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New perspectives on bioactivity of olive oil: evidence from animal models, human interventions and the use of urinary proteomic biomarkers

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Olive oil (OO) is the primary source of fat in the Mediterranean diet and has been associated with longevity and a lower incidence of chronic diseases, particularly CHD. Cardioprotective effects of OO consumption have been widely related with improved lipoprotein profile, endothelial function and inflammation, linked to health claims of oleic acid and phenolic content of OO. With CVD being a leading cause of death worldwide, a review of the potential mechanisms underpinning the impact of OO in the prevention of disease is warranted. The current body of evidence relies on mechanistic studies involving animal and cell-based models, epidemiological studies of OO intake and risk factor, small- and large-scale human interventions, and the emerging use of novel biomarker techniques associated with disease risk. Although model systems are important for mechanistic research nutrition, methodologies and experimental designs with strong translational value are still lacking. The present review critically appraises the available evidence to date, with particular focus on emerging novel biomarkers for disease risk assessment. New perspectives on OO research are outlined, especially those with scope to clarify key mechanisms by which OO consumption exerts health benefits. The use of urinary proteomic biomarkers, as highly specific disease biomarkers, is highlighted towards a higher translational approach involving OO in nutritional recommendations.

Olive oil: Phenolics: Coronary artery disease: Inflammation: Proteomic biomarkers

Relevance of the Mediterranean diet and olive oil to health

The olive tree, *Olea europaea* L., is one of the oldest agricultural tree crops and provides diversified products for human consumption such as table olives and olive oil (OO)[1]. The analytical parameters to ascertain OO quality and classify OO are defined by European Union regulations[2]. Oils obtained only by mechanical extraction are virgin olive oils (VOO) and further quality assessment can lead to a classification as extra virgin olive oil (EVOO)[3].

OO is the primary source of fat in the Mediterranean diet and has been associated with longevity and a lower incidence of chronic diseases, particularly CHD[4–7]. OO consumption is also associated with decreased rates

Abbreviations: CAD, coronary artery disease; CKD, chronic kidney disease; COX, cyclooxygenase; EFSA, European Food Safety Authority; EVOO, extra virgin olive oil; MMP, matrix metalloproteinases; OO, olive oil; VOO, virgin olive oils.
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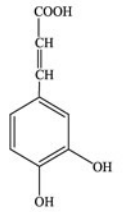
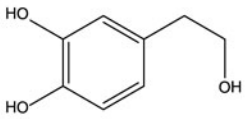
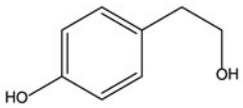
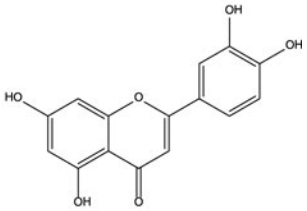
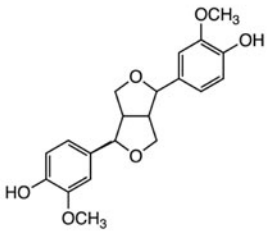
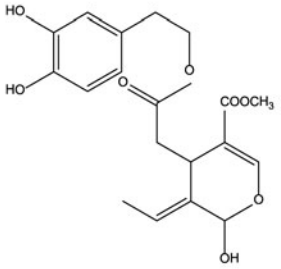
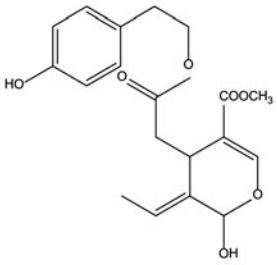
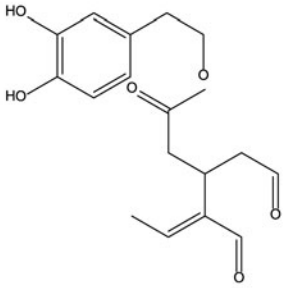
Table 1. Main classes of phenolic compounds in virgin olive oil

Phenolic acids	Phenolic alcohols		Flavonoids
			
Caffeic acid	Hydroxytyrosol	Tyrosol	Luteolin
Lignans	**Secoiridoids**		
			
(+) - Pinoresinol	Oleuropein aglycone (3,4-DHPEA-EA)	Ligstroside aglycone (*p*-HPEA-EA)	Dialdehydic form of deacetoxy oleuropein aglycone (3,4-DHPEA-EDA)

of cancer, diabetes and neurodegenerative diseases[8] as well as body weight reduction and obesity prevention[9,10]. The epidemiological evidence underpinning the relevance of the Mediterranean diet to health is strong with over seventeen studies including 2300 volunteers confirming that a Mediterranean diet decreases inflammation and improves endothelial function[11], and a meta-analysis of thirty-two cohort studies (>800 000 subjects) indicating that there is an inverse correlation between OO intake and CHD[12].

Olive oil bioactive components

The major components of OO are glycerols (saponifiable fraction) which represent more than 98 % of the total oil weight and are mainly TAG esters of oleic acid (55–83 %), palmitic acid (7·5–20 %), linoleic acid (3·5–21 %) and other fatty acids such as stearic acid (0·5–5 %)[13]. Minor components (the unsaponifiable fraction) include aliphatic and triterpenic alcohols, sterols, hydrocarbons as squalene, volatile compounds, tocopherols, carotenes, chlorophyll and phenolic compounds[13–15].

Special attention has been given to the phenolic compounds only found in VOO and EVOO. The agronomic and technological aspects of OO production have an impact on the concentration of phenolic compounds, as do the pedoclimatic conditions and agronomic techniques (e.g. irrigation)[4,14]. The main classes of phenolic compounds present in VOO are phenolic acids, phenolic alcohols (hydroxytyrosol and tyrosol), flavonoids, lignans and secoiridoids; Table 1.

Oleuropein and ligstroside, the most significant secoiridoids in *O. europaea* L., are esters of elenolic acid glucoside with hydroxytyrosol and tyrosol, respectively. During the mechanical extraction of the oil, fruit endogenous β-glucosidases[14,16] are released leading to the secoiridoid aglycones formation, accounting for more than 50 % of phenolic content of the oil[17,18]. The most abundant secoiridoids of VOO are the oleuropein and ligstroside aglycons and dialdehydic forms of deacetoxy of oleuropein and ligstroside aglycons[14] also named oleacein and oleocanthal, respectively[19].

Phenolic compounds bioavailability and bioactivity

Once OO has been ingested, it produces a micellar solution composed of a lipid and an aqueous phase. Chemical hydrolysis of secoiridoids can take place in the acidic medium of the stomach[20] or in alkaline conditions in the small intestine[21,22] leading to an increase of free phenolic alcohols released into the aqueous phase. As a result OO phenolic compounds are further absorbed in the small intestine[23]. Measuring the bioavailability of these compounds in plasma and urine reveals that OO phenolics undergo a conjugation process of methylation, glucuronidation and sulfation indicating that there is phase 2 metabolism involved during the absorption of these compounds[24–27]. The between-subjects variability in human absorption and metabolism of OO phenolics may explain differences in proportion of methyl, glucuronide and sulphate conjugates reported[28–30].

Bioavailability of OO phenolic compounds differs according to the intake matrix. OO as the intake vehicle promotes absorption of hydroxytyrosol: the corresponding bioavailability of hydroxytyrosol in rats for aqueous

and OO solutions were reported as 75 and 99 %[31], respectively. When a supplement containing hydroxytyrosol as a single oral dose (2·5 mg/kg) was fed to human subjets, the bioavailability was below 10 %[32], whereas previous studies showed higher bioavailability for hydroxytyrosol supplementation in lipid vehicles[33]. The addition of hydroxytyrosol to low fat yoghurt and administered to human subjects was also associated with a lower excretion of hydroxytyrosol when compared with OO[33]. As OO phenolic compounds are mainly absorbed in the small intestine[23] the increase of hydroxytyrosol bioavailability, in OO, might be related to the rate of gastric emptying[32] and slow release of hydroxytyrosol from the oil matrix[26,32]. The presence of other antioxidants in OO might prevent breakdown of hydroxytyrosol before absorption in the gastrointestinal tract[31].

Secoiridoids that are not absorbed in the small intestine are degraded by the colonic microbiota with oleuropein producing hydroxytyrosol as the major product[20]. *In vitro* colonic metabolism was evaluated on tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein showing an increase in phenolic acids, stability of hydroxytyrosol and tyrosol and degradation of hydroxytyrosol acetate and oleuropein mainly to hydroxytyrosol[34]. To evaluate OO phenolic metabolites produced from colonic fermentation, faecal samples were analysed before and after mid-term consumption of phenol-rich OO[34]. A significant increase in hydroxytyrosol concentration ($P < 0{\cdot}05$) was observed after phenol-rich OO intake. Although absorption of OO phenolic compounds mainly occurs in the small intestine a small proportion of hydroxytyrosol and its derivatives still pass into the large intestine[23]. This highlights the need to study the impact of OO phenolics in the colon, either with gut microbiota interaction or local activity due to its antioxidant and anti-inflammatory properties.

When assessing the chemical and *in vitro* biological antioxidant activities of these compounds, it is the glucuronide conjugates of hydroxytyrosol and tyrosol that must be assessed. These were tested in the range 0·01–10 μM against the radical 1,1-diphenyl-2-picrylhydrazyl. None of the glucuronides displayed significant antioxidant activities at the concentrations tested, whereas the parent aglycones did display antioxidant activity at these concentrations[35]. This conflicts with the results of others[36] with differences attributed to the fact that in one study reference standard material[35] was used and in the other the glucuronide conjugates were extracted from urine samples[36], and likely contained impurities that had antioxidant activity. Hydroxytyrosol metabolites might act as 'sinks' of hydroxytyrosol that could be locally released in the cells after enzymatic hydrolysis[37], thereby explaining the proposed hydroxytyrosol biological effects observed *in vivo*. Moreover, *in situ* deconjugation of hydroxytyrosol metabolites (into their free form) in erythrocytes was observed in rats after oral administration of an OO phenolic extract obtained from olive cake (1·5 g/kg body weight, equivalent to 34·4 mg hydroxytyrosol and derivatives), highlighting a potential protective mechanism against cell oxidative damage[38].

Although there are a number of biological effects for OO phenolic compounds, most cannot be achieved via normal dietary exposure to OO. This has led to development of enriched products with natural OO phenolic compounds. OO by-products such as olive mill wastewater[39] and olive pomace[40,41] are potential sources of natural bioactives which could be used to supplement OO. The development of new OO products such as pomace OO or refined olive oil enriched in natural bioactives opens new perspectives in the field.

Olive oil and inflammation

Inflammation involves a complex cascade of events partly related with the production of an excess of free radicals due to internal or environmental stress[42]. The inflammation process triggers signalling molecules such as NF-κB, which upregulates the production of inflammatory mediators, such as TNF-α[43], inducible NO synthase, cyclooxygenase (COX)-2 and IL-1β[42].

A number of phenolic compounds present in OO have anti-inflammatory properties, including oleocanthal, a secoiridoid (dose-dependent inhibition of COX-1 and -2 activities, similar to the anti-inflammatory drug ibuprofen[44]). However, to achieve comparable effect with the recommended daily dose of ibuprofen, 500 g EVOO would need to be consumed[45,46] making the dose–effect relationship out with any (acute) inflammatory benefits due to typical OO consumption.

Chronic inflammation

Rheumatoid arthritis is a major inflammatory, autoimmune, disease characterised by chronic joint inflammation[47,48]. Hydroxytyrosol has been studied for its anti-inflammatory effects in a rheumatoid arthritis animal model. We reported that it provided beneficial effects in the evolution of the disease[49], with 0·5 and 5 mg/kg doses in rats, after gavage administration, using refined olive oil as vehicle (human-equivalent of 4·9 and 49 mg/d, respectively, for a 60 kg adult), Fig. 1. Significant effects, on paw oedema reduction, were observed for a human-equivalent dose of 49 mg/d, a dose ten times higher than the approved European Food Safety Authority (EFSA) dose for phenolic compounds in relation to protection of lipid oxidation[50]. The same hydroxytyrosol dose was effective on colitis, another chronic inflammatory disease[51]. This dose would only be achievable through nutraceutical supplementation of OO with hydroxytyrosol, and the use of this functional food on a daily basis.

To further evaluate the anti-inflammatory mechanisms involved with hydroxytyrosol, we studied COX-2 and inducible NO synthase expression[49]. The treatment at 5 mg/kg dose significantly decreased histological damage, COX-2 and inducible NO synthase expression ($P < 0{\cdot}001$ *v.* positive control), markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation, improving articular function in the treated animals. Moreover, at the same dose there was a significant decrease

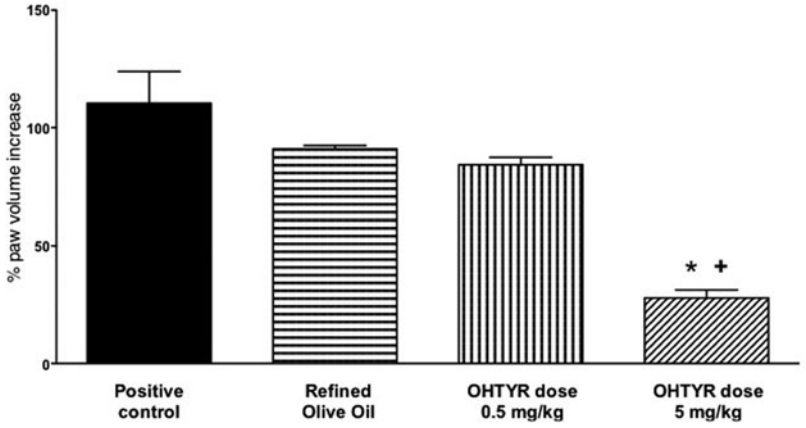


Fig. 1. Chronic inflammation model and impact on rats paw oedema. ANOVA, $^{*}P < 0{\cdot}001$ *v.* positive control – Rheumatoid Arthritis, $^{+}P < 0{\cdot}01$ *v.* Refined Olive Oil; OHTYR, hydroxytyrosol. Adapted from Silva *et al.*[49].

($P < 0{\cdot}005$ *v.* positive control and refined olive oil) in TNF-α serum levels. These results are in line with others that reported benefits on rheumatoid arthritis, in animal models, after oral administration of an EVOO extract[52], intraperitoneal administration of oleuropein aglycone[53] or polyphenol supplemented VOO diets[54]. The reports highlight the effects on rheumatoid arthritis of OO phenolic compounds either administered as isolated compounds or as an extract. However, dose comparison between animal studies have to take in consideration not only differences in species (rats *v.* mice), but also routes of administration. Compared with intraperitoneal administration, an oral dose has an extra pass through the liver with consequent metabolism through the first-pass effect.

Acute inflammation

Acute inflammation has been commonly induced using carrageenan in animals to evaluate the effects of non-steroid anti-inflammatory drugs[55]. We studied the effect of hydroxytyrosol-supplemented OO on acute inflammation, induced by carrageenan in rats, at 0·5 and 5 mg/kg[49] dose, after gavage administration which occurred 30 min before the challenge with carrageenan. Both doses significantly reduced paw oedema ($P < 0{\cdot}001$ *v.* positive control) with the lowest effective dose being achievable through OO daily intake. Previous studies in rats[56] also showed inhibition of carrageenan; acute inflammation of an aqueous hydroxytyrosol formulation (HT-20, 22 % hydroxytyrosol), and significant effects were obtained at a 22 mg/kg hydroxytyrosol dose. Differences in dose effect might be related to the administration vehicle with refined olive oil or OO being better vehicles than water.

Cardioprotection of olive oil

Most of the interventional studies focusing on the benefit of VOO intake on CVD have investigated the effect of phenolic compounds on the prevention of oxidation of LDL and HDL[57–64], two risk markers of CVD. A number of trials have also focused on cardioprotection against inflammation[65] mainly on antioxidant activity and inflammatory mediators.

Impact of olive oil constituents on lipoproteins and atherosclerosis

Fat content. LDL particles carry about two-thirds of plasma cholesterol and can infiltrate the arterial wall attracting macrophages, smooth muscle cells and endothelial cells[66] thus driving atherosclerosis.

LDL particle size is influenced by type and amount of dietary fat consumed[67]: Low-fat diets lead to a decrease in the size of LDL particles compared with high-fat diets[68]. The type of fat ingested is also important: LDL particles are larger with high-MUFA diets (such as those based on OO), compared with diets with a high PUFA intake, where LDL particles are smaller[69]. LDL particle size is especially relevant, since small-size particle are more prone to oxidation and can better enter into the arterial wall when compared with larger LDL particles[70]. Conversely, HDL particles are anti-atherogenic, as their primarily role is to deliver cholesterol to the liver to be metabolised and excreted or reused. HDL may also be able to dislodge cholesterol molecules from atheromas in arterial walls[66]. It has been reported in patients with peripheral vascular disease[71,72] that LDL particles are less susceptible to oxidation when the diet is enriched in VOO MUFA, compared with the PUFA of sunflower-oil enriched diets. Moreover, when compared with SFA intake, OO oleic acid reduces the level of LDL-cholesterol[63,64].

The health benefits associated with monounsaturated fat content in OO were recognised by the United States Food and Drug Administration in 2004, highlighting 'the benefits on the risk of CHD of eating about two tablespoons (23 g) of OO daily'[73]. Health benefits were related with a decrease of total and LDL-cholesterol in serum[73], diet improvement of endothelial dysfunction[74], coagulation activity[75] and reduced LDL susceptibility to oxidation[72].

Phenolic content. Antioxidants that can prevent lipid peroxidation, such as phenolic compounds, could play an important role in preventing oxidative modification of LDL[4], with the oxidative process an initiating factor for atherosclerotic plaques[76]. Once monocytes differentiate in macrophages on the endothelium they scavenge oxidised LDL, then becoming foam cells, leading to plaque formation[5].

The Effect of Olive Oil on Oxidative Damage in European Populations study was a cross-over fat replacement intervention[58], using OO with different phenolic content in healthy male volunteers. Its findings led to the current EFSA recommendation (Opinion of the Scientific Committee/Scientific Panel, *EFSA Journal*[50,77,78]). A linear increase in HDL-cholesterol levels after 3 weeks was observed after low-, medium- and high-polyphenol OO consumption: mean change from preintervention, 0·02 (95 % CI 0·00, 0·05) mM/l, 0·03 (95 % CI 0·00, 0·05) mM/l, and 0·04 (95 % CI 0·02, 0·06) mM/l, respectively. Total cholesterol: HDL-cholesterol ratio decreased linearly with the phenolic content of the OO. TAG levels decreased by an average of 0·05 mM/l for all OO[58]. Mean changes from preintervention for oxidised LDL levels (where U represents arbitrary units) were 1·21 (95 % CI −0·8, 3·6) U/l, −1·48 (95 % CI −3·6, 0·6) U/l and −3·21 (95 % CI −5·1, −0·8) U/l for the low-, medium- and high-polyphenol OO, respectively, showing a dose-dependent relation with VOO phenolic content[58]. The EFSA confirmed a cause–effect relationship between consumption of OO phenolics (standardised by the content of hydroxytyrosol and its derivatives) and protection of LDL-cholesterol particles against oxidative damage. To support the EFSA health claim, 5 mg hydroxytyrosol and its derivatives should be consumed daily in 20 g OO[50], but concentrations in some OO may be too low to achieve this target in the context of a balanced diet. Moreover, the EFSA Panel reported study design limitations as most human interventions with OO have been conducted in more homogeneous male populations[77] and not in general population.

The contribution of OO phenolics towards cardiovascular health benefits has been challenged with inconsistent results reported for *ex vivo* resistance of LDL to oxidation[79,80]. Seven human intervention studies with OO were compared for impact of phenolics on oxidised LDL, with no effect seen in five of them[79], possibly explained by artefacts generated during LDL isolation.

Since the approval of the EFSA claim, both terminology and analytical methodology supporting the dose calculation of hydroxytyrosol and derivatives have been appraised. Mastralexi *et al.*[81] commented on the weaknesses of the claim terminology namely the term 'olive oil polyphenols' is not entirely clear and accurate as 'olive oil' is a generic term for the type of oil, and the basic structure of OO phenolic compounds do not coincide with a 'polyphenolic' structure; accordingly 'virgin olive oil bioactive phenols' is a more appropriate term. Others also commented about the lack of robust and reliable methods for quantifying phenolic compounds in OO. A simple and robust method for routine analysis of hydroxytyrosol and tyrosol was proposed[81,82] based on hydrolysis of the polar fraction of OO. This was followed by development and validation of a ^{1}H NMR method enabling direct measurement of tyrosol and hydroxytyrosol derivatives, as well as oleocanthal and oleacein in OO, overcoming analytical issues such as chromatographic peak broadening[19].

Cardioprotective mechanisms of oleic acid

OO intake has been related with a decrease on blood pressure with oleic acid regarded as being a major contributor to this effect, as evidenced in animal models[83]. Chronic oral administration of VOO (rich in oleic acid), triolein (a TAG with three oleic acid moieties) or oleic acid over 14 d significantly reduced systolic blood pressure in rats (−26 (SEM 4) for VOO and −21 (SEM 3) mm Hg for triolein, $P < 0{\cdot}001$ and −17 (SEM 1·9) mm Hg for oleic acid $P < 0{\cdot}05$) when compared with the control group that received water. Similarly acute (2 h) treatments with either VOO or triolein also significantly reduced systolic blood pressure when compared with the control group (−20 (SEM 0) mm Hg, $P < 0{\cdot}001$ and −14 (SEM 2) mm Hg, respectively; $P < 0{\cdot}05$) with oleic acid again significantly reducing systolic blood pressure (−13·0 (SEM 0·3) mm Hg; $P < 0{\cdot}001$). In contrast, chronic treatment with the *trans*-MUFA elaidic (18 : 1*n*-9) or the SFA stearic acid (18 : 0) did not significantly affect blood pressure. Results show that saturation and *cis*/*trans* double-bond arrangement are implicated with the cardioprotective effect of the long-chain fatty acid in this animal model at high-dose levels[83]. Similar significant results were obtained after VOO and oleic acid intake in an animal model of hypertension using spontaneously hypertensive rats[83].

The molecular mechanisms were evaluated by measuring signalling proteins involved in the control of blood pressure in the aorta. OO intake increases oleic acid levels in membranes, which regulate membrane lipid structure and impact on G-protein-mediated signalling, causing a reduction in blood pressure[84]. Unlike its analogues elaidic and stearic acid, oleic acid, due to its *cis*-18 : 1*n*-9 structure, regulates cellular membrane lipid structure and the α_2 receptor system involved in the control of blood pressure ($\alpha_{2A/D}$ – adrenoreceptor/G protein/adenylyl cyclase-cAMP/PKA) as demonstrated *in vitro*[84] and *in vivo*[83]. Oleic acid can also contribute to heart health via intramyocardial TAG turnover[85], which is reduced in pressure-overloaded failing hearts. In this situation, oleate (derivative of oleic acid) upregulated TAG dynamics when compared with palmitate (derivative of palmitic acid and major SFA of palm oil). This result underscores the importance of the intracellular lipid storage type on nuclear receptor signalling and contractility[85] in diseased hearts.

An important driver of vasorelaxation is NO, a free radical that readily reacts with fats and proteins. Nitro-fatty acids are mediators of cardiovascular signalling actions[86] as these compounds relax blood vessels, attenuate platelet activation and reduce inflammation[87,88].

Both oleic acid and linoleic acid are unsaturated fatty acids that after reaction with nitrite may form nitro-fatty

acids. Nitro-oleic acid-mediated antihypertensive signalling actions were shown in a mouse model[89]. The mechanism was attributed to the inhibition of soluble epoxide hydrolase by nitro-fatty acids, thus lowering blood pressure in an angiotensin II-induced hypertension[89]. It is however unclear how the extent of nitrite in the human diet may contribute to nitration of dietary fat, and the physiological relevance of this finding.

Role of phenolic compounds on endothelium protection

Oxidative stress and reactive oxygen species have been implicated in endothelial damage, progression to atherosclerosis, injury in sustained myocardial infarction and ischaemia reperfusion[76,90–92]. Monocytes and macrophages are critical cells that are involved in atherosclerosis. These cells produce proinflammatory cytokines, such as IL-1β, TNF-α and C-reactive protein, which induce the expression of adhesion molecules such as intercellular adhesion molecule-1, vascular-cell adhesion molecule-1 and E-selectin[93].

Meanwhile, oxidative stress through reactive oxygen species production promotes the expression of the adhesion molecules on the endothelium[94].

Expression of adhesion molecules attracts circulating monocytes, inducing their adherence to the endothelium. OO phenolic compounds have been shown to act on endothelium protection as evidenced in *in vitro* assays with typical OO phenolic compounds and less on *in vivo* circulating metabolites. OO phenolic extract, oleuropein aglycone or homovanillic alcohol (metabolite of hydroxytyrosol) had inhibitory effects on vascular-cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin surface expression in human umbilical vascular endothelial cells, using TNF-α as pro-inflammatory stimulus[95].

Endothelium dysfunction refers to an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioactivity in the vessel wall. In animal models with rats oral hydroxytyrosol administration was tested on NO production and platetet function[96]. Results showed that hydroxytyrosol administration (100 mg/kg daily) increased vascular NO production by up to 34·2 % ($P < 0{\cdot}01$) and inhibited platelet aggregation for 50 % inhibitory dose of 48·25 mg/d for hydroxytyrosol ($P < 0{\cdot}01$) when compared with control group (treated with isotonic saline solution). Animal dose translation to human subjects allowed us to conclude that the effective hydroxytyrosol doses tested would be above the expected intake through OO daily. The reported benefits would only be achievable through nutraceutical supplementation.

Endothelium repair: matrix metalloproteinases and olive oil

Matrix metalloproteinases (MMP) play a role in endothelium repair. Macrophages resident in human and experimental atherosclerosis co-localise with and release active MMP, including the gelatinase MMP-9, which is specialised in the digestion of basement membrane collagens and elastin, and is implicated in atherogenesis, unstable coronary syndromes and in aortic aneurysms[97]. Accumulating evidence points to the MMP as major molecular mediators of arterial diseases[97]. Collagens, types 1 and 3, are the main proteins in arterial walls being also present in the thickened intima of atherosclerotic lesions[98,99]. Fragments of collagens found in urine are present as a result of proteolytic activity in arterial walls and other vascular structures. Collagen type 1 or 3 fragments were upregulated in urine in coronary artery disease (CAD) patients[100]. Increase in collagen degradation is related with an increase on collagenases circulation, such as MMP-9, as shown in patients with CAD[101].

In an *in vitro* study hydroxytyrosol (1–10 μM) reduced MMP-9 ($IC_{50} = 10$ μM/l, $P < 0{\cdot}05$) and COX-2 induction in activated human monocytes, with phorbol myristate acetate[102]. These effects were mediated by inhibition of transcription factor NF-κB and protein kinase Cα and protein kinase C β1 activation[102]. Results are in line with previous *in vitro* reports that showed inhibition of MMP-9 on endothelial cells by OO phenolics namely hydroxytyrosol in phorbol myristate acetate-induced cells[103] and oleuropein aglycone in TNF-α-induced cells by acting on NF-κB[94]. No hydroxytyrosol activity on MMP-9 was found in TNF-α-induced cells[94].

The discriminatory polypetides that increase in CAD includes collagen type 1 and 3 fragments with a C-terminal GxPGP motif[104]. Increase on these polypeptides would come from a protease decrease activity possibly related with chemical change of the substrate (e.g. oxidative damage) thus inhibiting it acting at a specific site, or a decrease in circulating levels by lack of enzyme activation. MMP-2 is secreted in an inactive form (pro-MMP-2) and several factors can promote its activation such as plasmin[105] and thrombin[106]. Other mechanisms that involve proteinases or oxidative stress can also activate MMP-2[107]. Therefore antioxidants, as phenolic compounds, might have a role on MMP-2 activation and published data indicate phenolic compounds from red wine[108] and green tea[109] as acting on prevention of thrombin-induced activation of MMP-2 in vascular smooth cells.

We evaluated the impact of a 6-week OO supplementation in healthy adults on urinary proteomic biomarkers of CAD in a randomised, parallel, controlled, double-blind study[110]. The present study was the first to describe the significant impact of daily OO supplementation on highly specific disease biomarkers for CAD. Analysis of urinary proteomic profiles at baseline and endpoint enabled the identification of twelve sequenced peptides that were significantly regulated towards healthy scoring. Eight of them included four collagen α−1(I) chain, one α−2 (1) chain, one α−2(V) chain and one α−2(VI) chain fragments. Changes in circulating concentrations of collagenases may mediate these changes in the urinary fingerprint. Therefore with more data or in future intervention studies with OO, it would be interesting to link urinary fragments to the proteases involved in their generation. This predictive analysis would enable looking at the peptide cleavage sites studying the MMP up or downregulated with OO intervention.

The majority of studies of dietary intake of proposed bioactive foods asses the activities of these foods based

on the major risk factors of CVD. However, markers such as lipoprotein profile, blood pressure, endothelial function, inflammation and oxidative stress have no direct link to the disease itself but are merely associated with it. There is a great need for more biomarkers that appear as a direct result of the disease itself[63,67].

Proteomics biomarkers as a mechanistic approach to explain olive oil health effects

The systems biology approach (encompassing genomics, transcriptomics, proteomics and metabolomics using urine, blood or saliva) could provide a greater understanding of disease development, treatment efficacy and evaluation of the influence of food bioactive compounds[46,111]. There is a need for biomarkers of practical value for clinical intervention, allowing disease risk prediction and more importantly early diagnosis. Accuracy, reproducibility, availability, feasibility of implementation into the clinical settings, sensitivity and specificity are additional characteristics to be fulfilled, and panels of biomarkers are gaining acceptance instead of individual molecules[112], as single biomarkers are often not available and lack the ability to adequately describe complex diseases[113]. Candidate biomarkers should be carefully validated in a wide and different cohort of samples from those used in the discovery phase as often overfitting of the biomarker model has occurred[114].

The proteome, corresponding to a set of expressed proteins, informs the current 'status' of an organism, constantly changing according to endogenous and exogenous factors[113]. Proteins are widely used in different clinical tests for both diagnosis and prognosis of diseases and to follow their evolutions[98]. They can be used to measure the extent of inflammation, calcification and the development of plaques on the arteries. Understanding what causes plaque rupture is of great importance. As previously mentioned, MMP could have a key role in this process[115]. The discovery of proteomic biomarkers may be useful in understanding the molecular mechanisms involved in the onset and progression of other vascular diseases[116]. Plasma, serum and urine are the most commonly used biological matrices in cardiovascular research, due to their perceived clinical relevance as a source of potential biomarkers[98]. However, proteomic studies have also been carried out on vascular tissues (arteries), artery layers, cells looking at proteomes and secretomes, exosomes, lipoproteins and metabolites[98]. Although sampling the tissue may seem an obvious method there are a number of difficulties, especially where the need for a biopsy would be required[117]. Recent advances in extraction processes and LC-MS/MS analysis has allowed the quantitative analysis of tissue samples in vascular research to be carried out[118,119].

Urine as a sample source is now recognised as the source of choice for proteomic biomarker investigations. It has a number of advantages such as being non-invasive and can be collected by untrained personnel. Urine is produced by renal filtration of the plasma and approximately 70 % of proteins in the normal human urinary proteome are of kidney origin, whereas the remaining 30 % are derived from plasma proteins[120,121]. It has high stability due to absence of proteolytic agents and the low dynamic range of analyte concentration facilitates the detection and quantification of peptides[113,122].

Using capillary electrophoresis coupled with MS[123] urinary biomarker classifiers for the diagnosis of diseases such as chronic kidney disease (CKD)[124], acute kidney injury[125], stroke[126] and CAD[104], were already identified, allowing classification of case–control groups with good accuracy[127].

Urinary peptides and protein fragments are the end products of proteolytic processes. The different pattern of urinary excretion of peptides when comparing controls and disease patients might indicate their role in the pathophysiology of disease. Therefore changes in the normal urine 'fingerprint' (e.g. presence of collagen fragments) can be used as biomarkers of disease. Besides collagens, common blood proteins (e.g. α1-antitrypsin, haemoglobin, serum albumin and fibrinogen), and uromodulin were also identified[128] in urine which provides additional proof of the suitability of this sample source for proteomic biomarker studies outwith the kidney and urinary tract. Collagens are the most abundant peptides sequenced so far in the CAD biomarker (66 % of all peptides)[104], with atherosclerosis associated with an increased synthesis of several extracellular matrix components, including collagen types 1 and 3, elastin and several proteoglycans[129]. Changes in the circulating levels of collagenases may mediate these changes in peptides represented in the fingerprint, as reported in coronary atherosclerosis[100] and CKD[128].

The progress in urinary proteomics and the use of multiple biomarker classifiers opens the possibility of establishing new tools adapted to different clinical needs[130], enabling direct monitoring of disease overcoming limitations of indirect measurements.

Proteomic in vitro *studies on olive oil phenolic compounds*

Proteomics has been applied in a number of studies of OO phenolic compounds on cardiovascular health using animal and *in vitro* studies. The *in vitro* effects of alperujo extract, an OO production waste product containing phenolic compounds present in olive fruits, were studied on platelet aggregation and changes in the platelet proteome[131]. Nine proteins were differentially regulated by the alperujo extract upon platelet aggregation underlying the anti-platelet effects of the extract. However, like a number of previously mentioned *in vitro* studies, the effective concentrations (40–500 mg/l) were far above the physiologically concentrations achievable by dietary intake.

The effects of EVOO, with low and high phenolic content, were evaluated in the hepatic proteome in ApoE$^{-/-}$ mice that spontaneously develop atherosclerosis[132]. For 10 weeks the mice were fed with a high-fat, high-cholesterol diet supplemented with 0·15 % (w/w) cholesterol and either 20 % (w/w) low phenolic EVOO or 20 % (w/w) high phenolic EVOO *v.* a control group fed

with 0·15 % (w/w) cholesterol and 20 % (w/w) palm oil. Within this work a range of hepatic antioxidant enzymes differentially regulated by OO[132] were identified. The authors concluded that the upregulation of a large array of antioxidant enzymes might explain anti-atherogenic mechanisms of EVOO[132]. Again the dose level was above what could be achieved through dietary intake and translation from an animal model to human use has also to be considered.

Urinary proteomics biomarkers, olive oil and CVD

Atherosclerosis is a process of chronic inflammation, characterised by the accumulation of lipids, cells, and fibrous elements in medium and large arteries[98]. The extent of inflammation, proteolysis, calcification and neovascularisation influences the development of advanced lesions (atheroma plaques) on the arteries[98].

Classical risk factors in atherosclerosis (hypertension, LDL-cholesterol, C-reactive protein, ageing, smoking, male gender, among others) do not actually measure disease initiation or progression. As such, they cannot be used directly to identify individuals who have developed atherosclerosis and prevent a fatal event[98,133]. Other, more recent markers that indicate changes in vascular structure can still only be detected once CVD has progressed to an advanced stage where drug or surgical intervention is required[134].

The analysis of urine samples from diseased and healthy individuals has been used to establish a database of naturally occurring urinary peptides, making a basis for the definition and validation of biomarkers for diagnosis/prognosis/monitoring of a wide range of diseases using proteomic biomarker patterns[128], such as CAD[100], emphasising that non-invasive proteomics analysis could become a valuable addition to assess CVD alongside other biomarkers that are indicators of cardiovascular risk.

The first time that urinary proteomics was applied to assess cardiovascular health improvements of OO consumption in human subjects, was in a randomised, parallel, controlled, double-blind study designed to evaluate the impact of a 6-week OO supplementation in healthy adults on urinary proteomic biomarkers of CAD[110]. The impact of the supplementation with OO was also studied on urinary proteomic biomarkers of CKD and diabetes.

The increase or decrease in the concentration of the peptides in the biomarker determines the scoring value of each disease biomarker. The CAD proteomic biomarker developed for clinical diagnosis produces a CAD scoring system from 1 (CAD case) to −1 (healthy artery). A scoring of disease absence, presence and severity is provided, based on the concentration of a group (panel) of urinary peptides measured by capillary electrophoresis coupled with MS, allowing monitoring of progression and/or effect of treatment[135,136]. In the present study, self-reported healthy participants were randomly allocated to supplementation with a daily dose of OO either low or high in phenolic compounds. For 6 weeks, they consumed a daily dose of 20 ml OO (not heated or cooked) as a supplement (no specific time during the day, single intake, equivalent to 6 mg hydroxytyrosol and derivatives for the high phenolic OO), in line with the EFSA and Food and Drug Administration recommendations. The impact of supplementation with OO was evaluated on urinary proteomic biomarkers of CAD with biomarkers being measured at baseline and 3 and 6 weeks. Consumption of both OO significantly improved the proteomic CAD score at endpoint compared with baseline, moving the CAD biomarker pattern in a healthy profile direction (Table 2). No differences were observed for CKD or diabetes proteomic biomarkers, Table 2.

Table 2. Scores of CAD, CKD and diabetes proteomic biomarkers at baseline, middle (3 weeks) and end of intervention (6 weeks)

		Low phenolic olive oil (*n* 34) score	High phenolic olive oil (*n* 28) score
CAD proteomic biomarker	Baseline	−0·5 (SD 0·2)	−0·6 (SD 0·4)
	3 weeks	−0·7 (SD 0·3)	−0·7 (SD 0·3)
	6 weeks	−0·8 (SD 0·3)**	−0·8 (SD 0·3)*
CKD proteomic biomarker	Baseline	−0·4 (SD 0·2)	−0·4 (SD 0·3)
	3 weeks	−0·4 (SD 0·2)	−0·4 (SD 0·3)
	6 weeks	−0·4 (SD 0·2)	−0·4 (SD 0·2)
Diabetes proteomic biomarker	Baseline	1·3 (SD 0·3)	1·3 (SD 0·3)
	3 weeks	1·3 (SD 0·4)	1·3 (SD 0·3)
	6 weeks	1·4 (SD 0·4)	1·2 (SD 0·3)

CAD, coronary artery disease; CKD, chronic kidney disease (adapted from Silva *et al.*[110]).
Compared with corresponding baseline value: *$P < 0·005$, **$P < 0·001$. There were no significant differences in changes between groups.
A repeated-measures ANOVA test was used with statistical significance at $P < 0·05$.

In a placebo-controlled intervention, irbesartan (angiotensin II receptor antagonist used for the treatment of hypertension) taken at 300 mg/d over 2 years in hypertensive type 2 diabetes patients, using the CAD 238 biomarker panel, led to a 0·35 point reduction in the CAD score for the drug-controlled group[104], which saw a significant reduction in incidents of CAD in this group. In the nutritional intervention[110], the CAD score change in the intervention was significant for both OO tested, using the same CAD 238 biomarker, leading to a similar degree of change as observed for irbersartan over a 6-week period. This evidence highlights the importance of the CAD biomarker as a tool for nutrition and health intervention studies. This type of urinary biomarker enabled the measurement of health effects induced by a change in diet that could not be detected by monitoring the conventional risk markers of CAD such as plasma TAG, oxidised LDL, and LDL-cholesterol. The overall change in CAD score in a short period of time is more likely due to OO major components, such as fatty acids. However, the role of other OO minor components other than phenolic compounds should also be taken into account. Squalene, a polyunsaturated triterpene which makes up 60–75 % of the unsaponifiable fraction of OO[137], reduced

atherosclerotic lesion size in male mice[138] and further investigation is needed to clarify its role on CVD.

Our results emphasise further the potential role of nutrition in the prevention or delay of CVD and offer new perspectives on OO applications. These results are highly translatable to guidelines for nutritional recommendations. The biomarkers were originally developed to detect early signs of diseases in clinical setting and to inform clinicians as to the effectiveness of treatment. However, the technology also provides a sensitive tool for the assessment of potential bioactive foods in cardiovascular health, CKD and diabetes, with a range of additional tests under development. Further testing of reportedly bioactive foods can now be carried out which will allow better nutritional health advice to be advanced and could also lead to better food labelling, so that the public can make informed choices on their food purchases.

Exploring olive oil health benefits: perspectives

Although strong evidence from heritability is related with CVD many forms of heart disease are not genome associated[139]. The epigenome is a possible link between genetics and environment[139] which includes impact of food components/diet. Omics techniques (such as genomics, transcriptomics, proteomics, epigenomics and metabolomics) have the potential, when integrated, to comprehensively demonstrate the contribution of diet towards the modulation of disease risk[140]. Some trials have shown the impact of OO on downregulation of atherosclerosis-related genes[140,141]. The effect of Mediterranean diet was studied on urinary metabolome[142] and related to compounds of the metabolism of carbohydrates, creatine, creatinine, amino acids, lipids and microbial cometabolites.

Phenolic compounds can interact with cellular signalling cascades regulating the activity of transcription factors with impact on gene expression. For instance, phenolic compounds have shown to affect the expression of microRNA[143]. microRNA are small, non-coding RNA implicated in the regulation of gene expression that control both physiological and pathological processes, influenced by external factors as diet components[144]. Most of the studies reported in this field are *in vitro* and more *in vivo* studies are needed to clarify microRNA targets of dietary phenolic compounds[144].

Interactions between genes and the bioactive components present in OO studied by nutrigenomics may help to explain its health benefits[145]. In this sense, besides their antioxidant and anti-inflammatory capacities, OO phenolic compounds are able to modify gene expression coding in a protective mode for proteins participating in the cellular mechanisms involved in oxidative stress resistance, inflammation or lipid metabolism amongst others[146].

Glycation, a non-enzymatic reaction between reducing sugars and proteins, is a proteome wide phenomenon, mainly observed in diabetes due to hyperglycaemia[147], but also relevant to end organ damage, disease pathogenesis and ageing[148] and OO phenolic compounds have been reported as potent inhibitors of the formation of advanced glycation end products[149]. Our human intervention trial with OO low or high in phenolics did not find a significant impact on the plasma fructosamine levels[110]. A key factor may be the duration of the study (6 weeks) not being sufficient to detect changes in protein modifications such as glycation, and may also be partly related to the quantity and quality of phenolic compounds, which exert differential antioxidant and antiglycative activities depending on structure[4,150]. Further studies should proceed in order to clarify antiglycation properties of OO phenolic compounds, given that glycation is a key driver for tissue damage and is present in all non-communicable disease scenarios.

Conclusion

Results outlined in the present review provide evidence of health benefits related with OO intake. The reported studies may allow the implementation of primary prevention programs of CVD, based on nutritional interventions, useful in non-regular OO consumers groups like the Northern European populations. Interventions in broad populations with highly specific disease biomarkers, as urinary proteomic biomarkers, will offer higher translational value, especially towards development and implementation of new nutritional recommendations.

Human intervention trials focusing on new outcomes related with proteomics and nutrigenomics are needed to better clarify pathways/mechanisms by which oleic acid, phenolic compounds or even other OO components act on CVD risk factors and affect the proteome.

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Conflict of Interest

T. K. is employed at Mosaiques Diagnostics, the company that developed the urinary proteomics for capillary electrophoresis coupled with MS technology for clinical application.

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Authorship

S. S. conducted the studies described and drafted the manuscript. E. C., M. E. F., W. M. and M. R. B. supervised the studies and contributed to the drafting of the manuscript. All authors were responsible for the critical review of the manuscript.

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