Impact of a 6-wk olive oil supplementation in healthy adults on urinary proteomic biomarkers of coronary artery disease, chronic kidney disease, and diabetes (types 1 and 2): a randomized, parallel, controlled, double-blind study[1–4]

Sandra Silva, Maria R Bronze, Maria E Figueira, Justina Siwy, Harald Mischak, Emilie Combet, and William Mullen

ABSTRACT

Background: Olive oil (OO) consumption is associated with cardiovascular disease prevention because of both its oleic acid and phenolic contents. The capacity of OO phenolics to protect against low-density lipoprotein (LDL) oxidation is the basis for a health claim by the European Food Safety Authority. Proteomic biomarkers enable an early, presymptomatic diagnosis of disease, which makes them important and effective, but understudied, tools for primary prevention.

Objective: We evaluated the impact of supplementation with OO, either low or high in phenolics, on urinary proteomic biomarkers of coronary artery disease (CAD), chronic kidney disease (CKD), and diabetes.

Design: Self-reported healthy participants (n = 69) were randomly allocated (stratified block random assignment) according to age and body mass index to supplementation with a daily 20-mL dose of OO either low or high in phenolics (18 compared with 286 mg caffeic acid equivalents per kg, respectively) for 6 wk. Urinary proteomic biomarkers were measured at baseline and 3 and 6 wk alongside blood lipids, the antioxidant capacity, and glycation markers.

Results: The consumption of both OOs improved the proteomic CAD score at endpoint compared with baseline (mean improvement: –0.3 for low-phenolic OO and −0.2 for high-phenolic OO; $P < 0.01$) but not CKD or diabetes proteomic biomarkers. However, there was no difference between groups for changes in proteomic biomarkers or any secondary outcomes including plasma triacylglycerols, oxidized LDL, and LDL cholesterol.

Conclusion: In comparison with low-phenolic OO, supplementation for 6 wk with high-phenolic OO does not lead to an improvement in cardiovascular health markers in a healthy cohort. This trial was registered at www.controlled-trials.com as ISRCTN93136746.

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Keywords Mediterranean diet, coronary artery disease, olive oil, phenolics, proteomic biomarkers

INTRODUCTION

Olive oil (OO)[5] is the primary source of fat in the Mediterranean diet and is associated with a lower incidence of chronic diseases, particularly cardiovascular diseases (1–3). The beneficial effects of OO consumption on cardiovascular disease risk factors were recognized by the Food and Drug Administration and European Food Safety Authority (EFSA) and attributed to the high amounts of MUFAs and phenolic compounds (2, 4–6). The EFSA claim, in particular, identified that the daily consumption of 5 mg hydroxytyrosol and derivatives (per 20-g OO dose) could protect LDL particles from oxidative damage if consumed daily (**Supplemental Table 1**) (4).

Phenolic compounds are minor components present in the nonsaponifiable fraction of OO (0.5–1.5% of the oil) along with a great variety of other components, namely hydrocarbons, carotenes, triterpenic compounds, and phytosterols. Health-beneficial properties of phenolic compounds have been attributed to their free-radical scavenging potential (7) and anti-inflammatory properties (3), and more recently, a significant role of these compounds was observed in human in the downregulation of atherosclerosis-related genes (8) and upregulation of genes involved in cholesterol efflux from cells to HDL (9), showing their nutrigenomic effects.

Primary outcomes such as total cholesterol, HDL cholesterol, LDL cholesterol, or oxidized LDL have been traditionally used to study the impact of OO consumption on cardiovascular disease

[1]From the Analytical Services Unit, Instituto de Biologia Experimental Tecnologica, Oeiras, Portugal (SS and MRB); the Analytical Chemistry Department, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal (SS and MRB); the Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal (SS and MRB); the Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal (MRB and MEF); Mosaiques Diagnostics AG, Hannover, Germany (JS and HM); and Human Nutrition, School of Medicine (EC) and the Institute of Cardiovascular and Medical Sciences (WM), College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom.

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[3]Supplemental Table 1 and Supplemental Methods 1 and 2 are available from the "Supplemental data" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[4]Address correspondence to E Combet, Human Nutrition, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom. E-mail: emilie.combet@glasgow.ac.uk.

[5]Abbreviations used: CAD, coronary artery disease; CE-MS, capillary electrophoresis coupled with mass spectrometric detection; CKD, chronic kidney disease; EFSA, European Food Safety Authority; OO, olive oil; ΔCAD, change in coronary artery disease score.

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risk. These markers are not optimal for nutritional primary prevention because improvement would typically be detected late in the disease progression. A new class of biomarkers, urinary proteomic biomarkers, enables early, presymptomatic detection of disease, which makes them a very important, effective set of tools for primary prevention (10). Proteomic biomarkers have been used to define specific diseases such as coronary artery disease (CAD), chronic kidney disease (CKD), and diabetes (types 1 and 2) (11, 12).

A scoring of disease absence, presence, and severity is provided on the basis of the concentration of a group (panel) of urinary peptides measured by capillary electrophoresis coupled with mass spectrometric detection (CE-MS), which allows for the monitoring of progression and effect of treatment (13, 14). An increase or decrease in the concentration of these peptides determines the scoring value of each disease biomarker. Urinary proteomic biomarkers, although offering a presymptomatic insight on disease-prevention strategies, have yet to be exploited in the context of nutrition and health claims.

The primary aim of this study was to evaluate the impact of a 6-wk supplementation with OOs (20 mL/d) either low or high in phenolics on highly specific urinary proteomic biomarkers of CAD, CKD, and diabetes in healthy adults. To the best of our knowledge, this is the first study to directly report changes in presymptomatic disease status and not on proxies associated with high disease risk (e.g., LDL and oxidized LDL). Secondary outcomes measured included plasma lipids profile, glucose, and fructosamine concentrations as well as total antioxidant status.

SUBJECTS AND METHODS

OO characteristics and methods

OOs with a low-phenolic content (refined OO) and high phenolic content (extra virgin OO), both with similar fatty acid profiles, were supplied by a Portuguese OO producer. An analysis of fatty acids, β sitosterol, free acidity, and peroxide values was carried out according to the European Union regulation for OOs (15). The phenolic fraction of the oil was extracted by liquid-liquid extraction on the basis of the protocol of Owen et al. (16) with modifications. The total phenolic content was assessed spectrophotometrically, and a phenolic composition analysis was carried out by using ultraperformance liquid chromatography (diode array and fluorescence detectors) and liquid chromatography–mass spectrometry methodologies (**Supplemental Methods 1**).

Recruitment

Participants (n = 78) aged 18–75 y were recruited via a poster advertisement in Glasgow, United Kingdom, between August and September 2012. Subjects were (self-reported) healthy adult, with no current diagnosed illness, who were nonregular OO consumers, not pregnant or lactating, and not allergic to olives or olive-derived products. Other exclusion criteria included a history of chronic disease of the gastrointestinal tract, taking any form of medication other than the contraceptive pill, having taken antibiotics in the 3 mo before recruitment, being pregnant, lactating, or trying to conceive. Smokers were not excluded from the study, and there was no restriction related to BMI.

The sample-size calculation (G Power 3.1.5 software; Universitat Dusseldorf) used a comparative case-control follow-up design with the change in coronary artery disease score (ΔCAD) as a readout variable. The study was powered to detect a difference in the mean ΔCAD of 0.15 in cases compared with control subjects, assuming a 0.25 (arbitrary units) SD for the ΔCAD (17). With the assumption that control subjects would show no changes in the CAD score (ΔCAD = 0.00) and a 10% dropout rate (consistent with similar nutrition studies), a total of 66 volunteers enabled us to detect a difference between groups (power: 90%; α = 0.05).

The study was approved by the Ethics Committee of the College of Medical, Veterinary & Life Sciences, University of Glasgow (reference 2012071; date: 31 August 2012). Protocols were according to the Declaration of Helsinki, and all participants provided informed consent at recruitment.

Random assignment

The trial followed a randomized, controlled, double-blind parallel design. A total of 78 participants were initially recruited; 9 of these withdrew from the study before random assignment (**Figure 1**). A stratified allocation list was drawn by EC by using block randomization within each stratum (18) according to age (>40 or <40 y of age) and BMI (in kg/m^2; >25 or <25) with block sizes of n = 6. The allocation sequence was concealed to recruiters (SS and GBC), with intervention assigned over the phone by EC. All investigators in Glasgow and participants were blinded to the sample type (samples A and B).

Study design

Participants were supplied with OO at baseline and midintervention visits. For 6 wk, 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 EFSA and Food and Drug Administration recommendations. Participants kept food diaries 2 d before the baseline visit and replicated their diet 2 d before days 21 (middle of intervention) and 42 (end of intervention). No dietary restrictions were put in place, and a simple food-frequency questionnaire, based on the European Prospective Investigation into Cancer and Nutrition consortium food-frequency questionnaire (19), was filled by all participants at recruitment. Compliance was assessed via scrutiny of intake logs alongside the amount of unconsumed oil returned at middle and end visits.

Sample collection

Fasting venous blood was collected at baseline (before ingestion of the first OO dose) and days 21 and 42 in two 12-mL tubes by using EDTA and heparin as an anticoagulant. After centrifugation at 2140 $\times$ g for 5 min at 4°C, plasma was split into aliquots and stored at −80°C until analysis. Spot urine samples (second urine of the day) were collected at the same time points, split into aliquots, and frozen at −20°C without any additional additives as recommended (www.eurokup.org) (20).

Anthropometric measures

Weight was measured to the nearest 100 g by using an electric scale (Seca), height was measured to the nearest millimeter by using a stadiometer (Tanita B.V.), and waist circumference was measured by using a nonelasticated tape at the smallest abdominal

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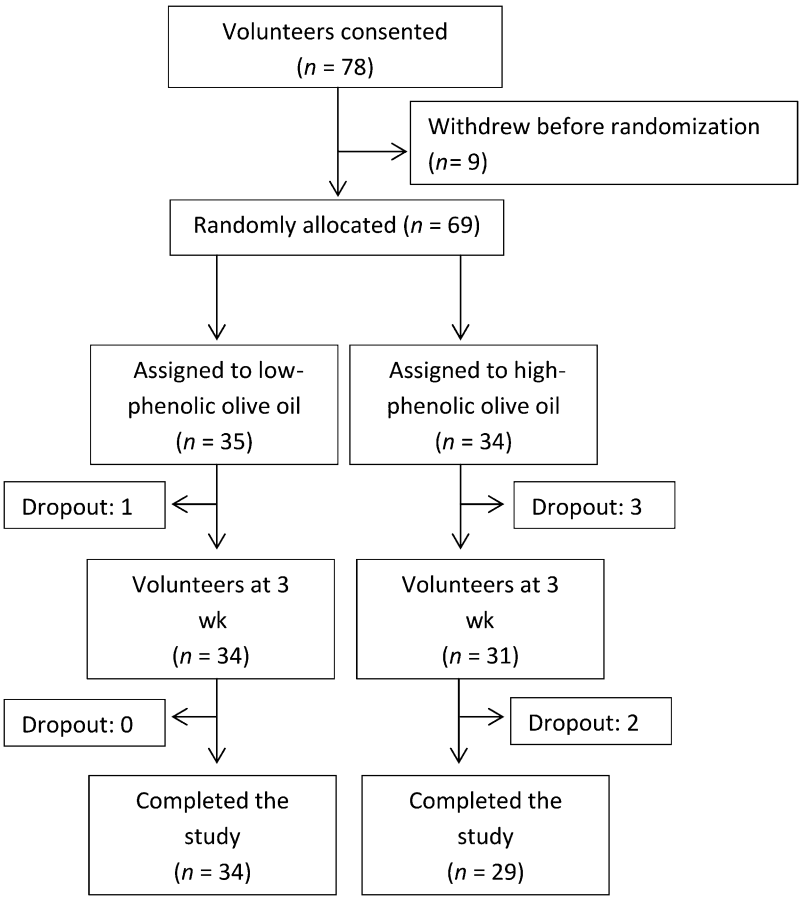

FIGURE 1 Study flow diagram.

position between the lowest rib and iliac crest (with the participant standing after an expiration). Blood pressure was measured by using a digital automatic blood pressure monitor (Omron) with the participant seated after 30 min of rest.

Proteomic analyses by CE-MS

A 0.7-mL aliquot of urine was thawed immediately before use and diluted with 0.7 mL 2 mol urea/L and 10 mmol NH_4OH/L containing 0.02% sodium dodecyl sulfate as described (21). The analysis was carried out as previously described (22) and as shown in **Supplemental Methods 2**. The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of CE-MS measurements were shown elsewhere (21). Of 189 urine samples available, one sample (from the high-phenolic OO group) was excluded because it did not pass quality-control criteria.

Plasma biomarkers

Commercially available kits were used to determine glucose, triacylglycerols, total cholesterol, and HDL cholesterol (Horiba) by using a semiautomatic analyzer (Cobas Mira Plus; ABX Diagnostics). LDL cholesterol was calculated by using the equation of Friedwald et al. (23). Oxidized LDL was analyzed by using an ELISA (Promokine). The ferric-reducing ability of plasma was determined as previously reported (23, 24). Total phenolic content measurements were determined by using the Folin-Ciocalteau method (23, 25) with gallic acid (Sigma Aldrich) as the standard. Samples were analyzed in duplicate with a single analyzer run for each subject. Plasma fructosamine, which is a marker of protein glycation, was analyzed in triplicate as previously described (26). All CVs (CV percentages) were $<10\%$.

Statistical analysis

Statistical analyses were performed with SPSS 22.0 software (SPSS Inc.). The normality of variables was assessed by using the Kolmogorov-Smirnoff test. Nonparametric data were log transformed, and values were expressed as antilogarithms. Differences between treatments for each time point were evaluated by using independent t tests and between groups by using a repeated-measure ANOVA and post hoc testing with Bonferonni correction (statistical significance: $P < 0.05$).

The association with the predefined proteomic biomarkers in each sample was assessed on the basis of the concentration of peptides detected calculated from their normalized logarithmic amplitude in the CE-MS analysis. The peptide amplitude between groups was assessed by using Wilcoxon's t test as previously described (27). All peptides detected in $>30\%$ of samples were individually investigated. Correction for multiple testing to ensure a low number of false positives was performed by assessing the false discovery rate as described by Benjamini and Hochberg (23) and previously described for proteomic data sets (27).

A multivariate linear regression analysis was used to describe the effect of selected independent variables on proteomic CAD results (CAD score). The dependent continuous variable was the ΔCAD at 6 wk with independent variables listed in **Table 1**.

RESULTS

OO characterization

The 2 OOs used in the study had a similar fatty acid profile but different phenolic composition (**Table 2**). The total phenolic content was 18 mg/kg caffeic acid equivalents for the low-phenolic OO compared with 286 mg/kg caffeic acid equivalents for the high-phenolic OO. Hydroxytyrosol and its derivatives were quantified by using liquid chromatography–mass spectrometry for the high-phenolic OO and were 6 mg/20g. Individual phenolic compounds for the high-phenolic OO were *p*-coumaric acid 0.1%, luteolin 0.8%, vanillin 0.9%, apigenin 1%, tyrosol 4%, hydroxytyrosol 13%, and secoiridoids 80%.

Study cohort

From the 69 participants included and allocated, 63 subjects completed the study ($n = 34$ for low-phenolic OO and $n = 29$ for high-phenolic OO), which led to a dropout of 9% (Figure 1). No harms or unintended effects were recorded.

Dietary and anthropometric measures

Baseline characteristics of study participants showed no significant differences between groups (**Table 3**). There was a mean increase ($P < 0.05$) of ~5 mm Hg in systolic blood pressure at endpoint for both oils.

No differences were observed for waist-circumference measurements at 3 wk (midpoint) and 6 wk (endpoint) (**Table 4**). There was no difference between groups in term of weight change (as kilograms of body mass or unit of BMI). The weight change was lower than expected for an energy load of 6880 kcal for the intervention on the basis of the energy provided by 20 mL oil (density: 0.91 kg/L, 9 kcal/g, every day for 6 wk), which would have led to an increase in body weight of 0.9 kg over 6 wk.

Proteomic biomarkers

All participants self-reported as healthy. There were no differences between groups at baseline for their proteomic CAD, CKD, or diabetes scores. A subanalysis by sex did not show a difference between groups for these biomarkers. All baseline CAD scores were below the disease threshold of −0.14 (17). Supplementation with both oils led to decreases of 0.3 (low phenolic) and 0.2 (high phenolic) units in the CAD score at endpoint ($P < 0.005$) (**Table 5**). There was no difference in CAD at endpoint between the 2 groups. Compiled patterns for urinary proteomic biomarkers were obtained from samples for each trial arm to obtain a typical fingerprint. The proteomic profile monitored comprises all urinary peptides, some of which belong to specific biomarkers studies. The CAD biomarker, for example, is composed of 238 peptides. With the comparison of overall proteomic profiles obtained presupplementation and postsupplementation, 112 peptides changed after low-phenolic OO supplementation, and 133 peptides changed for high-phenolic OO trial arm. Of these peptides, 22 peptides belonged to the 238-peptide CAD biomarker (17) as shown in **Table 6**, with 11 peptides changed after low-phenolic OO supplementation, 9 peptides changed after high-phenolic OO supplementation, and 2 peptides changed after supplementation with either oil. The direction of change of identified CAD peptides is presented in Table 6. Of

TABLE 1
Potential confounding variables ($n = 11$) for ΔCAD at 6 wk used in multiple regression analyses[1]

Explanatory variables	Individual factors	Definition of term
Participant characteristics	Age, sex	Age categorized as <40 or ≥40 y.
Anthropometric measures	BMI, waist circumference	BMI (in kg/m^2) categorized as <25 or ≥25; waist circumference (cm) categorized as ≤102 or >102 for men and ≤88 or >88 for women.
Blood pressure	Systolic and diastolic blood pressures	Systolic blood pressure (mm Hg) categorized as normal (90–119), prehypertension (120–139), stage 1 hypertension (140–159), stage 2 hypertension (160–179); diastolic blood pressure (mm Hg) categorized as normal (60–79), prehypertension (80–89), stage 1 hypertension (90–99), stage 2 hypertension (100–109).
Olive oil	Olive oil consumed during the study	Categorized as low- or high-phenolic olive oil.
Clinical plasma biomarkers	Total cholesterol, HDL cholesterol, LDL cholesterol, oxidized LDL, triacylglycerols	Total cholesterol (mmol/L) categorized as normal (<5.2), borderline (5.2–6.2), or high (>6.2); HDL cholesterol (mmol/L) categorized as low (<1.0 for men and <1.3 for women), medium (1.0–1.3 for men and 1.3–1.5 for women), or ideal (≥1.6); LDL cholesterol (mmol/L) ideal at high risk of heart disease (<1.8), ideal at risk of heart disease (<2.6), ideal at no risk of heart disease (2.6–3.3), borderline high (3.4–4.1), high (4.1–4.9), or very high (>4.9); oxidized LDL categorized as ≤287 or >287 μg/L; triacylglycerols (mmol/L) categorized as normal (<1.7), borderline high (1.7–2.2), high (2.3–5.6), or very high (>5.6).

[1] ΔCAD, change in coronary artery disease score.

TABLE 2
Characteristics of studied olive oils[1]

	Low-phenolic olive oil	High-phenolic olive oil
Free acidity,[2] percentage of oleic acid	0.03	0.35
Peroxide value, mEq O_2/kg	0.30	7.5
Fatty acids,[2] %		
14:0	0.02	0.01
16:0	12.1	11.7
16:1	1.1	0.7
17:0	0.1	0.1
17:1	0.2	0.2
18:0	3.0	2.7
18:1	73.2	73.5
18:2	8.7	8.6
18:3	0.7	0.8
20:0	0.4	0.4
20:1	0.3	0.3
22:0	0.1	0.1
24:0	0.1	0.0
β-Sitosterol, mg/kg	1149	1262
α-Tocopherol, mg/kg	160	234
Total polyphenols, mg CAEs/kg	18	286
Total polyphenols, mg GAEs/kg	21	338
Hydroxytyrosol and derivatives, mg/20 g	0.1	6.4

[1]CAE, caffeic acid equivalent; GAE, gallic acid equivalent.
[2]Percentage by mass.

11 peptides modified in the high-phenolic group, all of them were increased after the intervention. On the contrary, intake of the oil of low-phenolic content led to mixed effects on the 13 peptides significantly modified in the group. Supplementation with low- or high-phenolic OO did not have a significant effect on proteomic biomarker score for CKD or diabetes (Table 5).

Plasma biomarkers

There was no significant difference between groups for any of the plasma biomarkers measured. A number of significant changes within groups (between baseline and midpoint or endpoint) were observed (Table 3 and **Table 7**). At midpoint, fasted glucose increased in both low- and high-phenolic OO groups.

TABLE 3
Baseline characteristics of participants in the study[1]

	Low-phenolic olive oil (n = 34)	High-phenolic olive oil (n = 29)
Sex (M/F, n/n)	15/19	12/17
Ethnicity, n	32 Caucasian/2 Asian	26 Caucasian/1 Asian/2 mixed
Age, y	30.2 ± 12.1[2]	31.5 ± 11.9
Weight, kg	73.7 ± 20.0	72.0 ± 15.7
BMI, kg/m^2	23.9 ± 1.2[3]	24.2 ± 4.8
Waist circumference, cm	81.7 ± 14.2	80.5 ± 13.0
Systolic pressure, mm Hg	121.5 ± 15.9	122.5 ± 12.2
Diastolic pressure, mm Hg	73.2 ± 7.4	76.2 ± 8.6
Glucose, mmol/L	4.5 ± 1.2	4.5 ± 1.2
Triacylglycerol,[3] mmol/L	0.8 ± 1.6	0.8 ± 1.6
Total cholesterol, mmol/L	4.6 ± 1.5	4.3 ± 1.5
HDL cholesterol, mmol/L	1.4 ± 0.5	1.5 ± 0.4
LDL cholesterol, mmol/L	2.7 ± 1.1	2.4 ± 1.2
Total cholesterol:HDL cholesterol ratio	3.3 ± 1.0	3.0 ± 1.0
Oxidized LDL,[3] μg/L	128.1 ± 2.4	108.8 ± 2.8
Oxidized LDL:LDL ratio,[3] μg/mg	0.1 ± 2.8	0.1 ± 3.3
Plasma fructosamine (DMF), μmol/L	284.9 ± 38.7	293.2 ± 39.9
Plasma FRAP, Fe^{2+} mmol/L	0.4 ± 0.1	0.3 ± 0.1
Plasma total phenolic content (GAEs), μg/mL	377.7 ± 50.1	374.4 ± 64.8
Habitual fruit and vegetable intake, servings/wk	24.9 ± 16.0	21.8 ± 14.6
Habitual tea and coffee intake, servings/wk	18.5 ± 14.5	17.8 ± 14.6
Habitual fruit juice intake, servings/wk	6.6 ± 6.6	4.8 ± 4.5

[1]There were no significant differences between groups. DMF, 1-deoxy-1-morpholino-D-fructose; FRAP, ferric-reducing ability of plasma; GAE, gallic acid equivalent.
[2]Mean ± SD (all such values).
[3]For non-normally distributed data, antilogs are presented as means ± SDs.

TABLE 4
Anthropometric measures (weight, BMI, and waist circumference) and blood pressure after 3 and 6 wk of intervention[1]

	Low-phenolic olive oil (n = 34)		High-phenolic olive oil (n = 29)	
	Postintervention	Changes relative to baseline	Postintervention	Changes relative to baseline
Weight, kg				
3 wk	73.9 ± 19.7	0.2 ± 1.1 (−0.2, 0.6)	71.9 ± 15.8	−0.1 ± 2.1 (−0.9, 0.7)
6 wk	74.1 ± 19.7	0.4 ± 1.2 (0.1, 0.9)	72.3 ± 15.9	0.3 ± 2.0 (−0.5, 1.1)
BMI, kg/m^2				
3 wk	24.5 ± 5.1	0.1 ± 0.5 (−0.1, 0.3)	24.3 ± 4.8	0.0 ± 0.7 (−0.3, 0.3)
6 wk	24.6 ± 5.1	0.2 ± 0.5 (0.0, 0.4)	24.4 ± 4.8	0.1 ± 0.7 (−0.1, 0.4)
Waist circumference, cm				
3 wk	82.0 ± 13.9	0 3 ± 2.5 (−0.6, 1.1)	81.0 ± 12.1	0.5 ± 3.5 (−0.9, 1.9)
6 wk	82.2 ± 14.0	0.5 ± 2.3 (−0.3, 1.3)	80.9 ± 12.6	0.3 ± 3.3 (−0.9, 1.6)
Systolic pressure, mm Hg				
3 wk	122.0 ± 14.5	0.5 ± 10.6 (−3.2, 4.2)	127.1 ± 11.6	4.7 ± 7.8 (1.7, 7.6)
6 wk	127.4 ± 13.1	5.9 ± 12.6 (1.6, 10.3)*	127.5 ± 13.1	5.0 ± 12.0 (0.5, 9.6)*
Diastolic pressure, mm Hg				
3 wk	74.9 ± 7.1	1.7 ± 6.3 (−0.5, 3.9)	78.8 ± 9.3	2.6 ± 6.0 (0.3, 4.9)
6 wk	75.9 ± 7.9	2.7 ± 8.7 (−0.4, 5.7)	77.4 ± 8.7	1.2 ± 8.1 (−1.8, 4.3)

[1]All 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, $P < 0.05$. There were no significant differences in changes between groups.

HDL cholesterol and fasted glucose increased at endpoint in both low- and high-phenolic OO groups. There was no change in either LDL, oxidized LDL, fructosamine, or plasma total phenols within either group.

The linear regression model with independent variables listed in Table 1 explained ∼48% of the variance in ΔCAD at 6 wk ($r = 0.69$, $r^2 = 0.48$, $P = 0.001$). Age, the phenolic content of OO (high or low), total cholesterol, and LDL cholesterol at baseline were significant predictors ($P < 0.05$; **Table 8**). High-phenolic OO intake improved the ΔCAD at endpoint by 0.16 units, while total cholesterol concentrations above 5.2 mmol/L improved the ΔCAD at endpoint by 0.19 units. Being older (>40 y old) or with an LDL-cholesterol concentration >1.8 mmol/L reduced the CAD-score improvement at endpoint by 0.26 or 0.11, respectively.

DISCUSSION

To our knowledge, this work is the first to show that OO supplementation led to a marked improvement in the urinary proteomic biomarker of diseases over a relatively short period in a healthy population. Our results did not show an important contribution of OO phenolics toward a reduction of the CAD score. We could not detect any difference between groups, and the regression analysis only pointed at a small effect size for the phenolic content of OO as a predictor of the ΔCAD when baseline measurements were accounted for.

The Mediterranean diet, which is characterized by a relative high-fat consumption (mainly from OO), has been linked to reduced risk of cardiovascular mortality (28, 29) along with lower incidence of myocardial infarction in countries from Southern Europe (30). The United Kingdom population is not a big OO

TABLE 5
Changes in scores of CAD, CKD, and diabetes proteomic biomarkers at baseline and middle (3 wk) and end of intervention (6 wk)[1]

	Low-phenolic olive oil (n = 34)		High-phenolic olive oil (n = 28)	
	Score	Changes relative to baseline	Score	Changes relative to baseline
CAD proteomic biomarker				
Baseline	−0.5 ± 0.2	—	−0.6 ± 0.4	—
3 wk	−0.7 ± 0.3	−0.2 ± 0.3 (−0.3, −0.1)	−0.7 ± 0.3	−0.1 ± 0.4 (−0.3, 0.0)
6 wk	−0.8 ± 0.3	−0.3 ± 0.2 (−0.4, −0.2)**	−0.8 ± 0.3	−0.2 ± 0.3 (−0.4, −0.1)*
CKD proteomic biomarker				
Baseline	−0.4 ± 0.2	—	−0.4 ± 0.3	—
3 wk	−0.4 ± 0.2	0.0 ± 0.3 (−0.1, 0.1)	−0.4 ± 0.3	0.1 ± 0.3 (0.0, 0.2)
6 wk	−0.4 ± 0.2	0.0 ± 0.3 (0.0, 0.1)	−0.4 ± 0.2	0.0 ± 0.3 (−0.1, 0.1)
Diabetes proteomic biomarker				
Baseline	1.3 ± 0.3	—	1.3 ± 0.3	—
3 wk	1.3 ± 0.4	0.1 ± 0.4 (−0.1, 0.2)	1.3 ± 0.3	−0.1 ± 0.4 (−0.2, 0.1)
6 wk	1.4 ± 0.4	0.1 ± 0.4 (0.0, 0.2)	1.2 ± 0.3	0.0 ± 0.4 (−0.2, 0.1)

[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.

TABLE 6
Peptides from the CAD biomarker altered after olive oil supplementation (changes from baseline)[1]

Peptide identifier	Mass	CE migration time, min	Peptide sequence	Protein	Change of direction according to treatment group
5661	911.26	34.4	—	—	↓ in LPOO
11989	988.52	22.4	—	—	↑ in HPOO
16859	1082.49	20.8	—	—	↓ in LPOO
21147	1150.56	22.4	TDTEDPAKFK	Retinol-binding protein 4	↑ in HPOO
22625	1169.57	23.7	—	—	↓ in LPOO but ↑ in HPOO
24117	1194.55	26.7	SpGPDGKTGPpGP	Collagen α-1(I) chain	↑ in LPOO
31525	1312.62	22.5	—	—	↑ in HPOO
33135	1338.60	24.0	—	—	↑ in LPOO and ↑ in HPOO
34795	1368.58	21.9	—	—	↓ in LPOO
36988	1408.66	39.1	GPPGppGPpGPPGPPS	Collagen α-1(I) chain	↑ in HPOO
41514	1467.81	24.7	DQSRVLNLGPITR	Uromodulin	↑ in HPOO
42832	1495.68	39.4	GPpGPpGPpGPpGPPSA	Collagen α-1(I) chain	↑ in LPOO
43828	1512.69	26.6	SpGSDGPKGEKGDpGP	Collagen α-2(VI) chain	↑ in LPOO
45445	1539.73	40.3	GpEGPpGEPGpPGPPGP	Collagen α-2(V) chain	↑ in HPOO
46756	1565.69	26.3	—		↑ in LPOO
50212	1613.82	24.0	VGGGEQPPPAPAPRRE	Xylosyltransferase 1	↑ in LPOO
53035	1651.79	40.7	VGPpGPpGPpGPpGPPSAG	Collagen α-1(I) chain	↑ in HPOO
62387	1844.48	34.3	—	—	↓ in LPOO
67382	1936.87	34.8	—	—	↑ in LPOO
89083	2352.05	26.8	KGDRGETGpAGPPGApGAPGAPGPVGP	Collagen α-1(I) chain	↑ in LPOO
91044	2394.08	23.6	FFLPDEGKLQHLENELTHDI	α-1-Antitrypsin	↑ in HPOO
114086	2907.35	36.0	TGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGP	Collagen α-2(I) chain	↑ in HPOO

[1]Composed of 238 individual peptides. Of 22 peptides listed, only 12 have been sequenced. Arrows indicate the direction of change in urinary peptide concentration. CAD, coronary artery disease; CE, capillary electrophoresis; HPOO, high-phenolic olive oil; LPOO, low-phenolic olive oil.

consumer, and there is a potential for OO, as part of the diet, to improve cardiovascular disease risk factors irrespective of the phenolic content.

In this study, the overall ΔCAD was qualified as high for both oils with a highly significant change in such a short period of time with the major OO component of fatty acids as the most likely contributors to the effect observed. A previous placebo-controlled intervention with irbesartan (angiotensin II receptor antagonist used for the treatment of hypertension) taken at 300 mg/d over 2 y in hypertensive type 2 diabetes patients by using the same CAD 238 biomarker panel led to a 0.35-point reduction in the CAD score for the drug-controlled group (17). This decrease was associated with a reduction in the progression to diabetic nephropathy, which is a major vascular complication in diabetes patients. The OO intervention led to a similar change in the biomarker score over a 6-wk period. Our findings, although important, need to be carefully evaluated because an improvement of the biomarker score in a self-reported healthy population does not necessarily translate into a progression toward a healthier status. However, we conclude that OO has a major impact on the CAD biomarker, which warrants addition investigation into its benefits in a less healthy population. This finding also indicates that the CAD biomarker could become a significant tool in nutrition and health intervention studies.

The Mediterranean diet and its components are extensively studied in animal models, generating a hypothesis toward their mechanism of action, such as the interaction between nitrate and fatty acids as an antihypertensive strategy in mice (31). Although model systems have a place in food and nutrition research, there is a lack of methodologies with strong translational value that would allow investigations of nutritional supplementation in healthy free-living individuals. In this article, we position urinary proteomics as a very valuable tool to achieve this goal, since the methodology was to measure changes in the peptidome as a direct result of disease progression or treatment, accounting for pathophysiologic changes. Eight of the 12 sequenced peptides were significantly regulated toward healthy scoring, including 4 collagen α-1(I) chain, one α-2 (1) chain, oneα-2(V) chain, and one α-2(VI) chain fragments. Collagens are the most-abundant peptides sequenced thus far in the CAD biomarker (66% of all peptides) (17), with atherosclerosis associated with an increased synthesis of several extracellular matrix components, including collagen types I and III, elastin, and several proteoglycans (32). Changes in circulating concentrations of collagenases may mediate these changes in peptides represented in the fingerprint as reported in coronary atherosclerosis (10, 18), and CKD (33).

There was no significant difference between low- and high-phenolic OO groups for any of the plasma biomarkers, and differences were seen only within groups (HDL cholesterol and fasted glucose at endpoint for both groups). Increased HDL cholesterol was not detected in the high- compared with low-phenolic OO groups, contrary to the Eurolive study, in which a direct relation between plasma HDL cholesterol and the phenolic content of the OO administered was observed (34). We also did not observe a difference between groups for oxidized LDL concentrations, again in contrast with the findings of the Eurolive study, in which a linear decrease was observed with the increasing OO phenolic content (34). The reasons for these differences may be 3-fold and include power, design, and choice of target population. Our study was not powered to detect differences between (or within) groups for plasma biomarkers such as oxidized LDL. With an effect size of 0.17 for changes in

TABLE 7
Changes in plasma biomarkers at 3 and 6 wk after intervention[1]

	Low-phenolic olive oil (n = 34)				High-phenolic olive oil (n = 29)			
	Postintervention at 3 wk	Changes relative to baseline at 3 wk	Postintervention at 6 wk	Changes relative to baseline at 6 wk	Postintervention at 3 wk	Changes relative to baseline at 3 wk	Postintervention at 6 wk	Changes relative to baseline at 6 wk
Glucose, mmol/L	5.1 ± 1.2	0.5 ± 1.1 (0.1, 0.9)*	5.0 ± 1.2	0.50 ± 1.3 (0.02, 0.97)*	5.0 ± 1.2	0.5 ± 1.1 (0.03, 0.91)*	5.0 ± 1.3	0.6 ± 1.0 (0.2, 1.0)*
Triacylglycerol,[2] mmol/L	0.9 ± 1.5	1.0 ± 1.5 (0.9, 1.2)	0.9 ± 1.5	1.1 ± 1.5 (1.0, 1.3)	0.9 ± 1.6	1.04 ± 1.4 (0.9, 1.2)	0.9 ± 1.7	1.1 ± 1.4 (0.9, 1.3)
Total cholesterol, mmol/L	5.1 ± 1.8	0.5 ± 1.4 (−0.03, 1.0)	4.9 ± 1.8	0.4 ± 1.6 (−0.2, 0.9)	4.8 ± 1.9	0.4 ± 1.5 (−0.2, 1.0)	5.0 ± 2.1	0.7 ± 1.6 (0.01, 1.4)
HDL cholesterol, mmol/L	1.7 ± 0.7	0.2 ± 0.5 (0.06, 0.4)	1.7 ± 0.6	0.2 ± 0.4 (0.1, 0.4) *	1.7 ± 0.6	0.2 ± 0.6 (−0.1, 0.40)	1.7 ± 0.6	0.3 ± 0.6 (0.04, 0.48)*
LDL cholesterol, mmol/L	2.9 ± 1.2	0.2 ± 1.0 (−0.1, 0.6)	2.8 ± 1.4	0.1 ± 1.1 (−0.3, 0.5)	2.6 ± 1.4	0.2 ± 0.9 (−0.2, 0.6)	2.8 ± 1.6	0.4 ± 1.0 (−0.1, 0.8)
Total cholesterol: HDL cholesterol ratio	3.2 ± 0.8	−0.1 ± 0.6 (−0.4, 0.1)	3.1 ± 0.9	−0.3 ± 0.5 (−0.4 to −0.1)	2.9 ± 0.9	−0.1 ± 0.5 (−0.3, 0.1)	2.9 ± 1.0	−0.1 ± 0.4 (−0.3, 0.1)
Oxidized LDL,[2] μg/L	116.9 ± 2.2	0.9 ± 1.3 (0.9, 1.0)	126.4 ± 2.3	1.0 ± 1.4 (0.9, 1.1)	117.0 ± 3.1	1.0 ± 1.3 (0.9, 1.1)	117.7 ± 3.0	1.0 ± 1.4 (0.8, 1.1)
Oxidized-LDL:LDL ratio,[2] μg/mg	0.1 ± 2.4	0.9 ± 1.8 (0.7, 1.1)	1.3 ± 1.3	1.0 ± 1.3 (0.9, 1.1)	0.1 ± 3.5	0.9 ± 1.5 (0.8, 1.1)	0.1 ± 3.4	0.9 ± 1.5 (0.7, 1.0)
Fructosamine (DMF), μmol/L	272.0 ± 61.0	−14.9 ± 66.1 (−38.7, 9.0)	282.4 ± 44.7	−2.4 ± 51.4 (−20.3, 15.5)	282.6 ± 46.0	−6.2 ± 58.2 (−30.2, 17.9)	301.9 ± 41.8	8.5 ± 52.6 (−14.3, 31.2)
FRAP, mmol Fe^{2+}/L	0.4 ± 0.1	−0.05 ± 0.09 (−0.08, −0.01)*	0.4 ± 0.1	0.03 ± 0.11 (−0.01, 0.06)	0.3 ± 0.1	−0.02 ± 0.11 (−0.07, 0.03)	0.4 ± 0.1	0.03 ± 0.07 (−0.0, 0.1)
Total phenolic content (GAEs), μg/mL	390.2 ± 60.7	9.9 ± 74.4 (−16.9, 36.7)	394.2 ± 52.0	16.5 ± 74.6 (−9.5, 42.6)	356.0 ± 71.8	−17.4 ± 91.5 (−55.1, 20.4)	364.2 ± 79.6	−2.4 ± 100.7 (−45.9, 41.2)

[1]All 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, $P < 0.05$. There were no significant differences in changes between groups. DMF, 1-deoxy-1-morpholino-D-fructose; FRAP, ferric-reducing ability of plasma; GAE, gallic acid equivalent.

[2]For nonnormally distributed data, antilogs are presented as means ± SDs.

TABLE 8
Multiple regression analysis summary for participant characteristics, anthropometric measures, and clinical plasma biomarkers with ΔCAD levels at 6 wk[1]

	Unstandardized coefficient, β	SE	Standardized coefficient, β	P
Age	−0.258	0.093	−0.387	0.008*
Sex	0.081	0.068	0.142	0.237
Olive oil type	0.164	0.069	0.289	0.022*
Systolic blood pressure	0.067	0.061	0.171	0.274
Diastolic blood pressure	−0.114	0.086	−0.200	0.194
BMI	−0.075	0.095	−0.120	0.434
Waist circumference	−0.109	0.131	−0.138	0.410
Total cholesterol	0.193	0.086	0.472	0.029*
HDL cholesterol	0.013	0.048	0.033	0.789
LDL cholesterol	−0.112	0.044	−0.537	0.014*
Oxidized LDL	0.105	0.085	0.140	0.222
Triacylglycerols	0.150	0.127	0.186	0.245

[1]Correlation coefficient (r) = 0.69, determination coefficient (r^2) = 0.48, P = 0.001 (ANOVA). *Significant predictors, $P < 0.05$. ΔCAD, change in coronary artery disease score.

oxidized LDL between low- and high-phenolic OO groups in the Eurolive study, a total sample of 858 participants for a parallel design (80% power, 1 tail) would be required.

An alternative study design could have involved fat replacement, principally to avoid weight gain. However, we adopted a supplementation design to avoid metabolic changes potentially associated with decreased consumption of other fat types. The daily intake of 20 mL OO/d should have led to an extra 6880 kcal ingested over the duration of the study, equivalent to a projected 0.9-kg weight gain. OO consumption is associated with satiation effects (3), which may explain the lower weight gain observed in this study. The lack of significant weight gain in the high-phenolic OO group may have been related to the organoleptic characteristics of the high–phenolic-content OO. Participants provided feedback about the bitter and spicy taste of this OO because of its phenolic content (35) (the low-phenolic OO was characterized as sweet tasting). The taste might have led to lower compliance (not observed via scrutiny of intake logs and returned oil volumes) and also a decrease in other foods consumed by this group (satiation and a reduced desire to eat after intake of the bitter and spicy oil).

Our design was a randomized controlled intervention of supplementation, whereas the Eurolive study had a crossover design in men only, with inherent reduced variability, and OO as fat replacement. In our multiple regression modeling, sex did not predict the ΔCAD. A subanalysis of our results by sex did not highlight different outcomes for the trial. Our parallel design, which included a broad range of participants in term of ages and body composition with a low dropout rate (<10%), may offer better translational value, because it took in account the variability in the population, which is an important consideration for evidenced-based guideline preparation. A selection of a third, non-OO placebo would have potentially strengthened the design; however, finding an acceptable fat source that could be used in a blinded manner presents additional difficulties (e.g., structure and taste). The use of the general population overcame study-design limitations previously commented on by the EFSA panel (4) because most human interventions have been conducted in more-homogeneous male populations (4).

The regression analysis highlighted that some variables may modulate the impact of an OO supplementation in a very low or zero OO consumer population. These variables were age, baseline LDL-cholesterol concentrations, total-cholesterol concentrations, and the phenolic content of the oil. These variables are important to consider during implementation of future OO interventions for the primary prevention of diseases in such populations.

We measured the antioxidant capacity and total phenolic content of plasma and showed no impact of supplementation on these markers consistent with the following facts: *1*) these measurements are recognized as nonspecific markers of exposure to high-polyphenol diets, and *2*) it is unlikely that dietary supplementation is linked to a direct antioxidant effect (36).

Protein glycation is relevant to end organ damage, disease pathogenesis, and aging (37), and olive phenolic compounds have been reported as potent inhibitors of the formation of advanced glycation end products (38). With glycation occurring in short-lived plasma proteins and longer-lived intracellular proteins such as hemoglobin, a 6-wk (42-d) intervention should have been long enough to detect changes in fructosamine concentrations (39). Neither low nor high-phenolic OO had a significant impact on plasma fructosamine concentrations, which was indicative of the null or minimal contribution of the supplemented oil phenolics on pathways relevant to protein glycation, including radical scavenging or steric inhibition of protein glycation (40). This finding may have been due to quantity as much as quality because phenolics are known to exert differential antioxidant and antiglycative activities depending on the structure (1, 38, 41, 42). Additional minor variations were observed during and after supplementation with both oils, whereby a marginal increase (~0.5 SD, within the normoglycemic range) for fasted glucose concentrations (43) and blood pressure (~5 mm Hg, within the normal blood pressure range, and normal fluctuations for systolic blood pressure of ~6 mm Hg (44, 45). Some of these variations could be attributable to the background lifestyle, rest, and activity level of participants.

In conclusion, to our knowledge, this study is the first to describe the significant impact of daily OO supplementation on highly specific disease biomarkers for CAD, independent of the phenolic content of the oils. Although the study provides additional evidence of the beneficial impact of OO, which is a key ingredient of the Mediterranean diet, on cardiovascular health, it especially offers new perspectives on OO applications. The results, obtained in a broad population group by using a parallel design, are highly translatable for guidelines preparation and

nutritional recommendations and will be useful to inform the implementation of large primary-prevention programs in population groups where OO is not a staple.

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The authors' responsibilities were as follows—SS, MEF, and MRB: were responsible for the selection of study materials; EC and WM: designed the study; EC: supervised the recruitment, intervention, and collection of samples as well as plasma analyses and was responsible for the final draft of the manuscript; WM: supervised proteomic analyses and downstream data processing with critical input from JS and HM; SS: collected, assembled, and analyzed data and prepared the first draft of the manuscript; and all authors: were responsible for the interpretation of data and critical review of the manuscript. HM is the founder and co-owner of Mosaiques Diagnostics, which developed the CE-MS technology for clinical application, and JS is employed at Mosaiques Diagnostics. The Sovena Company had no input in the design of the study or its realization. SS, MRB, MEF, EC, and WM had no conflicts of interest.

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Errata

Erratum for Silva et al. Impact of a 6-wk olive oil supplementation in healthy adults on urinary proteomic biomarkers of coronary artery disease, chronic kidney disease, and diabetes (types 1 and 2): a randomized, parallel, controlled, double-blind study. Am J Clin Nutr 2015;101:44–54.

An author's name is spelled incorrectly. In the author list on page 44, "Justina Siwy" should be "Justyna Siwy."

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