vs high oleic sunflower oil	4 weeks	4 weeks usual diet	No	No	Systolic and Diastolic blood	Decrease with olive oil phenol content	Ruiz-Gutierrez et al.[70] (1996)
Hypertensive CHD patients (19 men)	Virgin vs Refined (raw) (50 mL/day)	3 weeks refined olive oil for cooking	10 days with refined olive oil for raw and cooking purposes	Yes	Yes	Systolic blood pressure	Decrease with olive oil phenol content	Fitó et al.[64] (2005)
Hyperlipemic Patients (22) (12 men and 10 women)	Virgin vs refined (raw) (40 mL/day)	7 weeks usual diet	4 weeks with usual diet	Yes	No	Serum TXB_2	Decrease with phenol content of olive oil	Visioli et al.[63] (2005)
Healthy volunteers (30 men)	Virgin vs Common vs Refined (raw) (25 mL/day)	3 weeks with refined olive oil for cooking	2 weeks with refined olive oil for raw and cooking purposes	Yes	Yes	HDL cholesterol	Increase after virgin olive oil	Marrugat et al.[37] (2004)
Healthy volunteers (12 men)	Virgin vs Common vs Refined (raw) (25 mL/day)	4 days with refined olive oil for cooking, very low-antioxidant diet	10 days with refined olive oil for raw and cooking purposes, very low-antioxidant	Yes	Yes	HDL cholesterol	Increase with olive oil phenol content	Weinbrenner et al.[38] (2004)

CHD, coronary heart disease; TXB_2, thromboxane B_2

for the baseline of each intervention period is necessary. Oxidative stress is a short-term response to several stimuli and influences the steady-state balance.[72] The biological variability of oxidative stress markers is high.[73] For this reason, comparison of the endpoint values for each intervention period with values obtained at the beginning of the study offers a long time span for interference with other confounding variables.

3. The measurements in plasma and/or urine of the phenolic compounds of olive oil, such as tyrosol and hydroxytyrosol, as compliance markers of the intervention are essential. In olive oil studies, some participants may identify the olive oil with either a low or very high content of phenolic compounds by their color and taste and, not liking them, may fail to observe full compliance with the scheduled protocol. The determination of compliance markers also permits the exclusion of noncompliant participants.
4. Biomarkers for secondary endpoints for risk of disease (i.e., oxidative damage) must be selected on the basis of their sensitivity and clinical significance. The sensitivity and specificity of some tests and ex vivo measurements for lipid and LDL oxidation are unknown.[73] In other cases, the molecules tested as biomarkers can be directly provided by food.[73] Concerning the clinical significance of the current biomarkers for oxidative damage, high levels of circulating oxidized LDL and F2-isoprostanes and low levels of GSH-Px have been shown to be predictors of cardiac events in coronary heart disease patients in several cohort and case-control studies.[74-76]

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