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Article

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Virgin olive oil enriched with its own phenolics or complemented with thyme phenols improves DNA protection against oxidation and antioxidant enzyme activity in hyperlipidemic subjects

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Abstract

The effect of virgin olive oil (VOO) enriched with its own polyphenols (PC) and/or thyme-phenols on the protection of oxidative DNA damage and antioxidant endogenous enzymatic system (AEES) were estimated in 33 hyperlipidemic subjects after the consumption of VOO, VOO enriched with its own PC (FVOO), or complemented with thyme PC (FVOOT). Compared to pre-intervention, 8-hydroxy-2'-deoxyguanosine (marker for DNA damage) decreased in the FVOO intervention and to a greater extent in the FVOOT with a parallel significant increase in olive and thyme phenolic biomarkers. Superoxide Dismutase (AEES enzyme) significantly increased in the FVOO intervention and to a greater extent in the FVOOT with a parallel significant increase in thyme phenolic metabolites. When comparing all three oils, FVOOT appeared to have the greatest effect in protecting against oxidative DNA damage and improving AEES. The sustained intake of a FVOOT improves DNA protection against oxidation and AEES probably due to a greater bioavailability of thyme PC in hyperlipidemic subjects.

Keywords:

virgin olive oil; phenol enrichment; thyme phenolics; hyperlipidemia; oxidative stress; enzymatic antioxidants

INTRODUCTION

Virgin olive oil (VOO) is a typical food found in the Mediterranean diet and several experimental and human studies have revealed that it has a unique phenolic composition with relevant biological properties related to its anti-oxidant capacity and also modulating gene expression [1]. The measurement of the antioxidant status of biological fluids is used as an early warning sign of possible disease onset and also as an indicator of the status of the antioxidant endogenous enzymatic system (AEES) [2].

The polyphenol content of commercial VOOs is influenced by multiple agronomic and technological factors. In this context, the enrichment of VOOs with its own phenolic compounds (PC) is an interesting strategy to increase and standardize the daily intake of PC in the real food matrix without increasing caloric intake. Additionally, flavoring olive oils with herbs and spices can improve their PC profile. The leafy parts of thyme and its essential oil have been used in foods for flavour, aroma, and preservation and also in traditional medicines. Thyme is rich in phenolics, e.g., favonoids, phenolic acids, and monoterpenes [3]. Thus, the enrichment of VOOs with complementary PC from thyme was proposed as a novel approach to investigate the combined or synergic beneficial effects of PC from different sources. In previous studies, we observed that when PC form olive and thyme in a combined extract were administered to rats, an enhanced bioavailability of olive PC occurred in the presence of thyme PC [4]. In agreement with these findings, when the volunteers from the VOHF Project (Virgin Olive oil and HDL Functionality (VOHF): a model for tailoring functional food) ingested VOO enriched with own PC plus complementary PC from thyme, an improved bioavailability of olive PC was also observed

[5]. The combination of different PC sources might, therefore, be a promising approach to improve not only the bioavailability but also a consequent enhancement of their biological effects.

Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT), which are of endogenous origin and constitute the first line of antioxidant defense, provide a real state of long-term defense against oxidative stress. The activity of this first line of antioxidants may be modulated by dietary bioactive compounds. Thus, PC provided by VOO can protect against systemic oxidation, which is modulated by the main antioxidant endogenous enzymatic system (AEES) [6]. The protection of body cells and molecules such as DNA, proteins and lipids from oxidative damage could be considered as a beneficial physiological effect. Different markers of oxidative damage or repair to molecules should preferably be determined in the same study and could be useful if appropriate techniques are used for its analysis [7]. In this regard, mass spectrometry determination of 8-hydroxy-2-deoxy-guanosin (oxidative damage to DNA), F2–isoprostanes (oxidative damage to lipids) and methionine sulfoxide (oxidative damage to proteins) are appropriate [8–10].

Our aim was to investigate the effect of two functional VOOs either enriched with its own PC (FVOO) or complemented with thyme PC (FVOOT), on the protection of oxidative stress, using urine and plasma oxidation biomarkers and erythrocyte antioxidant enzymes, simultaneously with the detection of urine, plasma and erythrocyte phenolic metabolites in hyperlipidemic subjects.

MATERIALS AND METHODS

Study participants and experimental design. The VOHF-sustained study was a randomized, double-blinded, crossover, controlled trial with 33 hypercholesterolemic volunteers (total cholesterol>200 mg/dL) (19 men and 14 women), aged 35 to 80. Exclusion criteria included the following: BMI>35Kg/m2, smokers (>7 cigarettes/week), athletes with high physical activity (>3000Kcal/day), diabetes, multiple allergies, intestinal diseases, or any other disease or condition that would worsen adherence to the measurements or treatment.

Subjects were randomized to one of 3 orders of administration of 25mL/day of (i) virgin olive oil (VOO; 2.88mg total phenols/day), (ii) VOO enriched with its own PC (FVOO; 12.59mg total phenols/ day), and (iii) VOO enriched with both its own PC and thyme PC (FVOOT; 12.10mg total phenols/ day). In the randomized, double blind, controlled crossover design, intervention periods were of 3 weeks with a daily ingestion of 25mL raw VOO distributed among meals and preceded by a 2 week wash-out with a common olive oil (Figure 1). The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the random sequence was done by a physician.

To avoid an excessive intake of antioxidants, such as PC, during the clinical trial period, participants were advised to limit the consumption of polyphenol-rich food. A 3-day dietary record was administered to the participants before and after each intervention period to control their habitual diet throughout the study. A set of portable containers with the corresponding 25mL of VOO for each day of consumption was delivered to the participants at the beginning of each VOO administration period. The participants were instructed to

return the containers to the center after the corresponding period in order to register the amount consumed. Subjects with less than 80% of treatment adherence (≥5 full VOO or FVOO or FVOOT containers returned) were considered non-compliance for this treatment.

24h/urine was collected in containers before each visit. Urine samples were stored at -80ºC prior to use. Blood samples were collected at fasting state. Plasma samples were obtained by centrifugation of whole blood directly after being drawn and were preserved at -80ºC until use. Erythrocytes were obtained by centrifugation, washed twice with saline and preserved at -80ºC until use.

The VOHF study was approved by the Clinical Research Ethical Committee of the Institut de Recerca Hospital del Mar (IMIM) (CEIC 2009/3347/I), and the study was listed on ISRCTR.org, ISRCTN77500181. Protocols were according to the Helsinki Declaration and good clinical practice guidelines of the International Conference of Harmonization (ICH GCP), the trial was conducted according to extended CONSORT 2010 guidelines.

Sample size and power analysis. The sample size of 30 individuals allows at least 80% power to detect a statistically significant difference among three groups of 3mg/dL of HDL-C and a standard deviation of 1.9, using an ANOVA test and assuming a dropout rate of 15% and a Type I error of 0.05.

Preparation and characterization of VOO. VOO with a low phenolic content (80mg total phenols/kg oil) was used as a control condition in the intervention and as an enrichment matrix for the preparation of the two phenol-enriched VOOs with the same amount of PC (500mg total phenols/kg oil) but with different phenolic composition. FVOO was enriched with its own PC by adding a phenol extract obtained from freeze-dried olive cake and

FVOOT was enriched with its own PC (50%) and complemented with thyme PC (50%) using a phenol extract made up of a mixture of olive cake and dried thyme. FVOOT contained 50% of olive PC (hydroxytyrosol derivates) and 50% thyme PC (flavonoids, phenolic acids and monoterpenes) (Table 1). The procedure for obtaining the phenolic extracts and enriched oils had been previously developed [11]. For the wash-out period, a commercial common olive oil kindly provided by Borges Mediterranean Group was used. The total phenolic content of the VOO was measured with the Folin–Ciocalteu method [12]. The phenolic profile of the VOOs was analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) using a previously described method [13]. Tocopherols and fatty acids in the VOOs were analyzed following the procedure described by Morelló et al. [14] and the carotenoid content was analyzed as previously described by Criado et al. [15].

Lipid profile. Blood samples were collected at fasting state at least 10 hours prior to the study, at the commencement of the study and before and after each treatment. EDTA-plasma glucose, total-cholesterol (TC), and triglyceride (TG) levels were measured using standard enzymatic automated methods, in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier,France). HDL-C was measured as soluble HDL-C determined by an accelerator selective detergent method (ABX-Horiba Diagnostics,Montpellier,France). LDL-C was calculated by the Friedewald equation whenever TGs were less than 300mg/dL.

LC-MS oxidative stress markers. A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, U.S.A.) was used for 8-hydroxy-2'-

deoxyguanosine (8-OHdG), Methionine (Met), Methionine sulfoxide (MetSO) and 8-iso Prostaglandin F2α (8-iso PGF2α) quantification. Ionization was carried out by electrospray ion source (ESI) and acquisition was done in multiple reaction monitoring (MRM) mode. ESI and MRM conditions are summarized in Supplementary Table 1 for all the compounds.

Chromatographic separation in both 8-OHdG method and Met and MetSO methods was performed in an Acquity UPLC BEH HILIC, 2.1x100mm, 1.8µm (Waters, Milford, U.S.A.), at a flow rate of 0.4mL/min, using 50mM NH4AcO in water (solvent A) and ACN (solvent B). Elution gradient for the 8-OHdG method was 0-2min 100%B isocratic, 2-4min 80%B, 4-5min 80%B isocratic, 5-7min 20%B, 7-9min 20%B isocratic and 9-10min 100%B, applying a post run of 1.5 min, and injecting a sample volume of 2µL. Retention time of 8-OHdG was at 4.37 min. Elution gradient for Met and MetSO was 0-1min 95%B isocratic, 1-6min 20%B, 6-10min 20%B isocratic, and 10-11min 95%B, with a post run of 1.5min, and a sample volume injection of 5µL. Retention times of Met and MetSO were of 3.51 and 4.30min, respectively.

For the 8-OHdG quantification, an aliquot of 50µL of freshly thawed urine sample was mixed with 20µL of 100ng/mL of 8OH-2'dOG-15N5 as internal standard in ACN. After a vortex of 10 sec and centrifugation at 15000 rpm for 10min at 4ºC, supernatant was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS).

For the Met and MetSO quantification, an aliquot of 50µL of freshly thawed plasma sample was mixed with 25µL of 25 µg/mL of L-methionine-13C,d3 as internal standard and 150µL of ACN/H_2O 50mM NH4AcO 95:5 (v/v). After a vortex of 10sec and centrifugation at 15000rpm for 10min at 4ºC, supernatant was analyzed by LC-MS.

For the 8-isoPGF2α, the chromatographic separation was carried out in an Eclipse XDB-C18, 2.1x150mm, 1.8µm (Agilent Technologies), at a flow rate of 0.4mL/min, using 0.2% acetic acid in water (solvent A) and ACN (solvent B). Elution gradient was 0-2 min 0%B isocratic, 2-10 min 50%B, 10-11 min 100%B, 13-14 min 100%B isocratic. A post run of 1.5 min was applied. Injected sample volume was of 20µL. Its retention time was at 9.97 min.

For the 8-iso PGF2α quantification, an aliquot of 250µL of freshly thawed urine sample was mixed with 20µL of 100ng/mL of 8iso PSF2α-d4 as internal standard in water/methanol 2:1 (v/v) to protein precipitation. After a vortex of 10 sec, extraction was done by the addition of 750µL of diethyl ether, agitation for 10 min at room temperature and centrifugation at 4000rpm for 10 min at 4ºC. A volume of 700µL of the upper organic phase was dried under a nitrogen gas flow and resuspended in 50µL of water/methanol 2:1 (v/v). After vortex and centrifugation at 15000rpm at 4ºC for 10 min, the supernatant was analyzed by LC-MS.

In the quantification of samples, standard solutions at different levels of concentration were used to obtain calibration curves, and compounds in the samples were quantified by interpoling the analyte/IS peak abundance ratio in these curves.

Antioxidant enzymes in erythrocytes. Determination of the hemoglobin (Hb) content of lysate erythrocytes was carried out by laser-impedance colorimetry. Superoxide dismutase (SOD) activity in erythrocytes was performed following McCord and Fridovich methodology [16] (Ransel RS 125, Randox Laboratories, Crumlin, United Kingdom) and was expressed in U/g of Hb. This method employs xanthine and xanthine oxidase to generate

172 superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-
173 phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured
174 by the degree of inhibition of this reaction. Glutathione peroxidase (GSH-Px) activity was
175 measured by a modification of the method of Paglia and Valentine [17] (Ransel RS 505,
176 Randox Laboratories, Crumlin, United Kingdom) and expressed in U/L. GSH-Px catalyses
177 the oxidation of Glutathione (GSH) by cumene hydroperoxide. Catalase (CAT) activity was
178 measured based on the method of Aebi [18] with slight modifications. Briefly, 70ml of
179 phosphate buffer, 50ml of erythrocyte lysate (5 mg protein per ml) and 50ml of 1%H_2O_2
180 were added in each well of a quartz microplate (Hellma, Müllheim, Germany). After
181 shaking for 1–2s in a plate reader (FisherScientic, Madrid, Spain), the absorbance at 240nm
182 was monitored for 1min in 15s intervals. The final value is expressed as U/mg protein.

183 **Analysis of phenolic metabolites in urine, plasma and erythrocytes.** The extraction of
184 the phenolic metabolites from urine and plasma samples was carried out as previously
185 reported [5]. The PC from erythrocytes samples were extracted with the Solid Phase
186 Extraction (SPE) system using OASIS HLB 200mg cartridges (Waters Corp., Milford,
187 MA). The conditioning of the SPE cartridges was done by adding sequentially 2mL of
188 methanol and 2mL of Milli-Q water acidified at pH 2 with acetic acid. Extractions were
189 performed by loading 1mL of washed erythrocytes, which had previously been mixed with
190 3mL of distilled water and 20µL of phosphoric acid at 85% to break the bonds between the
191 proteins and PC. The loaded cartridges were washed with 1mL of Milli-Q water and 1mL
192 of methanol at 5%. Finally, the retained PC were eluted using 3mL of methanol, which was
193 evaporated to dryness and reconstituted with 100µL of methanol.

194 The phenolic metabolites in biological fluids were selected based on our previous work in
195 which olive and thyme PC intake biomarkers were defined [5]. Thus, hydroxytyrosol sulfate
196 (HTS; urine, plasma and erythrocytes) and hydroxytyrosol acetate sulfate (HTAS; urine and
197 plasma) were analyzed as VOO phenol metabolites. Hydroxyphenylpropionic acid sulfate
198 (HPPAS; urine, plasma and erythrocytes), thymol sulfate (TS; urine, plasma and
199 erythrocytes) and p-cymene-diol glucuronide (PCymeneDG; urine) were analyzed as thyme
200 phenol metabolites. The analysis of the phenolic metabolites was carried out by Ultra
201 Performance Liquid Chromatography (UPLC) coupled to tandem MS (MS/MS) based on
202 the method described by Rubió et al. [5].

203 **Animals and experimental procedure.** Twenty Wistar rats were obtained from Charles
204 River Laboratories (Barcelona, Spain). They were separated into four groups of 5 rats in
205 each group (4 females and 1 male). Group 1: control diet (CON), group 2: secoiridoids
206 (SEC), group 3: secoiridoids combined with thyme phenols (SEC+THY) and group 4:
207 thyme phenols (THY). The diet preparation and characteristics is explained in more detail
208 in Supplementary Table 2. Rats were fed during 21 days at a dose of 5 mg of phenolic
209 compounds/kg rat weight/day. SEC extract and SEC+THY were the same phenolic extracts
210 used for the preparation of FVOO and FVOOT, respectively, as described previously [3].
211 Additionally, THY extract was used to investigate the effect of a comparable phenolic dose
212 exclusively from thyme. The animal procedures were conducted in accordance with the
213 guidelines of the European Communities Directive 86/609/EEC regulating animal research
214 and approved by the local ethical committee (CEEA-Universitat de Lleida, reference 7675).
215 The rats were sacrificed by intracardiac puncture after isoflurane anaesthesia (IsoFlo,
216 Veterinarian Esteve, Bologna, Italy). After blood collection, the rats were perfused with an

isotonic solution of sodium chloride (NaCl) 0.9 % to remove the remaining blood irrigating the tissues and their livers were excised. Tissue samples were stored at -80 ºC and freeze-dried.

NF-κB -DNA binding activity. NF-κB p65-DNA binding was assessed in rat hepatic tissue lysate using a Cayman kit (Cat. No. 10007889). A specific double-stranded DNA sequence containing the NF-κB response element was immobilized in the wells of a 96-well plate. NF-κB contained in whole-cell extract from tissue binds specifically to the NF-κB response element and was detected by addition of specific primary antibody directed against NF-κB (p65). Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provided sensitive colorimetric readout at 450nm. The activity of NF-κB p65-DNA binding was represented as relative absorbance at 450nm/μg of protein.

Data analysis and statistical procedures. Descriptive data were expressed as mean ± standard deviation and post-pre intervention changes were expressed as mean ± 95% confidence interval [95%CI]. Prior to all analyses, normality of data was assessed using Shapiro–Wilk's W test and those lacking a normal distribution were log-transformed to achieve normality. Linear regression models were used to adjust post-intervention values for pre-intervention values, age and sex. Comparisons among groups were analyzed by General Linear Models. Paired T-test was used to test the post-pre intervention period changes on oxidative biomarkers, AEES and PC biomarkers. Differences were considered statistically significant at $P<0.05$. Data were analyzed by SPSS version 20.0 (SPSS, Inc., IBM, Armonk, NY, USA).

RESULTS

Participants and compliance. The study was conducted at IMIM-Hospital del Mar Medical Research Institute (Barcelona, Spain) from April 2012 to September 2012 with 33 enrolled participants completing the intervention period. The participants' flow chart is described in Figure 2 and a discontinued single intervention occurred in three volunteers due to an investigator's decision. Participants had a BMI range indicative of normal weight to overweight and they were normotensive and hyperlipidemic (total cholesterol>200 mg/dL) according to established criteria. All 33 participants had borderline-high values of total cholesterol and LDL cholesterol. There were no statistically significant differences in baseline characteristics of the participants among sequences 1, 2 and 3 (Table 2). Compliance was monitored through the determination of biomarkers of intake analyzing the phenolic metabolites in the subject's biological fluids (urine and plasma) and a successful dietary intervention was guaranteed. No adverse side effects were reported by participants during any of the study treatments.

Olive oils characterization. Table 1 shows the chemical characterization of VOO, FVOO and FVOOT, including individual PC, fat soluble micronutrients and fatty acids composition. Only the phenolic composition differed among the three VOOs as they presented the same composition regarding fat-soluble micronutrients and fatty acids. In comparison to VOO, FVOO was basically enriched with HT and its derivatives providing 8.5mg/25mL oil/day. FVOOT enrichment consisted of a mixture of HT and its derivatives (4.3mg/25mL oil/day), phenolic acids (0.65mg phenols/25mL oil/day), flavonoids (2.95mg/25mL oil/day) and monoterpenes (0.86mg/25mL oil/day). Thus, FVOOT contained 50% of olive PC and 50% of thyme PC.

Olive and thyme phenolic metabolites in biological fluids. Results of the phenolic metabolites in urine and plasma are presented in table 3 and 4, respectively. Apart from urine and plasma, in the present work results of the phenolic metabolites detected in erythrocytes are presented (Table 5). When comparing all three VOOs, metabolites derived from olive PC were significantly higher in FVOO compared to VOO and FVOOT in urine, plasma and erythrocytes (Table 3, 4 and 5). Regarding the post-pre intervention changes, HTS and HTAS significantly increased after FVOO intervention in urine. HTAS was also significantly increased in plasma after FVOO. No post-pre intervention changes in FVOOT were observed in HT biomarkers in any biological fluid. The thyme phenolic metabolites detected in urine, plasma and erythrocytes were HPPAS, TS and PCymeneDG (only detected in urine). When comparing the three interventions HPPAS and TS levels were significantly higher in the FVOOT group compared to the VOO and FVOO in all biological fluids, and PCymeneDG also in urine (Table 3, 4 and 5). Regarding the post-pre intervention changes, HPPAS, TS and PCymeneDG significantly increased after the FVOOT. HPPAS appeared to be a clear erythrocyte biomarker for thyme phenolics, as it was only detected after FVOOT intervention (Table 5).

Effects of VOO PC enrichment on oxidative stress. The outcome measurements of urine oxidation biomarkers (8-iso PGF2α and 8-OHdG) and the post-pre intervention changes are presented in Table 3. When comparing the three VOOs interventions, FVOOT presented lower values of urinary 8-OHdG compared to FVOO and VOO after intervention. In addition, urinary 8-OHdG was also significantly lower in FVOO than VOO. Urinary 8-iso PGF2α did not differ when comparing the three VOOs interventions. Regarding the post-pre intervention changes, urinary 8-OHdG decreased in the FVOO and to a greater extent in

the FVOOT intervention group. No post-pre intervention changes were observed in urinary 8-iso PGF2α. The outcome measurements of plasma % of MetSO in total Met and the post-pre intervention changes are shown in Table 4. There were no differences between groups of administered olive oils in plasma % of MetSO. Compared to baseline values, % of MetSO was significantly increased in all groups (between 0.7-0.8 %).

Effects of VOO PC enrichment on erythrocyte antioxidant enzymes. The outcome measurements of erythrocyte GSH-Px, SOD and CAT activities after the three VOOs treatment and the post-pre intervention changes of each VOO group are shown in Table 5. When comparing the three interventions, the activities of all enzymes were significantly higher after the FVOOT and FVOO group compared to VOO. In addition, GSH-Px and SOD were also significantly higher after the FVOOT group compared to the FVOO ($P<0.05$). Regarding the post-pre intervention changes, SOD activity significantly improved after the FVOO intervention and significantly improved even to a greater extent after the FVOOT one ($P<0.05$). All the other measurements of antioxidant enzyme activities did not differ between post-pre interventions.

Animal experiment: NFkB-DNA binding activity. Thyme supplementation in rat feed (THY) significantly reduced the NFkB-DNA binding activity respect to control (CON) (Figure 3). As shown in Figure 3, it appears that supplementation with olive oil PC (SEC) and both thyme and olive oil PC (SEC+THY) starts a trend to reduced activity of NFkB, which is established as significant when rats are only supplemented with thyme PC (THY).

DISCUSSION

Our study demonstrates that a sustained intake of FVOOT, which provided the same amount of PC but different PC composition of FVOO, appeared to have a greater effect against oxidative stress in hyperlipidemic subjects. VOO presented the highest 8-OHdG values followed by FVOO and FVOOT, suggesting that FVOOT intervention provided major protection against oxidative DNA damage.

The antioxidant protection was also reflected in the activity of antioxidant enzymes in erythrocytes. In this sense, the SOD activity was also increased to a greater extent after the FVOOT than the FVOO and VOO interventions with a parallel increase in thyme phenolic metabolites detected both in urine and erythrocytes after FVOOT compared to FVOO. Our data therefore provide the first level of evidence for an antioxidant DNA action and antioxidant enzymatic induction through a combination of olive and thyme PC, after a sustained consumption of real-life doses of olive oil in hyperlipidemic subjects.

The 8-OHdG is a major base product formed after DNA oxidative damage and has been widely used as a DNA damage indicator in nutritional studies [19]. Large amounts of 8-OHdG are produced in mammalian cells, either as a by-product of normal oxidative metabolism or as a result of exogenous sources of reactive oxygen species (ROS). Increased levels of 8-OHdG in tissues represent a signal of a strong DNA damaging stimulus or the specific deficient DNA repair mechanism [20]. Oxidative damage to the DNA base produces a point mutation through an A-T substitution when incorporated into DNA, causing mutagenesis and carcinogenesis [21]. In a previous study the urinary excretion of oxidation products of guanine, the most commonly used markers for DNA oxidation, was not modified after 3-week consumption of 25mL olive oil with low (2.7mg/kg of caffeic

acid eq), medium (164 mg/kg), and high (366 mg/kg) PC in humans [22]. In the same way, no significant effect was detected in urinary excretion of DNA adducts after the consumption of phenol-rich olive oil (PC content from 2.7 to 366 mg/kg) [23]. In contrast, a decreased amount of 8-OHdG in urine after short-term consumption, 4-consecutive days intervention of 25mL of three VOO, with low (10mg/kg of caffeic acid eq), medium (133mg/kg), and high (486mg/kg) PC with a linear trend significantly correlated to the content of PC [24]. Similarly, 30% reduction of oxidative DNA damage in peripheral blood lymphocytes was observed after to substitute all types of fat and oils habitually consumed with the study oil (50 g/d) for two periods of 8 weeks intervention on postmenopausal women with VOO containing high amounts of phenols (592 mg total phenols/kg) compared to those that consumed lowest levels (147 mg/kg) in postmenopausal women [25]. Our results are in accordance with the latter 2 studies as a significant decrease in urinary 8-OHdG was observed after the sustained consumption of phenol-enriched olive oils, FVOO and FVOOT. Containing the same amount of PC, the 8-OHdG reduction was significantly 2-fold higher in the FVOOT compared to the FVOO, this reduction may be attributed to the different PC composition. Moreover, when comparing with VOO control group the 8-OHdG reduction was significantly 10-fold higher in the FVOOT and 5-fold higher in the FVOO.

In parallel to the oxidative DNA protection, the post-pre change values in 24h/urine of thyme phenolic biomarkers (HPPAS, TS and PCymeneDG) significantly increased in FVOOT group, which could be related to the significant reduction of 8-OHdG observed after the FVOOT intake. Thus, the significant decrease in urinary 8-OHdG after FVOOT consumption suggests that olive and thyme PC could act synergistically as bioactive

molecules protecting against oxidative DNA damage and improving oxidative systemic balance as reflected also in the increase of erythrocyte SOD activity.

The post-pre intervention increase in erythrocyte SOD activity was about 14-fold higher in the FVOOT group compared to the VOO and 2-fold higher compared to FVOO. These dada supports again that, olive and thyme PC may act synergistically as bioactive molecules improving the erythrocyte antioxidant enzymatic system, in which SOD plays the primary role [26].

Erythrocytes, oxygen carriers with high polyunsaturated fatty acid content in their membranes and high cellular concentration of hemoglobin, are particularly exposed to oxidative damage. The hemoglobin released from erythrocytes is potentially dangerous because when reacting with H_2O_2 it is converted into the oxidized forms with powerful promoters of oxidative processes [27]. For this reason, newer functional agents, such as PC from the diet can target oxidative stress in erythrocytes, as a valuable way to prevent or delay the development of organ complications [28].

In the present study, PC metabolites derived from olive or thyme were analyzed in erythrocytes for the first time after an oral administration of olive oil in humans. HTS was the only phenolic metabolite derived from olive PC detected in erythrocytes, whereas HPPAS and TS were detected in erythrocytes as thyme phenolic metabolites. Regarding the post-pre intervention changes, both erythrocyte HPPAS and TS significantly increased after intervention in FVOOT group. In this regard, the parallel significant augmentation in the SOD activity observed after the FVOOT intake could be attributed to the presence of these

371 metabolites in erythrocytes. This fact allows us to postulate that erythrocytes could be cell
372 targets for PC and its metabolites, which could exert an antioxidant effect in situ.

373 Thus, a clear parallelism appears between the modulations of antioxidant or oxidative
374 markers and PC metabolites observed in urine and in erythrocytes after VOO, FVOO or
375 FVOOT interventions.

376 In order to clarify the mechanistic pathways responsible for the higher protective
377 antioxidant effects observed after FVOOT compared to FVOO, a parallel experiment in
378 animals with the same phenolic compounds and similar doses administered to humans was
379 performed. It has seen that hydroxytyrosol act as a inhibitor of NF-kB activation, leading to
380 the inhibition of proliferation and promotion of apoptosis in human hepatocellular
381 carcinoma cells [29]. Furthermore, inhibiting NF-κB activation reduces ROS production and
382 oxidative damage to lipids and DNA [30]. In our animal experiment, results revealed that
383 after supplementation with olive oil PC and both thyme and olive oil PC, a reduction trend
384 in the activity of hepatic NF-κB is observed, which is established as significant when rats
385 are only supplemented with thyme PC. In that sense, the suppression of the NF-κB pathway
386 by thyme PC could be sufficient to reduce the endogenous DNA damage produced
387 naturally by cells. Further studies are needed to verify this mechanistic pathway responsible
388 for the protective antioxidant effect observed in humans.

389 Considering the described results, it is surprising that % of MetSO in total Met was
390 increased in all groups after intervention. The three intervention groups have ingested oils
391 with different phenolic profile, therefore, this cannot explain the similar increase of the
392 MetSO observed in all groups. The exogenous antioxidants, including PC, are considered

393 "double-edged swords" in the cellular redox state and several studies of exogenous
394 antioxidants had shown controversial results, especially when administered at high doses
395 [31,32]. However, in present study the data obtained from of the three intervention groups after
396 a regular consumption of phenol-enriched VOO did not go globally in this direction,
397 despite the increase in % of MetSO. On the other hand, no changes of 8-iso PGF2α were
398 observed in both the pre-post intervention levels and between VOO. As we are aware of the
399 limitations of the use of this biomarker, we have taken into account some important aspects
400 to use it in a reliable manner. We tried to prevent the ex vivo oxidation during processing
401 and storing of samples. In addition, the use of urine samples collected during 24 hours
402 globally reflect changes in lipid peroxidation and minimize the possible circadian variation
403 of 8-iso PGF2α.

404 One of the strengths of the present study was its design. Randomized, controlled, clinical
405 trials were those able to provide the first level of scientific evidence. The crossover design,
406 in which each subject acts as the corresponding control, minimizes the inter-variability. In
407 addition, the fatty acid composition, vitamin E content and parental matrix of the three
408 olive oils were similar whereas the only difference was the PC profile and amount.

409 One potential limitation of the study was that although the trial was blinded, some
410 participants might have identified the type of olive oil ingested by its organoleptic
411 characteristics. Another limitation was the inability to assess potential synergies and
412 interactions among the VOOs and other diet components. Nevertheless, the controlled diet
413 followed throughout the trial should have limited the scope of these interactions.

414 In conclusion, the sustained intake of a phenol-enriched VOO with its own PC and
415 complemented with thyme PC improves DNA protection against oxidation and antioxidant
416 endogenous enzymatic activity probably due to a greater bioavailability of thyme phenolic
417 compounds in hyperlipidemic subjects.

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SUPPORTING INFORMATION

ESI and MRM conditions are summarized in Supplementary Table 1 for all the compounds.

Diet characteristics of the animal experiment are detailed in Supplementary Table 2.

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FIGURE CAPTIONS

Figure 1. VOHF study design in human volunteers. This was a randomized, crossover, controlled trial with 30 hyperlipemic individuals comparing the effects of 3 types of virgin olive oil: control (VOO), enriched with its own phenolics (FVOO) and enriched not only with its own phenolics but also with phenolics from thyme (FVOOT).

Figure 2. VOHF Study flowchart.

Figure 3. Effect of phenolic compounds supplementation on NFkB activity in whole-cell extract from rat liver after 21 days of feeding at a dose of 5 mg of phenolic compounds/kg rat weight/day. Control Standard feed (CON), Secoiridoids (SEC), Secoiridoid combined with thyme phenols (SEC+THY) and Thyme phenols (THY). p values: respect to CON. Values are shown as mean ±SD.

TABLES

Table 1. Composition of the olive oils used in the study regarding phenolic compounds, fat soluble micronutrients and fatty acids profile.

	VOO	FVOO	FVOOT
PHENOLIC COMPOUNDS (mg/25 mL/day)			
hydroxytyrosol	0.01 ± 0.00	0.21 ± 0.02	0.12 ± 0.00
3,4-DHPEA-AC	n.d.	0.84 ± 0.06	0.39 ± 0.04
3,4-DHPEA-EDA	0.04 ± 0.00	6.73 ± 0.37	3.43 ± 0.29
3,4-DHPEA-EA	0.26 ± 0.04	0.71 ± 0.06	0.36 ± 0.03
Total HT derivates	***0.30***	***8.49***	***4.30***
p-hydroxybenzoic acid	n.d.	0.02 ± 0.00	0.06 ± 0.00
vanillic acid	n.d.	0.07 ± 0.00	0.13 ± 0.01
caffeic acid	n.d.	0.00 ± 0.00	0.06 ± 0.00
rosmarinic acid	n.d.	n.d.	0.41 ± 0.03
Total phenolic acids	***-***	***0.09***	***0.65***
thymol	n.d.	n.d.	0.64 ± 0.05
carvacrol	n.d.	n.d.	0.23 ± 0.02
Total monoterpenes	***-***	***-***	***0.86***
luteolin	0.04 ± 0.00	0.18 ± 0.02	0.21 ± 0.02
apigenin	0.02 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
naringenin	n.d.	n.d.	0.20 ± 0.02
eriodictyol	n.d.	n.d.	0.17 ± 0.01
thymusin	n.d.	n.d.	1.22 ± 0.09
xanthomicrol	n.d.	n.d.	0.53 ± 0.06
7-methylsudachitin	n.d.	n.d.	0.53 ± 0.09
Total flavonoids	***0.06***	***0.23***	***2.95***
pinoresinol	0.05 ± 0.00	0.12 ± 0.00	0.10 ± 0.05
acetoxipinoresinol	2.47 ± 0.19	3.66 ± 0.31	3.24 ± 0.28
Total lignans	***2.52***	***3.78***	***3.34***
FAT SOLUBLE MICRONUTRIENTS (mg/25 mL/day)			
α-tocopherol	3.27 ± 0.01	3.40 ± 0.02	3.44 ± 0.01
lutein	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
β-cryptoxanthin	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
β-carotene	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
FATTY ACIDS (relative area %)			
Palmitic acid	11.21	11.20	11.21
Stearic acid	1.92	1.92	1.92
Araquidic acid	0.36	0.36	0.36
Behenic acid	0.11	0.11	0.11
Total saturated	***13.75***	***13.74***	***13.75***
Palmitoleic acid	0.70	0.70	0.69
Oleic acid	76.74	76.83	76.75

Gadoleic acid	0.27	0.27	0.27
Total monounsaturated	***77.71***	***77.80***	***77.72***
Linoleic acid	7.43	7.36	7.43
Timnodonic acid	0.36	0.36	0.35
Linolenic acid	0.43	0.43	0.43
Total polyunsaturated	***8.22***	***8.15***	***8.22***

Values provide the individual phenolic characterization of the olive oils expressed as means ± SD of mg phenols/25 mL oil/day. Abbreviations: VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own phenolics; FVOOT: Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone; n.d.: not determined.

Table 2. Baseline characteristics of the participants in the chronic consumption study.

	Sequence 1 (n=11)	Sequence 2 (n=11)	Sequence 3 (n=11)
Gender, *male/female*	7/4	7/4	5/6
Age, *years*	55.45 ± 7.84	55.18 ± 11.88	54.91 ± 12.57
Body weight, *kg*	84.45 ± 17.74	74.60 ± 18.49	74.75 ± 16.80
BMI, kg/m^2	27.85 ± 4.71	26.33 ±5.29	25.63 ± 3.68
SBP, *mm Hg*†	130 (106 – 166)	128 (96 – 151)	125 (104 – 153)
DBP, *mm Hg*†	72 (44 – 90)	72 (52 – 85)	68 (52 – 101)
Glucose, *mg/dL*	90.91 ± 10.53	93.00 ± 13.33	88.55 ± 11.63
Total colesterol, *mg/dL*	218.82 ± 82	231.91 ± 32.70	228.36 ± 42.70
LDL colesterol, *mg/dL*	142.45 ± 25.64	152.00 ± 28.45	150.80 ± 34.08
HDL colesterol, *mg/dL*	53.39 ± 9.55	52.96 ±12.82	52.78 ±11.75
Tryglicerides, *mg/dL*	115.82 ± 32.49	134.36 ± 60.53	126 ± 86.68

Values are expressed as means ± SD; † Median (25^{th}-75^{th} percentile)

Sequence 1= FVOO, FVOOT and VOO; Sequence 2= FVOOT, VOO and FVOO; Sequence 3= VOO, FVOO and FVOOT. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own phenolics; FVOOT: Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme

Table 3. Post-intervention values and changes from baseline of oxidation biomarkers and phenolic metabolite biomarkers in urine

	VOO (n=33)			FVOO (n=33)			FVOOT (n=33)		
	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre
Post-intervention Urine HT biomarkers									
HTS, µmol/24h urine	9.6	(11.3)	0,660	18.0[a]	(21.3)	0,007	12.1[b]	(22.4)	0,350
HTAS, µmol/24h urine	10.7	(8.2)	0,231	13.0[a]	(7.5)	0,010	9.7[b]	(5.3)	0,412
Changes in Urine HT biomarkers (Post-Pre)									
HTS, µmol/24h urine	-0.8	[-4.7, 3.0]		8.1	[2.4, 13.8]		3.1	[-3.6, 9.7]	
HTAS, µmol/24h urine	3.9	[-2.6, 10.5]		6.0	[1.6, 10.5]		2.6	[-3.9, 9.1]	
Post-intervention Urine Thyme biomarkers									
HPPAS , µmol/24h urine	8.0	(4.3)	0,012	23.1[a]	(6.7)	0,707	324.7[a,b]	(73.6)	<0,001
TS, µmol/24h urine	58.8	(39.0)	0,068	65.9	(59.4)	0,116	539.0[a,b]	(287.9)	<0,001
PCymeneDG , µmol/24h urine	0.1	(0.16)	0,107	1.6[a]	(4.26)	0,351	53.4[a,b]	(25.1)	<0,001
Changes in Urine Thyme biomarkers (Post-Pre)									
HPPAS , µmol/24h urine	-22.3	[-39.2, -5.4]		-3.4	[-21.6, 14.9]		294.9	[187.6, 402.3]	
TS, µmol/24h urine	-29.1	[-60.4, 2.3]		-21.8	[-49.4, 5.8]		470.2	[291.7, 648.7]	
PCymeneDG , µmol/24h urine	-1.0	[-2.2, 0.2]		0.6	[-0.7, 1.8]		55.2	[35.2, 75.1]	
Post-intervention Urine Oxidation biomarkers									
8-OHdG , *nM*	15.3	(8.28)	0,796	12.9[a]	(5.48)	0,015	10.6[a,b]	(3.97)	0,008
8-iso PGF2α , *µg/L*	0.46	(0.12)	0,574	0.45	(0.13)	0,359	0.45	(0.18)	0,493
Changes in Urine Oxidation biomarkers (Post-Pre)									
8-OHdG , *nM*	0.4	[-2.4, 3.1]		-2.0	[-3.7, -0.4]		-4.4	[-7.6, -1.2]	
8-iso PGF2α , *µg/L*	-0.03	[-0.14, 0.08]		-0.03	[-0.09, 0.03]		-0.03	[-0.13, 0.06]	

Values are means and standard deviation (SD) for Post-intervention or 95% confidence interval [95%CI] for Changes Post-Pre. Post-intervention comparison between administered olive oils; [a]: P<0.05 compared to VOO; [b]: P<0.05 compared to FVOO. P-value: Paired T-test comparison between

Post-intervention and Pre-intervention. Abbreviations: VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own phenolics; FVOOT: Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; 8-iso PGF2α: 8-iso Prostaglandin F2α; HTS: Hydroxytyrosol sulfate; HTAS: Hydroxytyrosol acetate sulfate; HPPAS: Hydroxyphenylpropionic acid sulfate; TS: Thymol sulfate; PCymeneDG: p-cymene-diol glucuronide.

Table 4. Post-intervention values and changes from baseline of oxidation biomarkers and phenolic metabolite biomarkers in plasma

	VOO (n=33)			FVOO (n=33)			FVOOT (n=33)		
	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre
Post-intervention Plasma HT biomarkers									
HTS, µM	0.84	(0.69)	0.547	1.52[a]	(0.74)	0.099	1.23[a,b]	(0.85)	0.088
HTAS, µM	0.97	(0.69)	0.475	1.73[a]	(0.97)	0.002	1.14[b]	(0.75)	0.206
Changes in Plasma HT biomarkers (Post-Pre)									
HTS, µM	0.13	[-0.30, 0.56]		0.75	[-0.15, 1.66]		0.50	[-0.08, 1.09]	
HTAS, µM	0.15	[-0.28, 0.59]		0.92	[0.38, 1.46]		0.39	[-0.23, 1.01]	
Post-intervention Plasma Thyme biomarkers									
HPPAS, µM	0.12	(0.15)	0.018	1.12[a]	(0.62)	0.352	24.9[a,b]	(13.9)	<0.001
TS, µM	0.84	(0.26)	0.002	1.61[a]	(0.37)	0.221	26.7[a,b]	(9.5)	<0.001
Changes in Plasma Thyme biomarkers (Post-Pre)									
HPPAS, µM	-1.70	[-3.1, -0.31]		-0.56	[-1.8, 0.7]		24.2	[13.6, 34.9]	
TS, µM	-1.89	[-3, -0.73]		-0.78	[-2.1, 0.5]		24.7	[16.3, 33.1]	
Post-intervention Plasma Oxidation biomarkers									
MetSO in total Met, %	5.4	(0.58)	0.033	5.6[a]	(0.61)	0.006	5.5	(0.86)	0.016
Changes in Plasma Oxidation biomarkers (Post-Pre)									
MetSO in total Met, %	0.71	[0.06, 1.37]		0.85	[0.27, 1.43]		0.79	[0.6, 1.42]	

Values are means and standard deviation (SD) for Post-intervention or 95% confidence interval [95%CI] for Changes Post-Pre. Post-intervention comparison between administered olive oils; [a]: P<0.05 compared to VOO; [b]: P<0.05 compared to FVOO. P-value: Paired T-test comparison between Post-intervention and Pre-intervention. Abbreviations: VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own phenolics; FVOOT: Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme; LDL: low-density lipoprotein; Methionine

SO: methionine sulfoxide; Met: methionine; HTS: Hydroxytyrosol sulfate; HTAS: Hydroxytyrosol acetate sulfate; HPPAS: Hydroxyphenylpropionic acid sulfate; TS: Thymol sulfate.

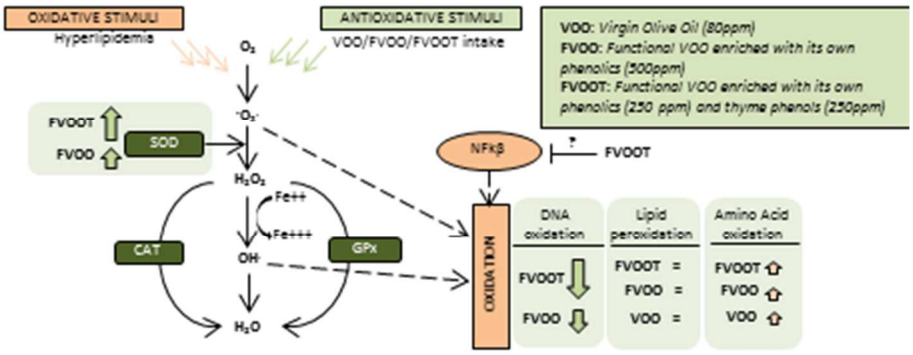
Table 5. Post-intervention values and changes from baseline of oxidation biomarkers and phenolic metabolite biomarkers in erythrocytes.

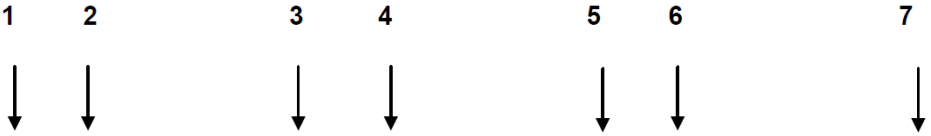
	VOO (n=33)			FVOO (n=33)			FVOOT (n=33)		
	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre	mean	(SD) [95%CI]	P-value Compared to Pre
Post-intervention Erythrocyte HT biomarkers									
HTS, nM	0.16	(0.67)	0.436	0.64[a]	(0.17)	0.171	1.55[a,b]	(1.28)	0.167
Changes in Erythrocyte HT biomarkers (Post-Pre)									
HTS, nM	0.09	[-0.15, 0.33]		0,44	[-0,21, 1.10]		1.44	[-0.65, 3.53]	
Post-intervention Erythrocyte Thyme biomarkers									
HPPAS, nM	n.d.	-		n.d.	-		28.5	(13.6)	0.007
TS, nM	n.d.	-		1.07	(1.31)	0.328	10.26[b]	(1.92)	0.006
Changes in Erythrocyte Thyme biomarkers (Post-Pre)									
HPPAS, nM	-	-		-	-		27.2	[8, 46.3]	
TS, nM	-	-		0,87	[-0,93, 2.67]		10.25	[3.25, 17.3]	
Post-intervention Erythrocytes Endogenous antioxidants									
GPx activity, nmol/min/ml	72.1	(9.90)	0.835	72.8[a]	(9.51)	0.329	74.3[a,b]	(8.83)	0.228
SOD activity, U/ml	716.6	(53.8)	0.875	739[a]	(76.7)	0.033	771[a,b]	(111.6)	0.043
CAT activity, U/ml	111.7	(22.9)	0.142	115[a]	(22.3)	0.308	115.2[a]	(23.4)	0.760
Changes in Erythrocytes Endogenous antioxidants (Post-Pre)									
GPx activity, nmol/min/ml	0.17	[-1.51, 1.85]		0,71	[-0,73, 2.14]		2.18	[-1.45, 5.82]	
SOD activity, U/ml	3.43	[-40.7, 47.6]		26,4	[2,14, 50.7]		48.1	[1.65, 94.6]	
CAT activity, U/ml	-6.49	[-15.28, 2.30]		-3,12	[-9,16, 2.93]		-2.17	[-16.65, 12.31]	

Values are means and standard deviation (SD) for Post-intervention or 95% confidence interval [95%CI] for Changes Post-Pre. Post-intervention comparison between administered olive oils; a: P<0.05 compared to VOO; b: P<0.05 compared to FVOO. P-value: Paired T-test comparison between

Post-intervention and Pre-intervention. Abbreviations: VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own phenolics; FVOOT: Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme; SOD: Superoxide Dismutase; CAT: Catalase; HTS: Hydroxytyrosol sulfate; HPPAS: Hydroxyphenylpropionic acid sulfate; TS: Thymol sulfate.

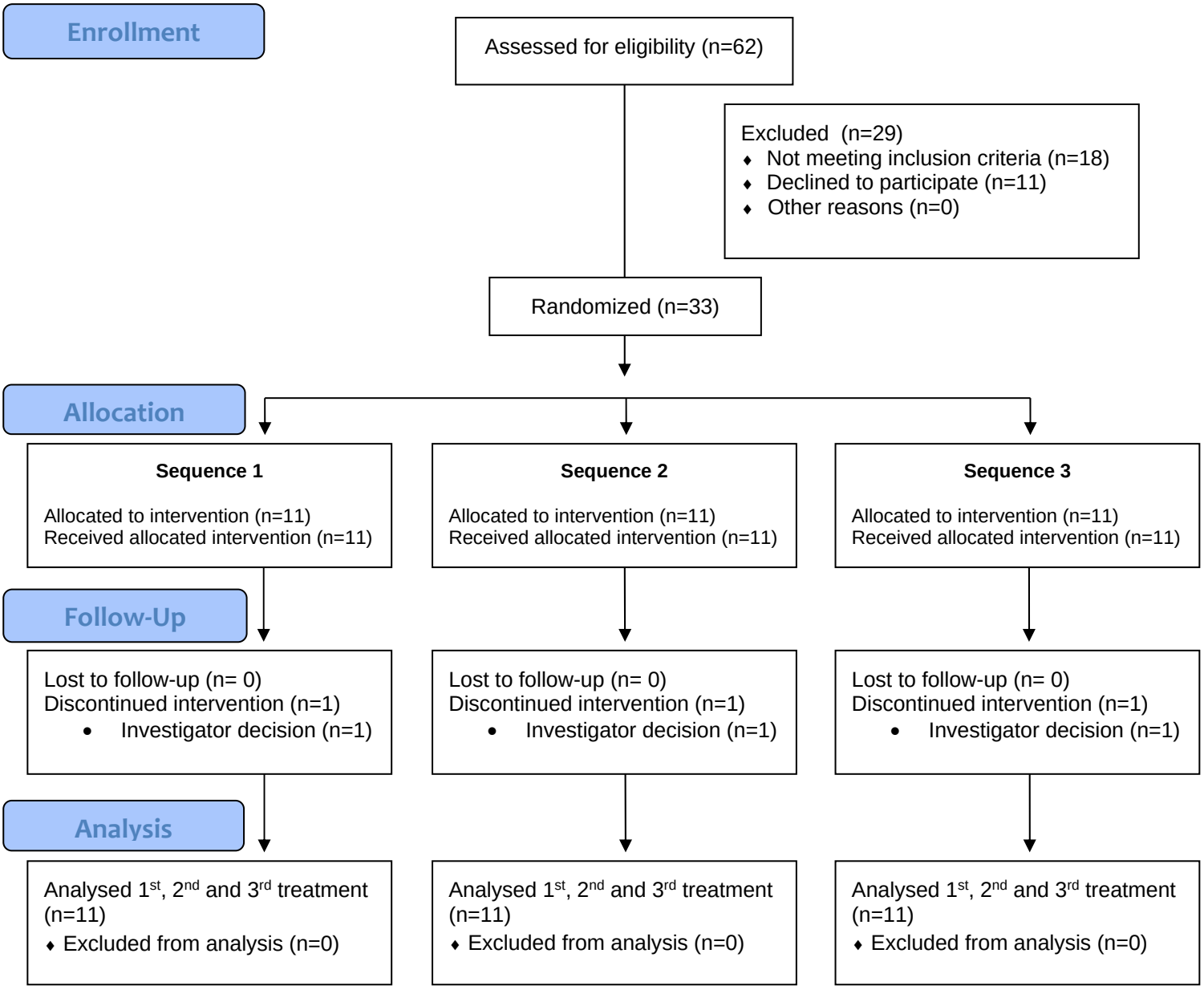
TOC Graphic

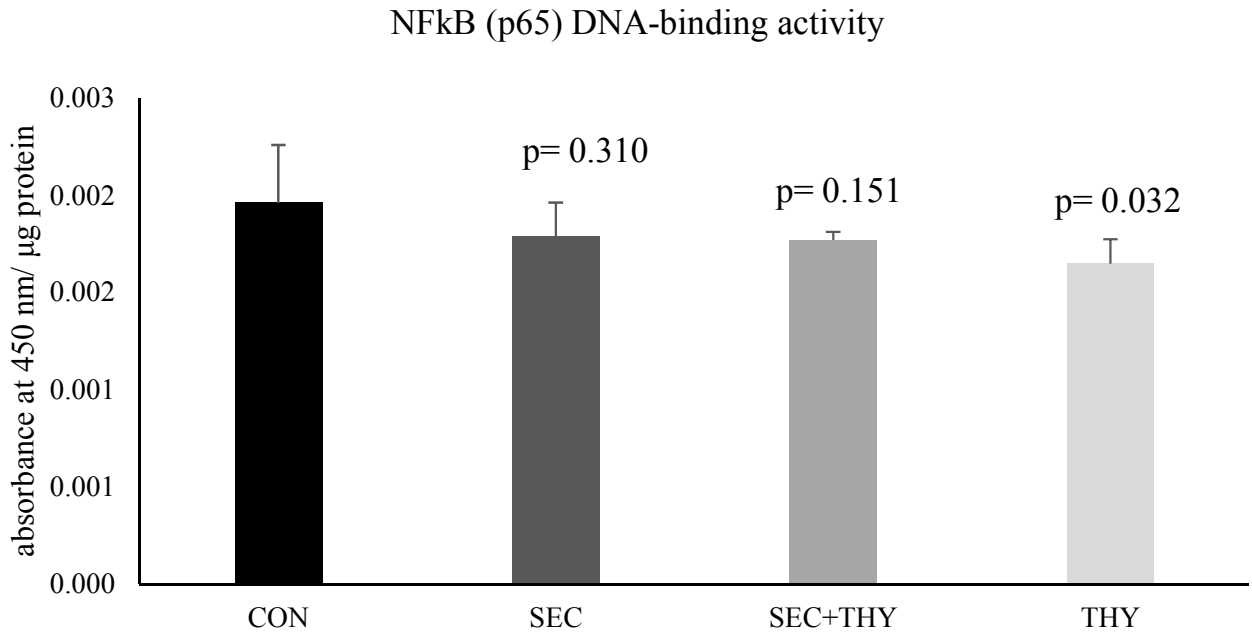

OXIDATIVE STIMULI
Hyperlipidemia
ANTIOXIDATIVE STIMULI
VOO/FVOO/FVOOT intake
VOO: Virgin Olive Oil (80ppm)
FVOO: Functional VOO enriched with its own phenolics (500ppm)
FVOOT: Functional VOO enriched with its own phenolics (250 ppm) and thyme phenols (250ppm)
FVOOT
FVOO
SOD
CAT
GPx
Fe++
Fe+++
OH
H2O
NFkβ
FVOOT
OXIDATION
DNA oxidation
Lipid peroxidation
Amino Acid oxidation
FVOOT
FVOO
VOO
FVOOT =
FVOO =
VOO =
FVOOT
FVOO
VOO



Order 1 (n=11)	WO	X	WO	Y	WO	Z
Order 2 (n=11)	WO	Y	WO	Z	WO	X
Order 3 (n=11)	WO	Z	WO	X	WO	Y

X = FVOO; Y = FVOOT; Z = VOO


Enrollment
Assessed for eligibility (n=62)
Excluded (n=29)
♦ Not meeting inclusion criteria (n=18)
♦ Declined to participate (n=11)
♦ Other reasons (n=0)
Randomized (n=33)
Allocation
Sequence 1
Allocated to intervention (n=11)
Received allocated intervention (n=11)
Sequence 2
Allocated to intervention (n=11)
Received allocated intervention (n=11)
Sequence 3
Allocated to intervention (n=11)
Received allocated intervention (n=11)
Follow-Up
Lost to follow-up (n= 0)
Discontinued intervention (n=1)
• Investigator decision (n=1)
Lost to follow-up (n= 0)
Discontinued intervention (n=1)
• Investigator decision (n=1)
Lost to follow-up (n= 0)
Discontinued intervention (n=1)
• Investigator decision (n=1)
Analysis
Analysed 1st, 2nd and 3rd treatment (n=11)
♦ Excluded from analysis (n=0)
Analysed 1st, 2nd and 3rd treatment (n=11)
♦ Excluded from analysis (n=0)
Analysed 1st, 2nd and 3rd treatment (n=11)
♦ Excluded from analysis (n=0)


NFkB (p65) DNA-binding activity
absorbance at 450 nm/ µg protein
0.003
0.002
0.002
0.001
0.001
0.000
p= 0.310
p= 0.151
p= 0.032
CON
SEC
SEC+THY
THY