Impact of a six-week olive oil supplementation in healthy adults on urinary proteomic biomarkers of coronary artery disease, chronic kidney disease and diabetes (types 1 & 2): a randomized, parallel, controlled, double-blind study.

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

[1] *From Analytical Services Unit, IBET, Oeiras, Portugal (SS, MRB), Analytical Chemistry Department, ITQB, Oeiras, Portugal (SS, MRB), Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal (SS, MRB), iMed.UL/FFUL Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal (MRB, MEF), Mosaiques Diagnostics AG, Hannover, Germany (JS, HM), Human Nutrition, School of Medicine, College of Medical, Veterinary and Medical Sciences, University of Glasgow, Glasgow, UK (EC), Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Medical Sciences University of Glasgow, Glasgow, UK (WM).*

[2] The project was funded by the University of Lisbon (QREN project Azeite+ Global nº 12228 to MRB), in collaboration with the Sovena company (Algés, Portugal), who produces the olive oil used in this study. The company had no input in the design of the study, or its realisation. HM acknowledges support from the EU-FP7 program MASCARA, HEALTH-2011-278249.

[3] Address reprint requests to Emilie Combet, Human Nutrition, School of Medicine, College of Medical, Veterinary and Medical Sciences, University of Glasgow, Glasgow, UK. emilie.combet@glasgow.ac.uk; phone number +44 (0) 141 201 8527.

[4] Abbreviations used: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BMI, Body mass index; CAD, coronary artery disease; CAE, caffeic acid equivalents; CE, capillary

electrophoresis; CI, confidence interval; CKD, chronic kidney disease; DMF, 1-deoxy-1-morpholino-D-fructose; EFSA, European Food Safety Authority; ESI, electrospray; GAE, gallic acid equivalents; HDL, high-density lipoprotein; HPLC, high performance liquid chromatography; LDL, low-density lipoprotein; LLE, liquid-liquid extraction; MS, mass spectrometry; MUFA, monounsaturated fatty acids; OO, olive oil; PVDF, polyvinylidene fluoride ; ox-LDL, oxidized low-density lipoprotein; SE, standard error; SD, standard deviation; SNR, signal to noise ratio; TOF, time of flight; UPLC, ultra performance liquid chromatography; UV, ultraviolet; VIS, visible.

Short running head: Olive oil and proteomic biomarkers of disease

Study ID number: ISRCTN93136746

Website of registration: www.controlled-trials.com

Names for PubMED indexing: Silva, Bronze, Figueira, Siwy, Mischak, Combet, Mullen.

ABSTRACT

Background: Olive oil (OO) consumption is associated with cardiovascular disease prevention due to both its oleic acid and phenolic content. 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 early, pre-symptomatic, diagnosis of disease, making them important and effective, yet understudied, tools for primary prevention**.**

Objective: To evaluate 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: Participants (n=69) were randomly allocated (stratified block randomization according to age and body mass index (BMI) to supplementation with a daily 20mL dose of OO either low or high in phenolics (18 mg/kg *vs.* 286 mg/kg caffeic acid equivalents) for 6 weeks. Urinary proteomic biomarkers were measured at baseline, 3 and 6 weeks, alongside blood lipids, antioxidant capacity, and glycation markers.

Results: Consumption of both OOs improved the proteomic CAD score at end-point compared to baseline (mean improvement – 0.3 for low phenolic and -0.2 for high phenolic olive oils, p<0.01), but not CKD or diabetes proteomic biomarkers. There was however no difference between groups for changes in proteomic biomarker, or any secondary outcomes including plasma triacylglycerols, oxidized LDL and LDL cholesterol.

Conclusions: compared to low phenolic OO, supplementation for 6 weeks with a high phenolic content OO does not lead to an improvement in cardiovascular health markers in a healthy cohort.

KEY WORDS: olive oil, phenolics, randomised controlled parallel design, Mediterranean diet, coronary artery disease, proteomic biomarkers, prevention.

INTRODUCTION

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

Phenolic compounds are minor components present in the non-saponifiable 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), anti-inflammatory properties (3) and more recently a significant role of these compounds was observed in human in the down regulation of atherosclerosis-related genes (8) and up-regulation 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 risk. These markers are not optimal for nutritional primary prevention, as improvement would typically be detected late in the disease progression. A new class of biomarkers, urinary proteomic biomarkers enable early, pre-symptomatic, detection of disease, making 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 (type 1 and type 2) (11,12).

A scoring of disease absence, presence and severity is provided, based on the concentration of a group (panel) of urinary peptides measured by capillary electrophoresis coupled with mass spectrometric detection (CE-MS), allowing monitoring of progression and/or effect of treatment (13, 14). Increase or decrease in the concentration of these peptides determines the scoring value of each disease biomarker.

Urinary proteomic biomarkers, while offering a presymptomatic insight on disease prevention strategies, are 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 weeks suplementation with olive oils (20 mL/day) with either low or high total phenolic content on highly specific urinary proteomic biomarkers of CAD, CKD and diabetes in healthy adults. As far as we are aware, 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, oxidized-LDL). Secondary outcomes measured included plasma lipids profile, glucose and fructosamine levels as well as total antioxidant status.

SUBJECTS AND METHODS

Olive oil characteristics and methods. Olive oils with low phenolic content (refined olive oil) and high phenolic content (extra virgin olive oil), both with similar fatty acid profile, were supplied by a Portuguese olive oil producer. Analysis of fatty acids, β-sitosterol, free acidity and peroxide value was carried out according to European Union regulation for olive oils (15). The phenolic fraction of the oil was extracted by liquid-liquid extraction (LLE) based on the protocol of Owen *et al.* (16) with modifications. Total phenolic content was assessed spectrophotometrically and phenolic composition analysis was carried out by, UPLC (diode array and fluorescence detectors) and LC-MS methodology (**Online Supplementary Method 1**).

Recruitment: Participants (*n*=78), aged 18 to 75, were recruited via poster advertisement in Glasgow, UK, between August and September 2012. They were apparently healthy adults, non-regular olive oil consumers, not pregnant or lactating, not allergic to olives or olive-derived products. Other exclusion criteria included history of chronic disease of the gastrointestinal tract, taking any form of medication other than the contraceptive pill, having taken antibiotics in the 3 months prior to recruitment, being pregnant, lactating or trying to conceive. Smokers were not excluded from the study.

The sample size calculation (G Power 3.1.5 software, Kiel, Germany) used a comparative case-control follow-up design with changes in CAD score (ΔCAD) as readout variable. The study was powered to detect a difference in mean ΔCAD of 0.15 in case compared to control subjects, assuming a 0.25 a.u standard deviation for ΔCAD (17) . Assuming that control subjects would show no changes in CAD score (ΔCAD=0.00) and a 10% drop-out rate (consistent with similar nutrition studies), a total of 66 volunteers would enable to detect 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 (ref 2012071, date: 31/08/2012). Protocols were according to the Declaration of Helsinki and all participants provided informed consent at recruitment.

Randomization: The trial followed a randomized, controlled, double blind parallel design. A total of 78 participants were initially recruited, 9 withdrew from the study prior to randomization (Figure 1). A stratified allocation list using block randomisation within each stratum was drawn by EC (18), according to age (above or below age 40) and body mass index (above or below BMI 25) with block sizes of n=6. The allocation sequence was

concealed to the recruiters (SS, GBC), with intervention assigned over the phone by EC. All investigators in Glasgow and participants were blinded to the sample type (sample A and B).

Study design: Participants were supplied with olive oil at baseline and mid-intervention visits. For 6 weeks, they consumed a daily dose of 20 mL olive oil (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. Participants kept food diaries two days prior to the baseline visit and replicated their diet two days prior to day 21 (middle of intervention) and day 42 (end of intervention). No dietary restrictions were put in place, and a simple food frequency questionnaire, based on the EPIC consortium FFQ (19) was filled by all participants at recruitment. Compliance was assessed via scrutiny of intake logs, alongside the amount of unconsumed oil returned at mid and end visits.

Sample collection: Fasting venous blood was collected at baseline (before ingestion of the first OO dose), days 21 and 42 in two 12 mL tubes, using EDTA and heparin as anti-coagulant. After centrifugation at 2140 ×*g* for 5 min at 4ºC, plasma was aliquoted and stored at -80ºC until analysis. Spot urine samples (second urine of the day) were collected at the same time points, aliquoted and frozen at -20 ºC without any further additives, as recommended (www.eurokup.org) (20).

Anthropometric measurements: Weight was measured to the nearest 100g using an electric scale (Seca, UK), height to the nearest mm using a stadiometer (Tanita B.V., The Netherlands) and waist circumference using a non-elasticated tape at the smallest abdominal position between the lowest rib and the iliac crest (participant standing, after an expiration). Blood pressure was measured using a digital automatic blood pressure monitor (Omron, UK) with the participant seated after 30 minutes 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 of 2 M urea, 10 mM NH_4OH containing 0.02% SDS, as described (21). The analysis was carried out as previously described (22) and in **Online Supplementary Method 2**. Accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements were demonstrated elsewhere (21). Of the 189 urine samples available, one (from the high phenolic OO group) was excluded as not passing the quality control criteria.

Plasma biomarkers: Commercially available kits were used to determine glucose, triacylglycerols, total and HDL cholesterol (Horiba, UK) using a semi-automatic analyzer (Cobas Mira Plus, ABX Diagnostics, France). LDL cholesterol was calculated using the equation of Friedwald (23). Oxidised LDL was analysed using an ELISA assay (Promokine, UK). Ferric reducing ability of plasma (FRAP) was determined as previously reported (23, 24). Total phenolic content measurements was by the Folin-Ciocalteau method (23, 25) using gallic acid (Sigma Aldrich (UK) as standard. Samples were analyzed in duplicate, with a single analyzer run for each subject. Plasma fructosamine, a marker of protein glycation, was analysed in triplicate, as previously described (26). All coefficients of variation (CV%) were below 10%.

Statistical analysis: Statistical analyses were performed by SPSS 22.0 software. Normality of variables was assessed with the Kolmogorov-Smirnoff test. Non-parametric data were log transformed and values expressed as antilogarithm. Differences between treatments for each time point were evaluated by independent t-tests and between groups using repeated measure ANOVA and post-hoc testing with Bonferonni correction (statistical significance: $p < 0.05$). Association with the predefined proteomic biomarkers in each sample was assessed based on the concentration of peptides detected, calculated from their normalized logarithmic amplitude in the CE-MS analysis. Peptide amplitude between groups was assessed with the

Wilcoxon t-test, as previously described (27). All peptides detected in over 30% of the samples were individually investigated. Correction for multiple testing to ensure a low number of false positives was performed assessing the false discovery rate, as described by Benjamini and Hochberg (23) as previously described for proteomic datasets (27). 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 ΔCAD score at 6 weeks, with the independent variables listed in **Table 1**.

RESULTS

Olive oils characterization

The two olive oils used in the study had a similar fatty acid profile but different phenolic composition, **Table 2**. Total phenolic content for the low phenolic olive oil was 18 mg/kg CAE, *versus* 286 mg/kg CAE for the high phenolic olive oil. Hydroxytyrosol and its derivatives were quantified by LC-MS for the high phenolic olive oil and were 6 mg/20g. Individual phenolic compounds for the high phenolic olive oil 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 completed the study (*n*=34 for low phenolic and *n*=29 for high phenolic OO) leading to a drop-out of 9% (Figure 1). No harms or unintended effects were recorded.

Dietary and anthropometric measurements

Baseline characteristics of the study participants showed no statistically significant differences between the groups (**Table 3**).

There was a mean increase (p<0.05) of approximately 5 mm Hg in systolic blood pressure at end-point for both oils.

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

Proteomic biomarkers

All participants self-reported as healthy. There were no differences between groups at baseline for their proteomic CAD, CKD or diabetes scores. Sub-analysis by gender did not show a difference between groups for these biomarkers. All baseline CAD scores were below the disease threshold of -0.14 (17). Neither supplementation with low or high phenolic olive oils had a significant effect on proteomic biomarkers score for CKD or diabetes (**Table 5**). 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). 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 the specific biomarkers studies. The CAD biomarker, for example, is composed of 238 peptides. Comparing the overall proteomic profiles obtained pre and post supplementation, 112 peptides changed after low phenolic OO supplementation, and 133 peptides for high phenolic OO trial arm. Of these, 22 belonged to the 238-peptide CAD biomarker (17), as shown in **Table 6**, with 11 changed after low phenolic OO supplementation, 9 after high phenolic OO supplementation and 2 changed after supplementation with either oil. The direction of change of the identified CAD peptides is

presented in Table 6. Of the 9 peptides modified in the high phenolic group, all were increased after intervention. On the contrary, low phenolic content oil intake led to mixed impact on the 11 peptides significantly modified in that group.

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 mid or end-points) were observed (**Tables 3** and **7)**. At mid-point, fasted glucose increased in both low and high OO groups. HDL cholesterol and fasted glucose increased at end-point in both low and high phenolic oil 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 approximately 48% of the variance in Δ CAD score at 6 weeks ($r = 0.69$, $r^2 = 0.48$, $p = 0.001$). Age, 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 CAD score change at endpoint by 0.16 units, while total cholesterol levels above 5.2 mmol/L improved the CAD score change at endpoint by 0.19 units. Being older (>40 years old), or with an LDL-cholesterol level above 1.8 mmol/L reduced the CAD score improvement at endpoint by 0.26 or 0.11 respectively.

DISCUSSION

This work is the first to demonstrate 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 demonstrate an important contribution of olive oil phenolics toward reduction of the CAD score. We could not detect any difference between groups, and regression analysis only pointed at a small effect size for phenolic content of OO as a predictor of CAD score change, when baseline measurements were accounted for.

The Mediterranean diet, characterized by a relative high-fat consumption (mainly from OO), has been linked to a reduced risk of cardiovascular mortality (28, 29), along with lower incidence of myocardial infarction in countries from Southern Europe (30). The UK population is not a big OO consumer and there is potential for OO, as part of the diet, to improve cardiovascular risk factors irrespective of phenolic content.

In this study, the overall change in CAD score can be qualified as high for both oils, and highly significant change in such a short period of time, with major OO components, the fatty acids, 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 300mg per day over 2 years in hypertensive type 2 diabetes patients, 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, a major vascular complication in diabetes patients. The OO intervention led to a similar change in the biomarker score over a 6 week period. Our findings, while important, need moderated as improvement of the biomarker score in a self-reported healthy population does not necessarily translate into progression toward a "healthier" status. We can however conclude that OO has a major impact on the CAD biomarker, warranting further investigation into its benefits in a less healthy population. It

244 also indicates that the CAD biomarker could become a significant tool in nutrition and health
245 intervention studies.
246 The Mediterranean diet and its components is extensively studied in animal models,
247 generating hypothesis toward their mechanism of action, such as the interaction between
248 nitrate and fatty acids as an anti-hypertensive strategy in mice (31). While model systems
249 have a place in food and nutrition research, there is a lack of methodologies with strong
250 translational value, allowing investigations of nutritional supplementation in health in free
251 living individuals. Here, we position urinary proteomics as a very valuable tool to achieve
252 this, since the methodology measures changes in the peptidome as a direct result of disease
253 progression and/or treatment, accounting for pathophysiological changes. Eight out of the 12
254 sequenced peptides were significantly regulated towards the "healthy scoring", including 4
255 collagen alpha-1(I) chain, , one alpha-2(1) chain, one alpha-2(V) chain and one alpha-2(VI)
256 chain fragments. Collagens are the most abundant peptides sequenced so far in the CAD
257 biomarker (66% of all peptides) (17), with atherosclerosis associated with an increased
258 synthesis of several extracellular matrix components, including collagen types I and III,
259 elastin, and several proteoglycans (32). Changes in the circulating levels of collagenases may
260 mediate these changes in peptides represented in the fingerprint, as reported in coronary
261 atherosclerosis (10, 18), and CKD (33).
262 There was no significant difference between the low and high phenolic OO groups for any of
263 the plasma biomarkers, and differences were seen only within groups (HDL cholesterol and
264 fasted glucose at end point for both groups). An increased HDL cholesterol was not detected
265 in the high phenolic content oil group compared to low phenolic content oil group, contrary to
266 the Eurolive study, where a direct relationship between plasma HDL cholesterol and phenolic
267 content of the OO administered was observed (34). Nor did we observe a difference between
268 groups for oxidized LDL levels, again contrasting with the findings for the Eurolive study,

where a linear decrease was observed with the increasing OO phenolic content (34). The reasons for these differences may be threefold 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 oxidized LDL between the low and high phenolic olive oil groups in the Eurolive study, a total sample of 858 participants for a parallel design (80% power, one 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 the metabolic changes potentially associated with decreased consumption of other fat types. The daily intake of 20 mL per day olive oil 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 content oil group may be related to the organoleptic characteristics of the high phenolic content OO. Participants fed-back about the bitter / spicy taste of this OO, due to 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), but also to a decrease in other foods consumed by this group (satiation, reduced desire to eat following intake of the bitter / spicy oil).

Our design was a randomised controlled intervention of supplementation, while the Eurolive study had a cross-over design in men only, with inherent reduced variability, and OO as fat replacement. In our multiple regression modeling, gender did not predict CAD score change. Sub-analysis of our results by gender 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, since it

takes in account the variability in the population, an important consideration for evidenced-based guideline preparation. Selection of a third, non-olive oil 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 general population overcomes study design limitations previously commented by the EFSA Panel (4) as most human interventions have been conducted in more homogeneous male populations (4).

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

We measured antioxidant capacity and total phenolic content of plasma, and showed no impact of supplementation on these markers, consistent with the facts that 1) these measurements are recognized as non-specific 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 haemoglobin, a 6 weeks (42 days) intervention should have been long enough to detect changes in fructosamine levels (39). Neither low nor high phenolic OO had a significant impact on plasma fructosamine levels, indicative of the null/minimal contribution of the supplemented oil phenolics on pathways relevant to protein glycation, including radical scavenging or steric inhibition of protein glycation (40). This may be a matter of quantity as much as quality, as phenolics are known to exert differential

antioxidant and antiglycative activities depending on structure (1),(38, 41)-(42). Further minor variations were observed during/after supplementation with both oils: a marginal increase (~ 0.5 SD, within the normoglycaemic range) for fasted glucose levels (43), and blood pressure (approximately 5 mm Hg, within the normal blood pressure range, and normal fluctuations for systolic blood pressure, ~ 6 mmHg (44, 45)). Some of these variations can be attributable to the participants' background lifestyle, rest and activity level.

In conclusion, 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. While providing additional evidence of the beneficial impact of OO, a key ingredient of the Mediterranean diet, on cardiovascular health, it especially offers new perspectives on olive oil applications. The results, obtained in a broad population group using a parallel design, are highly translatable for guidelines preparation, nutritional recommendations and will be useful to inform the implementation of large primary prevention programs in population groups where OO is not a staple.

Acknowledgements

The authors express their gratitude to all of the subjects for their time and dedication to the study and would like to thank Maria da Graça Beja da Costa for technical assistance during clinical trial intervention, Frances Cousins for her help with both sample management and blood lipid analysis and Dr. Angelique Stalmach for technical assistance in proteomic analysis using CE-MS.

The project was funded by the University of Lisbon, in collaboration with the Sovena company (Algés, Portugal), who produces the olive oil used in this study. The company had no input in the design of the study, or its realisation. Harald Mischak acknowledges supportfrom the EU-FP7 program MASCARA, HEALTH-2011-278249.

Conflict of interest: Harald Mischak is the founder and co-owner of Mosaiques Diagnostics, who developed the CE-MS technology for clinical application, and Justina Siwy is employed at Mosaiques Diagnostics. No other authors declare a conflict of interest.

Author contributions:

SS, MEF and MRB were responsible for selection of study materials. EC and WM designed the study. EC supervised the recruitment, intervention and collection of samples, as well as plasma analyses. WM supervised the proteomic analyses and down-stream data processing. SS collected, assembled, analysed the data, and prepared the first draft of the manuscript. All authors were responsible for the interpretation of the data and critical review of the manuscript.

References

1. Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S, La Guardia M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr Res Rev 2005;18(1):98-112. doi: S0954422405000089 [pii]

10.1079/NRR200495.

2. Covas MI. Olive oil and the cardiovascular system. Pharmacol Res 2007;55(3):175-86. doi: S1043-6618(07)00033-3 [pii]

10.1016/j.phrs.2007.01.010.

3. Lopez-Miranda J, Perez-Jimenez F, Ros E, De Caterina R, Badimon L, Covas MI, Escrich E, Ordovas JM, Soriguer F, Abia R, et al. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. Nutr Metab Cardiovasc Dis 2010;20(4):284-94. doi: S0939-4753(09)00316-0 [pii]

10.1016/j.numecd.2009.12.007.

4. European Comission, Comission Regulation (EC) No 432/2012 of 16 May 2012.

5. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), “anti-inflammatory properties” (ID 1882), “contributes to the upper respiratory tract health” (ID 3468), “can help to maintain a normal function of gastrointestinal tract” (3779), and “contributes to body defences against external agents” (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/20061. EFSA Journal 2011;9 (4)(2033).

6. Scientific Opinion on the substantiation of health claims related to olive oil and maintenance of normal blood LDL-cholesterol concentrations (ID 1316, 1332), maintenance of normal (fasting) blood concentrations of triglycerides (ID 1316, 1332), maintenance of normal blood HDL-cholesterol concentrations (ID 1316, 1332) and maintenance of normal blood glucose concentrations (ID 4244) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal 2011 9 (4)(2044).

7. Fito M, Covas MI, Lamuela-Raventos RM, Vila J, Torrents L, de la Torre C, Marrugat J. Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. Lipids 2000;35(6):633-8.

8. Martin-Pelaez S, Covas MI, Fito M, Kusar A, Pravst I. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol Nutr Food Res 2013;57(5):760-71. doi: 10.1002/mnfr.201200421.

9. Farras M, Valls RM, Fernandez-Castillejo S, Giralt M, Sola R, Subirana I, Motilva MJ, Konstantinidou V, Covas MI, Fito M. Olive oil polyphenols enhance the expression of cholesterol efflux related genes in vivo in humans. A randomized controlled trial. The Journal of nutritional biochemistry 2013;24(7):1334-9. doi: 10.1016/j.jnutbio.2012.10.008.

10. Zimmerli LU, Schiffer E, Zurbig P, Good DM, Kellmann M, Mouls L, Pitt AR, Coon JJ, Schmieder RE, Peter KH, et al. Urinary proteomic biomarkers in coronary artery disease. Molecular & cellular proteomics : MCP 2008;7(2):290-8. doi: 10.1074/mcp.M700394-MCP200.

11. Snell-Bergeon JK, Maahs DM, Ogden LG, Kinney GL, Hokanson JE, Schiffer E, Rewers M, Mischak H. Evaluation of urinary biomarkers for coronary artery disease, diabetes, and diabetic kidney disease. Diabetes technology & therapeutics 2009;11(1):1-9. doi: 10.1089/dia.2008.0040.

12. Maahs DM, Siwy J, Argiles A, Cerna M, Delles C, Dominiczak AF, Gayrard N, Iphofer A, Jansch L, Jerums G, et al. Urinary collagen fragments are significantly altered in diabetes: a link to pathophysiology. PloS one 2010;5(9). doi: 10.1371/journal.pone.0013051.

13. Fliser D, Novak J, Thongboonkerd V, Argiles A, Jankowski V, Girolami MA, Jankowski J, Mischak H. Advances in urinary proteome analysis and biomarker discovery. Journal of the American Society of Nephrology 2007;18(4):1057-71.

14. Julian BA, Suzuki H, Suzuki Y, Tomino Y, Spasovski G, Novak J. Sources of urinary proteins and their analysis by urinary proteomics for the detection of biomarkers of disease. Proteom Clin Appl 2009;3(9):1029-43. doi: 10.1002/prca.200800243.

15. European Comission, Comission Regulation (EC) No 2568/91 of 11 July 1991 and amendments.

16. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 2000;36(10):1235-47. doi: S0959-8049(00)00103-9 [pii].

17. Delles C, Schiffer E, von Zur Muhlen C, Peter K, Rossing P, Parving HH, Dymott JA, Neisius U, Zimmerli LU, Snell-Bergeon JK, et al. Urinary proteomic diagnosis of coronary artery disease: identification and clinical validation in 623 individuals. Journal of hypertension 2010;28(11):2316-22. doi: 10.1097/HJH.0b013e32833d81b7.

18. Altman DG, Bland JM. How to randomise. BMJ 1999;319(7211):703-4.

19. Kroke A, Klipstein-Grobusch K, Voss S, Moseneder J, Thielecke F, Noack R, Boeing H. Validation of a self-administered food-frequency questionnaire administered in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study: comparison of energy, protein, and macronutrient intakes estimated with the doubly labeled water, urinary nitrogen, and

repeated 24-h dietary recall methods. The American journal of clinical nutrition 1999;70(4):439-47.

20. Mischak H, Kolch W, Aivaliotis M, Bouyssie D, Court M, Dihazi H, Dihazi GH, Franke J, Garin J, Gonzalez de Peredo A, et al. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. Proteomics Clin Appl 2010;4(4):464-78. doi: 10.1002/prca.200900189.

21. Theodorescu D, Fliser D, Wittke S, Mischak H, Krebs R, Walden M, Ross M, Eltze E, Bettendorf O, Wulfing C, et al. Pilot study of capillary electrophoresis coupled to mass spectrometry as a tool to define potential prostate cancer biomarkers in urine. Electrophoresis 2005;26(14):2797-808. doi: 10.1002/elps.200400208.

22. Mullen W, Gonzalez J, Siwy J, Franke J, Sattar N, Mullan A, Roberts S, Delles C, Mischak H, Albalat A. A pilot study on the effect of short-term consumption of a polyphenol rich drink on biomarkers of coronary artery disease defined by urinary proteomics. J Agric Food Chem 2011;59(24):12850-7. doi: 10.1021/jf203369r.

23. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18(6):499-502.

24. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry 1996;239(1):70-6. doi: 10.1006/abio.1996.0292.

25. Rossi Sa. Colorimetric of total phenolics with phosphomolybdate - phosphotungstic acid reagent. Am J Enol Vitic 1965:144-58.

26. Vlassopoulos A, Lean ME, Combet E. Role of oxidative stress in physiological albumin glycation: a neglected interaction. Free radical biology & medicine 2013;60:318-24. doi: 10.1016/j.freeradbiomed.2013.03.010.

27. Dakna M, Harris K, Kalousis A, Carpentier S, Kolch W, Schanstra JP, Haubitz M, Vlahou A, Mischak H, Girolami M. Addressing the challenge of defining valid proteomic biomarkers and classifiers. BMC Bioinformatics 2010;11:594. doi: 1471-2105-11-594 [pii]

10.1186/1471-2105-11-594.

28. Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. Adherence to Mediterranean diet and health status: meta-analysis. BMJ 2008;337:a1344. doi: 10.1136/bmj.a1344.

29. Urpi-Sarda M, Casas R, Chiva-Blanch G, Romero-Mamani ES, Valderas-Martinez P, Arranz S, Andres-Lacueva C, Llorach R, Medina-Remon A, Lamuela-Raventos RM, et al. Virgin olive oil and nuts as key foods of the Mediterranean diet effects on inflammatory biomakers related to atherosclerosis. Pharmacol Res 2012;65(6):577-83. doi: 10.1016/j.phrs.2012.03.006.

30. Tunstall-Pedoe H, Kuulasmaa K, Mahonen M, Tolonen H, Ruokokoski E, Amouyel P. Contribution of trends in survival and coronary-event rates to changes in coronary heart disease mortality: 10-year results from 37 WHO MONICA project populations. Monitoring trends and determinants in cardiovascular disease. Lancet 1999;353(9164):1547-57.

31. Charles RL, Rudyk O, Prysyazhna O, Kamynina A, Yang J, Morisseau C, Hammock BD, Freeman BA, Eaton P. Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase. Proceedings of the National Academy of Sciences of the United States of America 2014. doi: 10.1073/pnas.1402965111.

32. Lee RT, Libby P. The unstable atheroma. Arteriosclerosis, thrombosis, and vascular biology 1997;17(10):1859-67.

33. Coon JJ, Zurbig P, Dakna M, Dominiczak AF, Decramer S, Fliser D, Frommberger M, Golovko I, Good DM, Herget-Rosenthal S, et al. CE-MS analysis of the human urinary proteome for biomarker discovery and disease diagnostics. Proteomics Clin Appl 2008;2(7-8):964. doi: 10.1002/prca.200800024.

34. Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Baumler H, et al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Annals of internal medicine 2006;145(5):333-41.

35. Favati F, Condelli N, Galgano F, Caruso MC. Extra virgin olive oil bitterness evaluation by sensory and chemical analyses. Food Chem 2013;139(1-4):949-54. doi: S0308-8146(13)00133-7 [pii]

10.1016/j.foodchem.2013.01.098.

36. Hollman PC, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, Serafini M, Scalbert A, Sies H, Vidry S. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. J Nutr 2011;141(5):989S-1009S. doi: 10.3945/jn.110.131490.

37. Levi B, Werman MJ. Long-term fructose consumption accelerates glycation and several age-related variables in male rats. J Nutr 1998;128(9):1442-9.

38. Kontogianni VG, Charisiadis P, Margianni E, Lamari FN, Gerothanassis IP, Tzakos AG. Olive leaf extracts are a natural source of advanced glycation end product inhibitors. Journal of medicinal food 2013;16(9):817-22. doi: 10.1089/jmf.2013.0016.

39. Reaven PD, Herold DA, Barnett J, Edelman S. Effects of Vitamin E on susceptibility of low-density lipoprotein and low-density lipoprotein subfractions to oxidation and on protein glycation in NIDDM. Diabetes care 1995;18(6):807-16.

40. Vlassopoulos A, Lean ME, Combet E. Protein-phenolic interactions and inhibition of glycation - combining a systematic review and experimental models for enhanced physiological relevance. Food Funct 2014. doi: 10.1039/c4fo00568f.

41. Ayse Karadag BO, Samim Saner. Review of Methods to Determine Antioxidant Capacities. Food Anal Methods 2009;2:41-60.

42. Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M, Gallina-Toschi T, Lercker G, Compagnone D, Fernandez-Gutierrez A. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. J Agric Food Chem 2005;53(23):8918-25. doi: 10.1021/jf0515680.

43. Association AD. Standards of Medical Care in Diabetes - 2014. Diabetes care 2014;37(Supplement 1):S14-S80.

44. Lawes C VHS, Law M, Elliott P, MacMahon S, Rodgers A (2004) High blood pressure. In: Ezzati M, Lopez AD, Rodgers A, Murray CJL. Edtion ed. In: WHO, ed. Comparative quantification of health risks: Global and regional burden of disease attributable to selected major risk factors. Geneva, 2004:281–389.

45. Brookes L. The Updated WHO/ISH Hypertension Guidelines. Medscape 2004.

TABLE 1 Potential Confounding Variables (n = 11) for Δ CAD score at 6 weeks used in multiple regression analyses

Explanatory variables	Individual factors	Definition of term
Participant characteristics	Age, gender	Age categorized as: < 40 or ≥ 40 years.
Anthropometric measurements	BMI, waist circumference	BMI (kg/m^2) categorized as < 25 or ≥ 25; Waist circumference (cm) categorized as: ≤ 102 or > 102 for men, ≤ 88 or > 88 for women.
Blood pressure	Systolic and diastolic blood pressure	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 type	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, men or < 1.3, women), medium (1.0-1.3, men or 1.3-1.5, women); 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 for no risk of heart disease (2.6-3.3), borderline high (3.4-4.1), high (4.1-4.9), 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), very high (> 5.6).

TABLE 2 Characteristics of the studied olive oils

	Low phenolic olive oil	**High phenolic olive oil**
Free acidity (% oleic acid)[1]	0.03	0.35
Peroxide value (mEq O_2/kg)	0.30	7.5
Fatty acids (%)[1]		
C14:0	0.02	0.01
C16:0	12.1	11.7
C16:1	1.1	0.7
C17:0	0.1	0.1
C17:1	0.2	0.2
C18:0	3.0	2.7
C18:1	73.2	73.5
C18:2	8.7	8.6
C18:3	0.7	0.8
C20:0	0.4	0.4
C20:1	0.3	0.3
C22:0	0.1	0.1
C24:0	0.1	0.0
β-sitosterol (mg/kg)	1149	1262
α-tocopherol (mg/kg)	160	234
Total polyphenols (mg/kg CAE)	18	286
Total polyphenols (mg/kg GAE)	21	338
Hydroxytyrosol and derivatives (mg/20 g)	0.1	6.4

[1]percentage by mass.

TABLE 3 Baseline characteristics of participants in the study

	Low phenolic olive oil (*n* = 34)	**High phenolic olive oil (*n* = 29)**
Gender (Male/Female) (n/n)	15/19	12/17
Ethnicity (n/n)	32 Caucasian / 2 Asian	26 Caucasian / 1 Asian / 2 Mixed
Age, years	30.2 ± 12.1	31.5 ± 11.9
Weight, kg	73.7 ± 20.0	72.0 ± 15.7
BMI, kg/m^2	23.9 ± 1.2[1]	24.2 ± 4.8
Waist circumference, cm	81.7 ± 14.2	80.5 ± 13.0
Systolic pressure, mmHg	121.5 ± 15.9	122.5 ± 12.2
Diastolic pressure, mmHg	73.2 ± 7.4	76.2 ± 8.6
Glucose (mmol/L)	4.5 ± 1.2	4.5 ± 1.2
Triacylglycerol (mmol/L)[1]	0.8 ± 1.6	0.8 ± 1.6
Total cholesterol (mmol/L)	4.6 ± 1.5	4.3 ± 1.5
HDL (mmol/L)	1.4 ± 0.5	1.5 ± 0.4
LDL (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 (µg/L)[1]	128.1 ± 2.4	108.8 ± 2.8
Oxidized LDL/LDL ratio (µg/mg)[1]	0.1 ± 2.8	0.1 ± 3.3
Plasma Fructosamine (µM, DMF)	284.9 ± 38.7	293.2 ± 39.9
Plasma FRAP (Fe^{2+} mM)	0.4 ± 0.1	0.3 ± 0.1
Plasma Total phenolic content (µg/mL, GAE)	**377.7 ± 50.1**	**374.4 ± 64.8**
Habitual fruit & vegetable intake (servings / week)	24.9 ±16.0	21.8 ± 14.6
Habitual tea and coffee intake (servings / week)	18.5 ± 14.5	17.8 ± 14.6
Habitual fruit juice intake (servings / week)	6.6 ± 6.6	4.8 ± 4.5

Data are expressed as mean ± SD. [1]For non-normally distributed data antilog are presented as mean ± SD. There were no significant differences between groups. BMI = Body mass index. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

TABLE 4 Changes in anthropometric measurements (weight, BMI and waist circumference) and blood pressure at 3 and 6 weeks after intervention

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

Data are expressed as mean ± SD (95% Confidence Interval). Repeated measure ANOVA test (statistical significance: $p < 0.05$). *p<0.05 *versus* its corresponding baseline. There were no significant differences in changes between groups.

TABLE 5 Changes in CAD, CKD and diabetes proteomic biomarkers scoring at baseline, mid (3-weeks) and end-of-intervention (6 weeks).

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

Data are expressed as mean ± SD (95% Confidence Interval). *p<0.005 *versus* its corresponding baseline. **p<0.001 *versus* its corresponding baseline. Repeated measure ANOVA test (statistical significance: $p < 0.05$). There were no significant differences in changes between groups.

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

Peptide ID	Mass	CE migration time. (min)	Peptide sequence[2]	Protein[2]	Change of direction [3] according to treatment group [4]
5661	911.26	34.4			↓ in LPOO
11989	988.52	22.4			↑ in HPOO
16859	1,082.49	20.8			↓ in LPOO
21147	1,150.56	22.4	TDTEDPAKFK	Retinol-binding protein 4	↑ in HPOO
22625	1,169.57	23.7			↓ in LPOO but ↑ in HPOO
24117	1,194.55	26.7	SpGPDGKTGPpGP	Collagen alpha-1(I) chain	↑ in LPOO
31525	1,312.62	22.5			↑ in HPOO
33135	1,338.60	24.0			↑ in LPOO and ↑ in HPOO
34795	1,368.58	21.9			↓ in LPOO
36988	1,408.66	39.1	GPPGppGPpGPPGPPS	Collagen alpha-1(I) chain	↑ in HPOO
41514	1,467.81	24.7	DQSRVLNLGPITR	Uromodulin	↑ in HPOO
42832	1,495.68	39.4	GPpGPpGPpGPpGPPSA	Collagen alpha-1(I) chain	↑ in LPOO
43828	1,512.69	26.6	SpGSDGPKGEKGDpGP	Collagen alpha-2(VI) chain	↑ in LPOO
45445	1,539.73	40.3	GpEGPpGEPGpPGPPGP	Collagen alpha-2(V) chain	↑ in HPOO
46756	1,565.69	26.3			↑ in LPOO
50212	1,613.82	24.0	VGGGEQPPPAPAPRRE	Xylosyltransferase 1	↑ in LPOO
53035	1,651.79	40.7	VGPpGPpGPpGPpGPPSAG	Collagen alpha-1(I) chain	↑ in HPOO
62387	1,844.48	34.3			↓ in LPOO
67382	1,936.87	34.8			↑ in LPOO
89083	2,352.05	26.8	KGDRGETGpAGPPGApGAPGAPGPVGP	Collagen alpha-1(I) chain	↑ in LPOO
91044	2,394.08	23.6	FFLPDEGKLQHLENELTHDI	Alpha-1-antitrypsin	↑ in HPOO
114086	2,907.35	36.0	TGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGP	Collagen alpha-2(I) chain	↑ in HPOO

[1] Composed of 238 individual peptides
[2] Out of the 22 peptides listed, only 12 have been sequenced
[3] The arrows indicate direction of change in urinary peptide concentration.
[4] LPOO: low polyphenol content olive oil; HPOO: high polyphenol content olive oil

TABLE 7 Changes in plasma biomarkers at 3 and 6 weeks after intervention

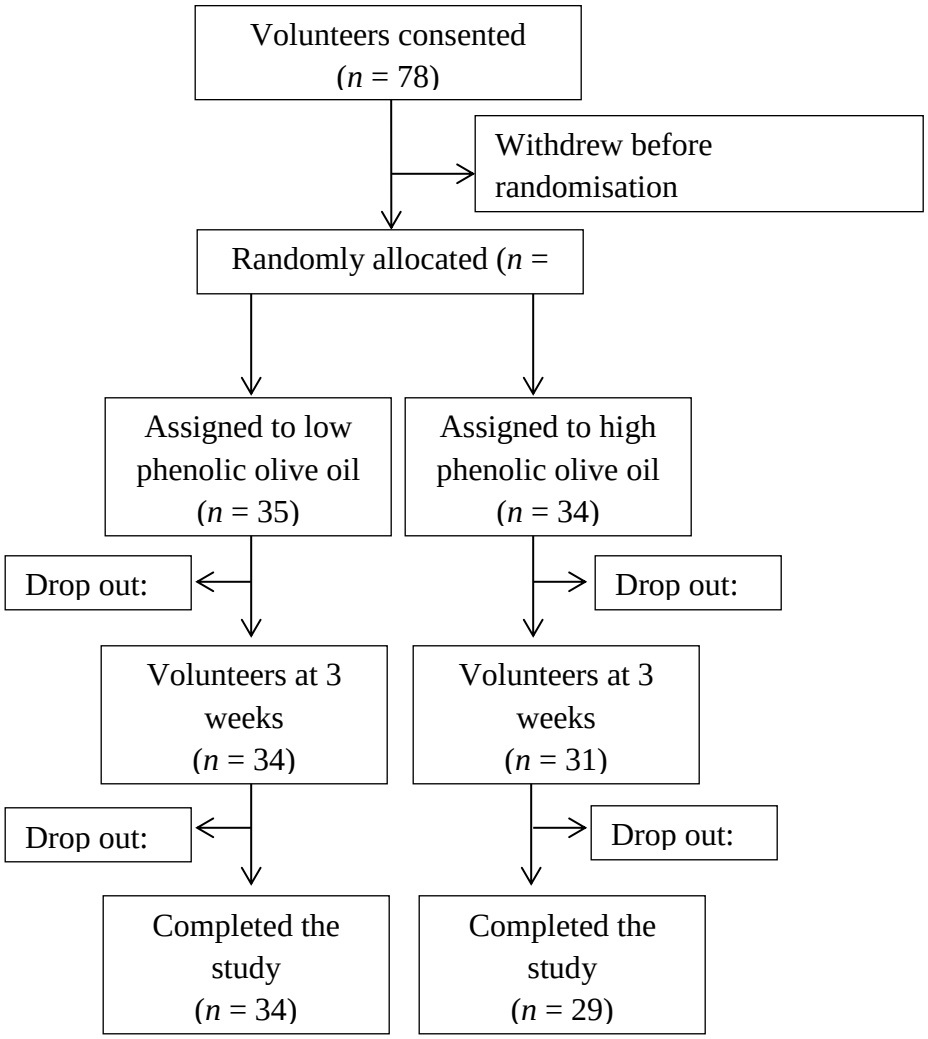
	Low phenolic olive oil (n = 34)				High phenolic olive oil *(n = 29)*			
	Post intervention 3 weeks	**Changes relative to baseline 3 weeks**	**Post intervention 6 weeks**	**Changes relative to baseline 6 weeks**	**Post intervention 3 weeks**	**Changes relative to baseline 3 weeks**	**Post intervention 6 weeks**	**Changes relative baseline 6 weeks**
Glucose (mmol/L)	5.1 ± 1.2	0.5 ± 1.1 (0.1 to 0.9)*	5.0 ± 1.2	0.50 ± 1.3 (0.02 to 0.97)*	5.0 ± 1.2	0.5 ± 1.1 (0.03 to 0.91)*	5.0 ± 1.3	0.6 ± 1.0 (0.2 to 1.0)*
Triacylglycerol (mmol/L)[1]	0.9 ± 1.5	1.0 ± 1.5 (0.9 to 1.2)	0.9 ± 1.5	1.1 ± 1.5 (1.0 to 1.3)	0.9 ± 1.6	1.04 ± 1.4 (0.9 to 1.2)	0.9 ± 1.7	1.1 ± 1.4 (0.9 to 1.3)
Total cholesterol (mmol/L)	5.1 ± 1.8	0.5 ± 1.4 (-0.03 to 1.0)	4.9 ± 1.8	0.4 ± 1.6 (-0.2 to 0.9)	4.8 ± 1.9	0.4 ± 1.5 (-0.2 to 1.0)	5.0 ± 2.1	0.7 ± 1.6 (0.01 to 1.4)
HDL (mmol/L)	1.7 ± 0.7	0.2 ± 0.5 (0.06 to 0.4)	1.7 ± 0.6	0.2 ± 0.4 (0.1 to 0.4) *	1.7 ± 0.6	0.2 ± 0.6 (-0.1 to 0.40)	1.7 ± 0.6	0.3 ± 0.6 (0.04 to 0.48) *
LDL (mmol/L)	2.9 ± 1.2	0.2 ± 1.0 (-0.1 to 0.6)	2.8 ± 1.4	0.1 ± 1.1 (-0.3 to 0.5)	2.6 ± 1.4	0.2 ± 0.9 (-0.2 to 0.6)	2.8 ± 1.6	0.4 ± 1.0 (-0.1 to 0.8)
Total Cholesterol:HDL cholesterol ratio	3.2 ± 0.8	-0.1 ± 0.6 (-0.4 to 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 to 0.1)	2.9 ± 1.0	- 0.1 ± 0.4 (-0.3 to 0.1)
Oxidized LDL (µg/L)[1]	116.9 ± 2.2	0.9 ± 1.3 (0.9 to 1.0)	126.4 ± 2.3	1.0 ± 1.4 (0.9 to 1.1)	117.0 ± 3.1	1.0 ± 1.3 (0.9 to 1.1)	117.7 ± 3.0	1.0 ± 1.4 (0.8 to 1.1)
Oxidized LDL/LDL ratio (µg/mg)[1]	0.1 ± 2.4	0.9 ± 1.8 (0.7 to 1.1)	1.3 ± 1.3	1.0 ± 1.3 (0.9 to 1.1)	0.1 ± 3.5	0.9 ± 1.5 (0.8 to 1.1)	0.1 ± 3.4	0.9 ± 1.5 (0.7 to 1.0)
Fructosamine (µM, DMF)	272.0 ± 61.0	-14.9 ± 66.1 (-38.7 to 9.0)	282.4 ± 44.7	-2.4 ± 51.4 (-20.3 to 15.5)	282.6 ± 46.0	-6.2 ± 58.2 (-30.2 to 17.9)	301.9 ± 41.8	8.5 ± 52.6 (-14.3 to 31.2)
FRAP (Fe2+ mM)	0.4 ± 0.1	-0.05 ± 0.09 (-0.08 to -0.01)*	0.4 ± 0.1	0.03 ± 0.11 (-0.01 to 0.06)	0.3 ± 0.1	-0.02 ± 0.11 (-0.07 to 0.03)	0.4 ± 0.1	0.03 ± 0.07 (-0.0 to 0.1)
Total phenolic content (µg/mL, GAE)	390.2 ± 60.7	9.9 ± 74.4 (-16.9 to 36.7)	394.2 ± 52.0	16.5 ± 74.6 (-9.5 to 42.6)	356.0 ± 71.8	-17.4 ± 91.5 (-55.1 to 20.4)	364.2 ± 79.6	-2.4 ± 100.7 (-45.9 to 41.2)

Data are expressed as mean ± SD (95% Confidence Interval).[1] For non-normally distributed data antilog are presented as mean ± SD. *p<0.05 versus its corresponding baseline. Repeated measure ANOVA test (statistical significance: $p < 0.05$). There were no significant differences in changes between groups.

TABLE 8 Multiple regression analysis summary for participants' characteristics, anthropometric measurements and clinical plasma biomarkers with Δ CAD score levels at 6 weeks

Δ CAD score 6 weeks	**Correlation coefficient (*r*)**	**Determination coefficient (r^2)**	**ANOVA significance (*p*)**	
Model	0.690	0.476	0.001	
	Unstandardized Coefficient (B)	**Standard error**	**Standardized Coefficient (β)**	***p*-value**
Age	-0.258	0.093	-0.387	***0.008***
Gender	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

FIGURE 1 – Study flow diagram



Supplementary Method 1

Liquid-liquid extraction (LLE), based on the protocol of Owen *et al.* (1) with modifications, for total phenolic content, individual phenolic compounds percentage and hydroxytyrosol and derivatives determination

Methanol *p.a.* (Fischer, UK) (3 mL) was added to 2 g of olive oil and homogenized for 5 min with a vortex. After that, two phases were obtained by centrifugation at 3214 ×*g* for 30 min and the alcoholic phase was separated. This step was repeated twice and the extracts were combined. Then, the alcoholic extracts were evaporated to dryness at 35 ºC and dissolved in 2 ml methanol:water (80:20, v/v) using water Milli-Q quality (18.2 MΩ.cm, Millipore Corp, Bedford, MA, USA).

Total phenolic content was determined in triplicate, assessed using methodology based on previous work (2) and expressed as caffeic acid (CAE) and gallic acid (GAE) equivalents. Briefly, 3.5 mL of caffeic or gallic acid (Sigma, USA) standard solutions (1-6 mg/L) or diluted sample was mixed with 0.1 mL of Folin–Ciocalteu reagent (Fluka, Switzerland). The solution was kept for 3 min without agitation. Sodium carbonate (Panreac, Spain) 35% was added to the solution. After 60 min of incubation at room temperature, the absorbance was read against a reagent blank at 750 nm on a Beckmann DU-70 (USA) spectrophotometer, with 1cm glass cuvette. Phenolic composition of samples was determined analyzing the LLE extract by UPLC after filtering using PVDF 0.22 µm filters. Standard phenolic compounds were used to obtain calibration curves to quantify the compounds in the olive oils. Apigenin, luteolin, hydroxytyrosol and tyrosol were purchased from Extrasynthese (Genay, France). Vanillin was purchased from

Fluka (Switzerland) and caffeic acid, *p*-coumaric acid and vanillic acid were purchased from Sigma (USA). Standard stock solutions of each compound were prepared in methanol and dilutions in methanol:water (80:20, v/v). The UPLC system consisted of an AcQuity UPLC® Class H equipped with a quaternary pump system Waters (Milford, MA, USA) coupled to PDA and fluorescence detectors AcQuity UPLC® Class H. For phenolic compounds analysis an AcQuity UPLC® BEH Shield RP18 column (1.7 µm, 150 mm × 2.1 mm i.d.) with a VanGuard pre-column AcQuity UPLC® BEH C18 (2.1 mm×5 mm, 1.7 µm) also from Waters was used. During the analysis, the column was kept at 35 ºC and the flow-rate was 0.4 mL/min. The mobile phase was composed by MilliQ water and acetic acid Panreac (Spain): eluent A, water/acetic acid (100/0.01, v/v) and eluent B, acetonitrile (Carlo Erba, Italy). The elution started at 10% of eluent B, then was linearly increased to 34% of eluent B in 10min, kept isocratic for 2.0 min, further increased to 90% of eluent B in 6 min and then to 100% B in 0.5 min and kept isocratic for 6.5 min. The injection volume was 2.5 µL. For α-tocopherol determination by UPLC, samples were weighed (5.00 ± 0.01 g) and dissolved in 10 mL hexane (Carlo Erba, Italy) 0.1% BHT (Sigma, USA). A dilution (factor of 2.5) was then prepared with 2-propanol (Carlo Erba, Italy). The final solution was filtered through 0.22 µm filters, before UPLC analysis with fluorescence detection. The α-tocopherol (Sigma, USA) content was determined in duplicate against a standard calibration curve prepared in 2-propanol (10-90 mg/L). The chromatographic column used was an AcQuity UPLC® BEH Shield RP18 column (1.7 µm, 50 mm × 2.1 mm i.d.) equipped with a VanGuard pre-Column AcQuity UPLC® BEH C18 (2.1 mm×5 mm, 1.7 µm) also from Waters. During the analysis, the column was kept at 35 ºC and the flow-rate was 0.8 ml/min. The mobile phase was a mixture of acetonitrile, 2-propanol and water (85:7.5:7.5, v/v)

and it was run isocratic for 4 minutes with an injection volume of 2.5 µL. The fluorescence detector was set to λ_{exc} 296 nm/ λ_{em} 330 nm.

For hydroxytyrosol and derivatives assessment in olive oils HPLC-PDA-Exact Mass-MS analysis was carried out on a Thermo Accela HPLC system comprising an autosampler with a sampler cooler maintained at 15 °C, a photodiode array detector scanning from 200 to 600 nm. Samples (5 µL) were injected onto a 150 × 4.6 mm C18 Kinetex column (Phenomenex) maintained at 40 °C and eluted with a 5 −90% gradient of 0.01% acetic acid and acetonitrile at 250 µL/min over 25 min. The eluant was directed to the electrospray interface of an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Samples were run in negative ionization mode, and the scan range was from 130 to 2000 amu with resolution set to 50,000. Quantification of hydroxytyrosol derivatives was by exact mass measurements, using hydroxytyrosol and tyrosol as standards, and expressed as hydroxytyrosol and tyrosol equivalents.

Supplementary Method 2

Urinary proteomic analysis

To remove higher molecular mass proteins, such as albumin and immunoglobulin G, the sample was filtered using Centrisart centrifugation filter devices (20 kDa MW), supplied by Sartorius Stedim Limited (U.K.) at 2000 ×*g* until 1.1 mL of filtrate was obtained. This filtrate was then applied onto a disposable PD-10 desalting column supplied by GE Healthcare Biosciences AB (Bedford, USA) equilibrated in 0.01% NH_4OH (Sigma Aldrich,UK) in HPLC-grade H_2O to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich polypeptides present. Finally, all samples were lyophilized, stored at 4 ºC, and suspended in HPLC-grade H_2O, shortly before CE-MS analyses, as described (3). CE-MS analyses were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, USA) online coupled to a micrOTOF MS (Bruker Daltonic, Germany) as described previously (3, 4). The ESI sprayer (Agilent Technologies, USA) was grounded, and the ion spray interface potential was set between -4 and -4.5 kV. Data acquisition and MS acquisition methods were automatically controlled by the CE via contact close- relays. Spectra were accumulated every 3 s, over a range of *m/z* 350 to 3000.

Data processing and cluster analysis: Mass spectra were processed using MosaiquesVisu software which includes peak picking, deconvolution and deisotoping and has been previously described (5,6).

Supplementary Table 1 - EFSA health claims approved for olive oil components (7)

Compound(s)	Claim	Requirements
Oleic acid	Replacing the saturated fats with unsaturated fats in the diet contributes to maintaining normal levels of blood cholesterol. Oleic acid is an unsaturated fat.	The claim can only be used for foods containing at least 45% of unsaturated fatty acids
Phenolic compounds	The olive oil polyphenols contribute to the protection of blood lipids against oxidation reactions	The claim can be used only for oil containing at least 5 mg of hydroxytyrosol and its derivatives (e.g., tyrosol and oleuropein complex) per 20 g of oil. In order to bear the claim, the consumer must receive information that the beneficial effect is obtained with a daily intake of 20 g of olive oil
Vitamin E	Vitamin E helps protect cells against oxidation reactions	The claim may be used only for food which is at least a source of vitamin E (at least 18 mg vitamin E per L/kg)

References

1. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 2000;36(10):1235-47. doi: S0959-8049(00)00103-9 [pii].

2. Stamatakis G, Tsantila N, Samiotaki M, Panayotou GN, Dimopoulos AC, Halvadakis CP, Demopoulos CA. Detection and isolation of antiatherogenic and antioxidant substances present in olive mill wastes by a novel filtration system. J Agric Food Chem 2009;57(22):10554-64. doi: 10.1021/jf9016288.

3. Theodorescu D, Wittke S, Ross MM, Walden M, Conaway M, Just I, Mischak H, Frierson HF. Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. Lancet Oncol 2006;7(3):230-40. doi: S1470-2045(06)70584-8 [pii]

10.1016/S1470-2045(06)70584-8.

4. Wittke S, Mischak H, Walden M, Kolch W, Radler T, Wiedemann K. Discovery of biomarkers in human urine and cerebrospinal fluid by capillary electrophoresis coupled to mass spectrometry: towards new diagnostic and therapeutic approaches. Electrophoresis 2005;26(7-8):1476-87. doi: 10.1002/elps.200410140.

5. Mullen W, Gonzalez J, Siwy J, Franke J, Sattar N, Mullan A, Roberts S, Delles C, Mischak H, Albalat A. A pilot study on the effect of short-term consumption of a polyphenol rich drink on biomarkers of coronary artery disease defined by urinary proteomics. J Agric Food Chem 2011;59(24):12850-7. doi: 10.1021/jf203369r.

6. Neuhoff N, Kaiser T, Wittke S, Krebs R, Pitt A, Burchard A, Sundmacher A, Schlegelberger B, Kolch W, Mischak H. Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2004;18(2):149-56. doi: 10.1002/rcm.1294.

7. European Comission, Comission Regulation (EC) No 432/2012 of 16 May 2012.