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

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Abstract

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 coronary heart disease. 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 cardiovascular disease 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. While model systems are important for mechanistic research nutrition, methodologies and experimental designs with strong translational value are still lacking. This 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 highlited towards a higher translational approach involving olive oil in nutritional recommendations.

59

1. 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 OOs are defined by European Union (EU) regulations[2]. Oils obtained only by mechanical extraction are virgin olive oils (VOOs) 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 coronary heart disease (CHD)[4-7]. OO consumption is also associated with decreased rates 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 coronary heart disease[12].

75

Olive oil bioactive components

The major components of OO are glycerols (saponifiable fraction) which represent more than 98% of of the total oil weight and are mainly triglyceride esters of oleic acid (55 to 83%), palmitic acid (7.5 to 20%), linoleic acid (3.5 to 21%) and other fatty acids such as stearic acid (0.5 to 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 does 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 *Olea europaea* L., are esters of elenolic acid glucoside with hydroxytyrosol and tyrosol, respectively. During the mechanical

91   extraction of the oil, fruit endogenous β-glucosidases[14, 16] are released leading to the secoiridoid

92   aglycones formation, accounting for more than 50% of the phenolic content of the oil[17, 18]. The

93   most abundant secoiridoids of VOO are the oleuropein and ligstroside aglycons and dialdehydic

94   forms of deacetoxy of oleuropein and ligstroside aglycons[14] also named oleacein and

95   oleocanthal, respectively[19].

96

97   **Phenolic compounds bioavailability and bioactivity**

98   Once OO has been ingested, it produces a micellar solution composed of a lipid and an aqueous

99   phase. Chemical hydrolysis of secoiridoids can take place in the acidic medium of the

100   stomach[20] or in alkaline conditions in the small intestine[21, 22] leading to an increase of free

101   phenolic alcohols released into the aqueous phase. As a result OO phenolic compounds are

102   further absorbed in the small intestine[23]. Measuring the bioavailability of these compounds in

103   plasma and urine reveals that OO phenolics undergo a conjugation process of methylation,

104   glucuronidation and sulfation indicating that there is phase 2 metabolism involved during the

105   absorption of these compounds[24-27]. The between-subjects variability in human absorption and

106   metabolism of OO phenolics may explain differences in proportion of methyl, glucuronide and

107   sulfate conjugates reported[28-30].

108

109   Bioavailability of OO phenolic compounds differs according to the intake matrix. OO as the

110   intake vehicle promotes absorption of hydroxytyrosol: the corresponding bioavailability of

111   hydroxytyrosol in rats for aqueous and OO solutions were reported as 75 and 99%[31],

112   respectively. When a supplement containing hydroxytyrosol as a single oral dose (2.5 mg/kg)

113   was fed to humans, the bioavailability was below 10%[32], while previous studies showed higher

114   bioavailability for hydroxytyrosol supplementation in lipid vehicles[33]. The addition of

115   hydroxytyrosol to low fat yogurt and administered to humans was also associated with a lower

116   excretion of hydroxytyrosol when compared with OO[33]. As OO phenolic compounds are mainly

117   absorbed in the small intestine[23] the increase of hydroxytyrosol bioavailability, in OO, might be

118   related to the rate of gastric emptying[32] and slow release of hydroxytyrosol from the oil

119   matrix[26, 32]. The presence of other antioxidants in OO might prevent breakdown of

120   hydroxytyrosol before absorption in the gastrointestinal tract[31].

121

122 Secoiridoids that are not absorbed in the small intestine are degraded by the colonic microbiota

123 with oleuropein producing hydroxytyrosol as the major product[20]. *In vitro* colonic metabolism

124 was evaluated on tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein showing an

125 increase in phenolic acids, stability of hydroxytyrosol and tyrosol and degradation of

126 hydroxytyrosol acetate and oleuropein mainly to hydroxytyrosol[34]. In order to evaluate OO

127 phenolic metabolites produced from colonic fermentation, faecal samples were analysed before

128 and after mid-term consumption of phenol-rich OO[34]. A significant increase in hydroxytyrosol

129 concentration ($p < 0.05$) was observed after phenol-rich OO intake. Although absorption of OO

130 phenolic compounds mainly occurr in the small intestine a small proportion of hydroxytyrosol

131 and its derivatives still pass into the large intestine[23]. This highlights the need to study the

132 impact of OO phenolics in the colon, either with gut microbiota interaction or local activity due

133 to its antioxidant and anti-inflammatory properties.

134

135 When assessing the chemical and *in vitro* biological antioxidant activities of these compounds, it

136 is the glucuronides conjugates of hydroxytyrosol and tyrosol that must be assessed. These were

137 tested in the range 0.01–10 μM against the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). None

138 of the glucuronides displayed significant antioxidant activities at the concentrations tested,

139 whereas the parent aglycones did display antioxidant activity at these concentrations[35]. This

140 conflicts with the results of others[36] with differences attributed to the fact that in one study

141 reference standard material[35] was used and in the other the glucuronide conjugates were

142 extracted from urine samples[36], and likely contained inpurities that had antioxidant activity.

143 Hydroxytyrosol metabolites might act as "sinks" of hydroxytyrosol that could be locally released

144 in the cells after enzymatic hydrolysis[37], therby explaining the proposed hydroxytyrosol

145 biological effects observed *in vivo*. Moreover, *in situ* deconjugation of hydroxytyrosol

146 metabolites (into their free form) in red blood cells was observed in rats after oral administration

147 of an OO phenolic extract obtained from olive cake (1.5 g/kg body weight, equivalent to 34.4 mg

148 of hydroxytyrosol and derivatives), highlighting a potential protective mechanism against cell

149 oxidative damage[38].

150

151 Although there are a number of biological effects for OO phenolic compounds, most cannot be

152 achieved via normal dietary exposure to OO. This has led to development of enriched products

153 with natural OO phenolic compounds. OO by-products such as olive mill wastewater[39] and

154 olive pomace[40, 41] are potential sources of natural bioactives which could be used to supplement

155 OO. The development of new OO products such as pomace OO or refined olive oil (ROO)

156 enriched in natural bioactives opens new perspectives in the field.

157

158 **2. Olive oil and inflammation**

159 Inflammation involves a complex cascade of events partly related with the production of an

160 excess of free radicals due to internal or environmental stress[42]. The inflammation process

161 triggers signaling molecules such as nuclear factor-kappa-B (NF-$_k$B), which up-regulates the

162 production of inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α)[43] inducible

163 NO synthase (iNOS), cyclooxygenase-2 (COX-2), and interleukin-1beta (IL-1β)[42].

164 A number of phenolic compounds present in OO have anti- inflammatory properties, including

165 oleocanthal, a secoiridoid (dose-dependent inhibition of COX-1 and COX-2 activities, similar to

166 the anti-inflammatory drug ibuprofen[44]). However, to achieve comparable effect to the

167 recommended daily dose of ibuprofen, 500 g of EVOO would need to be consumed[45, 46] making

168 the dose/effect relationship outwith any (acute) inflammatory benefits due to typical OO

169 consumption.

170

171 **Chronic inflammation**

172 Rheumatoid arthritis (RA) is a major inflammatory, autoimmune, disease characterized by

173 chronic joint inflammation[47, 48]. Hydroxytyrosol has been studied for its anti-inflammatory

174 effects in a RA animal model. We reported that it provided beneficial effects in the evolution of

175 the disease[49], with 0.5 and 5 mg/kg doses in rats, after gavage administration, using ROO as

176 vehicle (human-equivalent of 4.9 and 49 mg/day, respectively, for a 60 kg adult), **Figure 1**.

177 Significant effects, on paw edema reduction, were observed for a human-equivalent dose of

178 49 mg/day, a dose 10 times higher than the approved European Food Safety Authority (EFSA)

179 dose for phenolic compounds in relation to protection of lipid oxidation[50]. The same

180 hydroxytyrosol dose was effective on colitis, another chronic inflammatory disease[51]. This dose

181 would only be achievable through nutraceutical supplementation of OO with hydroxytyrosol, and

182 the use of this functional food on a daily basis.

183 To further evaluate the anti-inflammatory mechanisms involved with hydroxytyrosol, we studied

184 COX-2 and iNOS expression[49]. The treatment at 5 mg/kg dose significantly decreased

185 histological damage, COX-2 and iNOS expression ($p<0.001$ *vs.* positive control), markedly

6

186 reduced the degree of bone resorption, soft tissue swelling and osteophyte formation, improving
187 articular function in treated animals. Moreover at the same dose there was a significant decrease
188 ($p<0.005$ *vs.* positive control and ROO) in TNF-α serum levels. These results are in line with
189 others that reported benefits on RA, in animal models, after oral administration of an EVOO
190 extract[52], intraperitoneal administration of oleuropein aglycone[53] or polyphenol supplemented
191 VOOs diets[54]. The reports highlight effects on RA of OO phenolic compounds either
192 administered as isolated compounds or as an extract. However, doses comparison between
193 animal studies have to to take in consideration not only differences in species (rats *vs.* mice) but
194 also routes of administration. Compared to intraperitoneal administration, an oral dose has an
195 extra pass through the liver with consequent metabolism through the first-pass effect.
196

197 **Acute inflammation**
198 Acute inflammation has been commonly induced using carrageenan in animals in order to
199 evaluate the effects of non steroid anti-inflammatory drugs (NSAIDs)[55]. We studied the effect
200 of hydroxytyrosol-supplemented OO on acute inflammation, induced by carrageenan in rats, at
201 0.5 and 5 mg/kg[49] dose, after gavage administration which occurred 30 min before the challenge
202 with carrageenan. Both doses significantly reduced paw edema ($p<0.001$ *vs.* positive control)
203 with the lowest effective dose being achievable through OO daily intake. Previous studies in
204 rats[56] also showed inhibition of carrageenan - acute inflammation of an aqueous hydroxytyrosol
205 formulation (HT-20, 22% hydroxytyrosol), and significant effects were obtained at a 22 mg/kg
206 hydroxytyrosol dose. Differences in dose effect might be related to the administration vehicle
207 with ROO or OO being better vehicles than water.
208

209 **3. Cardioprotection of olive oil**
210 Most of the interventional studies focusing on the benefit of VOO intake on cardiovascular
211 disease have investigated the effect of phenolic compounds on the prevention of oxidation of
212 low-density (LDL) and high-density (HDL) lipoproteins[57-64], two risk markers of cardiovascular
213 disease. A number of trials have also focused on cardioprotection against inflammation[65]
214 mainly on antioxidant activity and inflammatory mediators.
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216
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218

Impact of olive oil constituents on lipoproteins and atherosclerosis

220

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 to high-fat diets[68]. The type of fat ingested is also important: LDL particles are larger with high-monounsaturated fatty acid diets (such as those based on OO), compared to diets with a high polyunsaturated fatty acids 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 antiatherogenic, as their primarily role is to deliver cholesterol to the liver to be metabolized 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 monounsaturated fatty acids, compared to the polyunsaturated fatty acids of sunflower oil enriched diets. Moreover when compared to saturated fatty acids 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 (FDA) in 2004, highligthing "the benefits on the risk of coronary heart disease 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].

243

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 oxidized LDL (ox-LDL), then becoming foam cells, leading to plaque formation[5].

250 The Effect of Olive Oil on Oxidative Damage in European Populations (EUROLIVE) study was

251 a cross-over fat replacement intervention[58], using OOs with different phenolic content in

252 healthy male volunteers. Its findings led to the current EFSA recommendation (Opinion of the

253 Scientific Committee/Scientific Panel, *EFSA Journal*[50, 77, 78]). A linear increase in HDL-

254 cholesterol levels after 3 weeks was observed after low-, medium-, and high-polyphenol OO

255 consumption: mean change from preintervention, 0.02 mmol/L (95% CI, 0.00 to 0.05 mmol/L),

256 0.03 mmol/L (95% CI, 0.00 to 0.05 mmol/L) , and 0.04 mmol/L (95% CI, 0.02 to 0.06 mmol/L),

257 respectively. Total cholesterol:HDL cholesterol ratio decreased linearly with the phenolic

258 content of the OO. Triglyceride levels decreased by an average of 0.05 mmol/L for all OOs[58].

259 Mean changes from preintervention for ox-LDL levels were 1.21 U/L (95% CI, -0.8 to 3.6 U/L),

260 -1.48 U/L(95% CI, -3.6 to 0.6 U/L) and -3.21 U/L (95% CI, -5.1 to -0.8 U/L) for the low-,

261 medium-, and high-polyphenol OO, respectively, showing a dose-dependent relation with VOO

262 phenolic content[58]. The EFSA confirmed a cause effect relationship between consumption of

263 OO phenolics (standardized by the content of hydroxytyrosol and its derivatives) and protection

264 of LDL cholesterol particles against oxidative damage. To support the EFSA health claim, 5 mg

265 of hydroxytyrosol and its derivatives should be consumed daily in 20 g OO[50], but

266 concentrations in some OOs may be too low to achieve this target in the context of a balanced

267 diet. Moreover, the EFSA Panel commented study design limitations as most human

268 interventions with OO have been conducted in more homogeneous male populations[77] and not

269 in general population.

270

271 The contribution of OO phenolics toward cardiovascular health benefits has been challenged

272 with inconsistent results reported for *ex vivo* resistance of LDL to oxidation[79, 80]. Seven human

273 intervention studies with OO were compared for impact of phenolics on ox-LDL, with no effect

274 seen in five of them[79], possibly explained by artifacts generated during LDL isolation.

275

276 Since the approval of the EFSA claim, both terminology and analytical methodology supporting

277 the dose calculation of hydroxytyrosol and derivatives have been appraised. Mastralexi *et al.*[81]

278 commented on the weaknesses of the claim terminology namely the term "olive oil polyphenols"

279 is not entirely clear and accurate as "olive oil" is a generic term for the type of oil, and the basic

280 structure of OO phenolic compounds do not coincide with a "polyphenolic" structure;

281 accordingly "virgin olive oil bioactive phenols" is a more appropriate term. Others also

282 commented about the lack of robust and reliable methods for quantifying phenolic compounds in
283 OO. A simple and robust method for routine analysis of hydroxytyrosol and tyrosol was
284 proposed[81, 82] based on hydrolysis of the polar fraction of OO. This was followed by
285 development and validation of a ^{1}H NMR method enabling direct measurement of tyrosol and
286 hydroxytyrosol derivatives, as well as oleocanthal and oleacein in OO, overcoming analytical
287 issues such as chromatographic peak broadening[19].

288

289 **Cardioprotective mechanisms of oleic acid**
290 OO intake has been related with a decrease on blood pressure with oleic acid regarded as being a
291 major contributor to this effect, as evidenced in animal models[83]. Chronic oral administration of
292 VOO (rich in oleic acid), triolein (a triacylglyceride with three oleic acid moieties) or oleic acid
293 over 14 days significantly reduced systolic blood pressure in rats (-26 ± 4 for VOO and -21 ± 3
294 mm Hg for triolein, $p<0.001$, and -17 ± 1.9 mm Hg for oleic acid $p<0.05$) when compared to the
295 control group that received water. Similarly acute (2 h) treatments with either VOO or triolein
296 also significantly reduced systolic blood pressure when compared to the control group
297 (-20 ± 0 mm Hg, $p < 0.001$, and -14 ± 2 mm Hg, respectively, $p < 0.05$) with oleic acid again
298 significantly reducing systolic blood pressure (-13.0 ± 0.3 mm Hg; $p < 0.001$). In contrast,
299 chronic treatment with the trans-monounsaturated fatty acid elaidic (18:1n-9) or the saturated
300 fatty acid stearic acid (18:0) did not significantly affect blood pressure. Results show that
301 saturation and cis/trans double bond arrangement are implicated with the cardioprotective effect
302 of the long chain fatty acid in this animal model at high dose levels[83]. Similar significant results
303 were obtained after VOO and oleic acid intake in an animal model of hypertension using
304 spontaneously hypertensive rats[83].
305 The molecular mechanisms were evaluated by measuring signaling proteins involved in the
306 control of blood pressure in the aorta. OO intake increases oleic acid levels in membranes, which
307 regulate membrane lipid structure and impact on G protein-mediated signaling, causing a
308 reduction in blood pressure[84]. Unlike its analogues elaidic and stearic acid, oleic acid, due to its
309 cis-18:1n-9 structure, regulates cellular membrane lipid structure and the α_2 receptor system
310 involved in the control of blood pressure ($\alpha_{2A/D}$ - adrenoreceptor/G protein/adenylyl cyclase-
311 cAMP/PKA) as demonstrated *in vitro*[84] and *in vivo*[83]. Oleic acid can also contribute to heart
312 health via intramyocardial triglyceride turnover[85], which is reduced in pressure-overloaded
313 failing hearts. In this situation oleate (derivative of oleic acid) upregulated triglyceride dynamics

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314 when compared to palmitate (derivative of palmitic acid and major saturated fatty acid of palm

315 oil). This result underscores the importance of the intracellular lipid storage type on nuclear

316 receptor signaling and contractility[85] in diseased hearts.

317 An important driver of vasorelaxation is nitric oxide, a free radical which readily reacts with fats

318 and proteins. Nitro-fatty acids are mediators of cardiovascular signaling actions[86] as these

319 compounds relax blood vessels, attenuate platelet activation, and reduce inflammation[87, 88].

320 Both oleic acid and linoleic acid are unsaturated fatty acids that after reaction with nitrite may

321 form nitro-fatty acids. Nitro-oleic acid mediated antihypertensive signaling actions were shown

322 in a mouse model[89]. The mechanism was attributed to the inhibition of soluble epoxide

323 hydrolase by nitro-fatty acids, thus lowering blood pressure in an angiotensin II-induced

324 hypertension[89]. It is however unclear how the extent of nitrite in the human diet may contribute

325 to nitration of dietary fat, and the physiological relevance of this finding.

326

327 **Role of phenolic compounds on endothelium protection**

328 Oxidative stress and reactive oxygen species (ROS) have been implicated in endothelial damage,

329 progression to atherosclerosis, injury in sustained myocardial infarction and ischemia

330 reperfusion[76, 90-92]. Monocytes and macrophages are critical cells that are involved in

331 atherosclerosis. These cells produce proinflammatory cytokines, such as IL-1β, TNF-α and

332 C-reactive protein (CRP), which induce the expression of adhesion molecules like intercellular

333 adhesion molecule-1 (ICAM-1), vascular-cell adhesion molecule-1 (VCAM-1), and E-selectin[93].

334 Meanwhile, oxidative stress through ROS production promotes the expression of the adhesion

335 molecules on the endothelium[94].

336 Expression of adhesion molecules attracts circulating monocytes inducing their adherence to the

337 endothelium. OO phenolic compounds have been shown to act on endothelium protection as

338 evidenced in *in vitro* assays with typical OO phenolic compounds and less on *in vivo* circulating

339 metabolites. OO phenolic extract, oleuropein aglycone or homovanillic alcohol (metabolite of

340 hydroxytyrosol) had inhibitory effects on VCAM-1, ICAM-1 and E-selectin surface expression

341 in human umbilical vascular endothelial cells, using TNF-α as pro-inflammatory stimulus[95].

342

343 Endothelium dysfunction refers to an impairment of endothelium-dependent vasorelaxation

344 caused by a loss of NO bioactivity in the vessel wall. In animal models with rats oral

345 hydroxytyrosol administration was tested on NO production and platetet function[96]. Results

346　showed that hydroxytyrosol administration (100 mg/kg/day) increased vascular NO production

347　by up to 34.2% ($p < 0.01$) and inhibited platelet aggregation for 50% inhibitory dose of

348　48.25 mg/day for hydroxytyrosol ($p<0.01$) when compared to control group (treated with

349　isotonic saline solution). Animal dose translation to humans allowed us to conclude that the

350　effective hydroxytyrosol doses tested would be above the expected intake through OO daily. The

351　reported benefits would only be achievable through nutraceutical supplementation.

352

353　**Endothelium repair: matrix metalloproteinases and olive oil**

354　Matrix metalloproteinases (MMPs) play a role in endothelium repair. Macrophages resident in

355　human and experimental atherosclerosis co-localize with and release active MMPs including the

356　gelatinase MMP-9, which is specialized in the digestion of basement membrane collagens and

357　elastin, and is implicated in atherogenesis, unstable coronary syndromes, and in aortic

358　aneurysms[97]. Accumulating evidence points to the MMPs as major molecular mediators of

359　arterial diseases[97]. Collagens, types 1 and 3, are the main proteins in arterial walls being also

360　present in the thickened intima of atherosclerotic lesions[98, 99]. Fragments of collagens found in

361　urine are present as a result of proteolytic activity in arterial walls and other vascular structures.

362　Collagen type 1 or 3 fragments were up-regulated in urine in coronary artery disease (CAD)

363　patients[100]. Increase in collagen degradation is related with an increase on collagenases

364　circulation, such as MMP-9, as shown in patients with CAD[101].

365　In an *in vitro* study hydroxytyrosol (1-10 μM) reduced MMP-9 ($IC_{50} = 10$ μmol/L, $p< 0.05$) and

366　COX-2 induction in activated human monocytes, with phorbol myristate acetate (PMA)[102].

367　These effects were mediated by inhibition of transcription factor NF-κB and protein kinase C

368　(PKC) α and PKCβ1 activation[102]. Results are in line with previous *in vitro* reports that showed

369　inhibition of MMP-9 on endothelial cells by OO phenolics namely hydroxytyrosol in PMA

370　induced cells[103], and oleuropein aglycone in TNF-α induced cells by acting on NF-κB[94]. No

371　hydroxytyrosol activity on MMP-9 was found in TNF-α induced cells[94].

372　The discriminatory polypetides that increase in CAD includes collagen type 1 and 3 fragments

373　with a C-terminal GxPGP motif[104]. Increase on these polypeptides would come from a protease

374　decrease activity possibly related with chemical change of the substrate (e.g.: oxidative damage)

375　thus inhibiting it acting at a specific site, or a decrease in circulating levels by lack of enzyme

376　activation. MMP-2 is secreted in an inactive form (pro MMP-2) and several factors can promote

377   its activation such as plasmin[105] and thrombin[106]. Other mechanisms that involve proteinases or

378   oxidative stress can also activate MMP-2[107]. Therefore antioxidants, as phenolic compounds,

379   might have a role on MMP-2 activation and published data indicate phenolic compounds from

380   red wine[108] and green tea[109] as acting on prevention of thrombin-induced activation of MMP-2

381   in vascular smooth cells.

382

383   We evaluated the impact of a 6-week OO supplementation in healthy adults on urinary proteomic

384   biomarkers of CAD in a randomized, parallel, controlled, double-blind study[110]. This study was

385   the first to describe the significant impact of daily OO supplementation on highly specific

386   disease biomarkers for CAD. Analysis of urinary proteomic profiles at baseline and endpoint

387   enabled the identification of 12 sequenced peptides that were significantly regulated toward

388   healthy scoring. Eight of them included four collagen α-1(I) chain, one α-2 (1) chain, one α-2(V)

389   chain, and one α-2(VI) chain fragments. Changes in circulating concentrations of collagenases

390   may mediate these changes in the urinary fingerprint. Therefore with more data or in future

391   intervention studies with OO it would be interesting to link urinary fragments to the proteases

392   involved in their generation. This predictive analysis would enable looking at the peptide

393   cleavage sites studying the MMPs up or downregulated with OO intervention.

394

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

396   foods based on the major risk factors of cardiovascular disease. However marker such as

397   lipoprotein profile, blood pressure, endothelial function, inflammation and oxidative stress have

398   no direct link to the disease itself but are merely associated with it. There is a great need for more

399   biomarkers that appear as a direct result of the disease itself[63, 67].

400

401

4. 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, MMPs 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 recognized as the source of choice for proteomic biomarker investigations. It has a number of advantages such as being noninvasive and can be collected by untrained personnel. Urine is produced by renal filtration of the plasma and approximately 70%

14

433 of proteins in the normal human urinary proteome are of kidney origin, whereas the remaining

434 30% are derived from plasma proteins[120, 121]. It has high stability due to absence of proteolytic

435 agents and the low dynamic range of analyte concentration facilitates the detection and

436 quantification of peptides[113, 122].

437 Using capillary electrophoresis coupled with mass spectrometry (CE-MS)[123] urinary biomarker

438 classifiers for the diagnosis of diseases like chronic kidney disease[124], acute kidney injury[125],

439 stroke[126], and coronary artery diseases[104], were already identified, allowing classification of

440 case *versus* control groups with good accuracy[127].

441 Urinary peptides and protein fragments are the end products of proteolytic processes. The

442 different pattern of urinary excretion of peptides when comparing controls and disease patients

443 might indicate their role in the pathophysiology of disease. Therefore changes in the normal

444 urine "fingerprint" (e.g.: presence of collagen fragments) can be used as biomarkers of disease.

445 Besides collagens, common blood proteins (e.g., alpha-1-antitrypsin, hemoglobin, serum

446 albumin, and fibrinogen), and uromodulin were also identified[128] in urine which provides

447 additional proof of the suitability of this sample source for proteomic biomarker studies out with

448 the kidney and urinary tract. Collagens are the most abundant peptides sequenced so far in the

449 CAD biomarker (66% of all peptides)[104], with atherosclerosis associated with an increased

450 synthesis of several extracellular matrix components, including collagen types 1 and 3, elastin,

451 and several proteoglycans[129]. Changes in the circulating levels of collagenases may mediate

452 these changes in peptides represented in the fingerprint, as reported in coronary

453 atherosclerosis[100], and chronic kidney disease[128].

454 The progress in urinary proteomics and the use of multiple biomarker classifiers opens the

455 possibility of establishing new tools adapted to different clinical needs[130], enabling direct

456 monitoring of disease overcoming limitations of indirect measurements.

457

458 **Proteomic *in vitro* studies on olive oil phenolic compounds**

459 Proteomics has been applied in a number of studies of OO phenolic compounds on

460 cardiovascular health using animal and *in vitro* studies. The *in vitro* effects of alperujo extract, an

461 OO production waste product containing phenolic compounds present in olive fruits, were

462 studied on platelet aggregation and changes in the platelet proteome[131]. Nine proteins were

463 differentially regulated by the alperujo extract upon platelet aggregation underlying the anti-

464 platelet effects of the extract. However, like a number of previously mentioned *in vitro* studies,

465 the effective concentrations (40-500 mg/L) were far above the physiologically concentrations

466 achievable by dietary intake.

467 The effects of EVOOs, with low and high in phenolic content, were evaluated in the hepatic

468 proteome in Apoe$^{-/-}$ mice that spontaneously develop atherosclerosis[132]. For 10 weeks the mice

469 were fed with a high fat high cholesterol diet supplemented with 0.15% (w/w) cholesterol and

470 either 20% (w/w) low phenolic EVOO or 20% (w/w) high phenolic EVOO *versus* a control

471 group fed with 0.15% (w/w) cholesterol and 20% (w/w) palm oil. Within this work a range of

472 hepatic antioxidant enzymes differentially regulated by OO[132] were identified. The authors

473 concluded that the up-regulation of a large array of antioxidant enzymes might explain anti-

474 atherogenic mechanisms of EVOOs[132]. Again the dose level was above what could be achieved

475 through dietary intake and translation from an animal model to human has also to be considered.

476

477 **Urinary proteomics biomarkers, olive oil and cardiovascular disease**

478 Atherosclerosis is a process of chronic inflammation, characterized by the accumulation of

479 lipids, cells, and fibrous elements in medium and large arteries[98]. The extent of

480 inflammation, proteolysis, calcification, and neovascularization influences the development of

481 advanced lesions (atheroma plaques) on the arteries[98].

482 Classical risk factors in atherosclerosis (hypertension, LDL-cholesterol, C-reactive protein,

483 aging, smoking, male gender, among others) do not actually measure disease initiation or

484 progression. As such, they cannot be used directly to identify individuals who have developed

485 atherosclerosis and prevent a fatal event[98, 133]. Other, more recent markers that indicate changes

486 in vascular structure can still only be detected once cardiovascular disease has progressed to an

487 advanced stage where drug or surgical intervention is required[134].

488 The analysis of urine samples from diseased and healthy individuals has been used to establish a

489 database of naturally occurring urinary peptides, making a basis for the definition and validation

490 of biomarkers for diagnosis/prognosis/monitoring of a wide range of diseases using proteomic

491 biomarker patterns[128], such as CAD[100], emphasizing that non-invasive proteomics analysis

492 could become a valuable addition to assess cardiovascular disease alongside to other biomarkers

493 which are indicators of cardiovascular risk.

The first time that urinary proteomics was applied to assess cardiovascular health improvements of OO consumption in humans, was in a randomized, 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 chronic kidney disease (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 CE-MS, allowing monitoring of progression and/or effect of treatment[135, 136]. In this 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 of hydroxytyrosol and derivatives for the high phenolic OO), in line with the EFSA and FDA 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 OOs 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.

In a placebo-controlled intervention, Irbesartan (angiotensin II receptor antagonist used for the treatment of hypertension) taken at 300 mg per day 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 OOs 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 triacylglycerols, oxidized LDL, and LDL cholesterol. The overall change in CAD score in a

525 short period of time is more likely due to OO major components, such as fatty acids. However
526 the role of other OO minor components other than phenolic compounds should also be taken into
527 account. Squalene, a polyunsaturated triterpene which makes up 60–75% of the unsaponifiable
528 fraction of OO[137], reduced atherosclerotic lesion size in male mice[138] and further investigation
529 is needed to clarify its role on cardiovascular disease.

530

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

541

5. Exploring olive oil health benefits: perspectives

Although strong evidence from heritability is related with cardiovascular disease 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 (genomics, transcriptomics, proteomics, epigenomics, metabolomics) have the potential, when integrated, to paint a comprehensive picture of the contribution of diet toward the modulation of disease risk[141]. Some trials have shown the impact of OO on down-regulation 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 signaling cascades regulating the activity of transcription factors with impact on gene expression. For instance, phenolic compounds have shown to affect the expression of microRNAs (miRNA)[143]. miRNAs are small, noncoding RNAs 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 miRNA 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 hyperglycemia[147], but also relevant to end organ damage, disease pathogenesis and aging[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 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 anti-glycation properties of OO phenolic compounds, given that glycation is a key driver for tissue damage and is present in all non-communicable disease scenarios.

6. Conclusion

Results outlined in this review provide evidence of health benefits related with OO intake. The reported studies may allow the implementation of primary prevention programs of cardiovascular disease, 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 toward 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 cardiovascular disease risk factors and affect the proteome.

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8. Conflict of Interest

Conflict of interest: Thomas Koeck is employed at Mosaiques Diagnostics, the company that developed the urinary proteomics for CE-MS technology for clinical application. No other authors declare a conflict of interest.
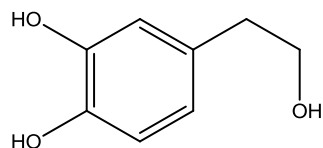
Table 1 – Main classes of phenolic compounds in virgin olive oil
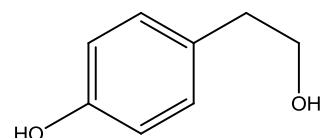
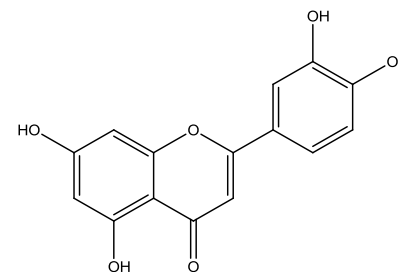**Phenolic acids**
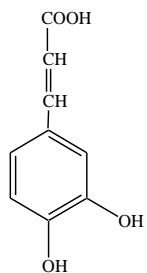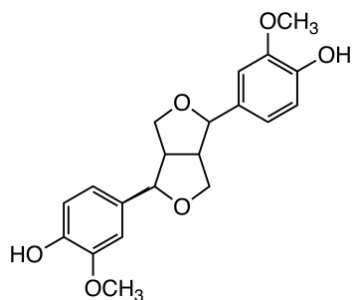
Caffeic acid

Phenolic alcohols

Hydroxytyrosol

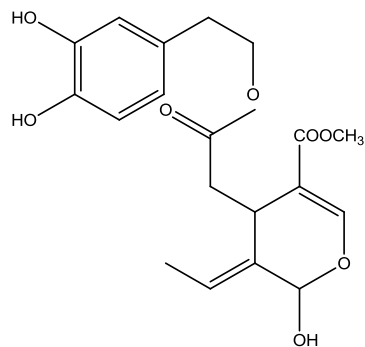**Tyrosol**

Flavonoids
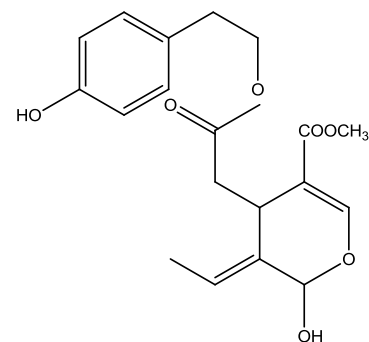
Luteolin

Lignans
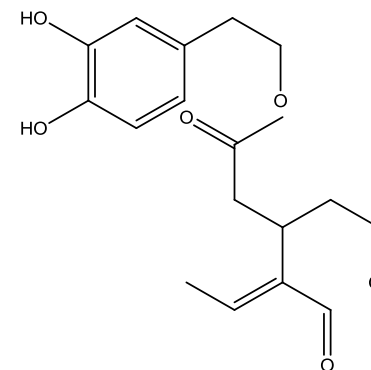
(+) - Pinoresinol

Secoiridoids

Oleuropein aglycone

(3,4-DHPEA-EA)

Ligstroside aglycone

(*p*-HPEA-EA)

Dialdehydic form of deacetoxy

oleuropein (3,4-DHPEA-EDA)

Table 2 Changes in scores of CAD, CKD and diabetes proteomic biomarkers at baseline, middle (3-weeks) and end of intervention (6 weeks)[1]

		Low phenolic olive oil (n = 34)	High phenolic olive oil (*n* = 28)
		Score	Score
CAD proteomic biomarker	baseline	-0.5 ± 0.2	-0.6 ± 0.4
	3 weeks	-0.7 ± 0.3	-0.7 ± 0.3
	6 weeks	-0.8 ± 0.3**	-0.8 ± 0.3*
CKD proteomic biomarker	baseline	-0.4 ± 0.2	-0.4 ± 0.3
	3 weeks	-0.4 ± 0.2	-0.4 ± 0.3
	6 weeks	-0.4 ± 0.2	-0.4 ± 0.2
Diabetes proteomic biomarker	baseline	1.3 ± 0.3	1.3 ± 0.3
	3 weeks	1.3 ± 0.4	1.3 ± 0.3
	6 weeks	1.4 ± 0.4	1.2 ± 0.3

[1]Values are means ± SDs; 95% CIs in parentheses. A repeated-measures ANOVA test was used with statistical significance at $p < 0.05$. ***Compared with corresponding baseline value: *$p < 0.005$, **$p < 0.001$. There were no significant differences in changes between groups. CAD, coronary artery disease; CKD, chronic kidney disease (adapted from Silva *et al.*[104]).

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Figure 1 – Chronic inflammation model and impact on rats paw edema (ANOVA, * $p < 0.001$ *vs.* postive control – Rheumatoid Arthritis, + $p < 0.01$ *vs.* Refined Olive Oil; OHTYR = hydroxytyrosol) (adapted from Silva *et al.*[49])