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Oleocanthal-rich extra virgin olive oil demonstrates acute anti-platelet effects in healthy men in a randomized trial

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ABSTRACT

The phenolic profiles of extra virgin olive oils (EVOOs) may influence their cardiovascular benefits. In a randomized crossover of acute EVOO intake on platelet function, participants (n = 9) consumed 40 mL of EVOO weekly. EVOOs were matched for total phenolic content and were either tyrosol-poor with 1:2 oleacein/oleocanthal ($D2_i0.5$), or 2:1 oleacein/oleocanthal ($D2_i2$), or predominantly tyrosol ($D2_i0$). Ibuprofen provided a platelet inhibition control. Blood was collected pre- and 2 h post-EVOO intake. $D2_i0.5$ and $D2_i2$ reduced 1 μg/mL collagen-stimulated maximum platelet aggregation (Pmax), with effects best correlated to oleocanthal intake ($R = 0.56$, $P = 0.002$). Total phenolic intake was independently correlated to eicosanoid production inhibition, suggesting that cyclooxygenase blockade was not responsible for the Pmax inhibition. Five participants exhibited >25% ΔPmax declines with $D2_i0.5$ and $D2_i2$ intake and plasma metabolomic profiles discriminated subjects by oil responsivity. Platelet responses to acute EVOO intake are associated with oil phenolic composition and may be influenced by diet.

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1. Introduction

Recent dietary guidelines have moved towards promoting healthy dietary patterns that encourage the intake of a synergy of nutrients for the prevention of chronic disease (Mozaffarian, 2016). While the potential benefit of dietary patterns such as a Mediterranean diet are suggested from epidemiological observations and large controlled dietary interventions (Estruch et al., 2013; Mozaffarian, 2016), adaptation of these results to recommendations for diverse population groups can have several challenges, which include assessing the role of diet coupled to genetic variation and environmental influences. Further refinement of dietary recommendations is also complicated by the knowledge that varietal differences, as well as agricultural and manufacturing practices can affect the final nutrient content of the food product of interest (Mozaffarian, 2016).

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AICc, corrected Akaike Information Criterion; BIC, Bayesian Information Criterion; COX, cyclooxygenase; D1i, sum of an extra virgin olive oil's oleocanthal and oleacein concentrations; D2i, oleocanthal/oleacein ratio for an extra virgin olive oil; D2i0, extra virgin olive oil with undetectable oleocanthal and oleacein; D2i0.5, extra virgin olive oil with 1:2 oleacein/oleocanthal; D2i2, extra virgin olive oil with 2:1 oleacein/oleocanthal; DAD, diode array detector; EVOO, extra virgin olive oil; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; PG, prostaglandin; PLS-DA, partial least squares-discriminant analysis; Pmax, maximum platelet aggregation; qNMR, quantitative 1H nuclear magnetic resonance spectroscopy; TXB2, thromboxane B2; UPLC, ultra-performance liquid chromatography; Δ[COX + LOX], changes in sum of cyclooxygenase- and lipoxygenase-derived lipid mediators.

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Olive oil has long been considered a key component of a Mediterranean dietary pattern. Beyond the potential benefits of its major components such as monounsaturated fatty acids, the minor components of olive oil are also thought to be of benefit (Covas, Fito, & de la Torre, 2015). Extra virgin olive oils (EVOO) contain an array of phenolic antioxidants, from three major chemical classes which include the simple phenolics tyrosol and hydroxytyrosol, the secoiridoids oleuropein aglycon and oleocanthal, and lignans (Bendini et al., 2007). Phenolic content forms the basis of the European Food Safety Authority guidance on olive oil health claims for the prevention of low density lipoprotein oxidation. Specifically, oils must contain at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 mg of olive oil to make such claims (EFSA Panel on Dietetic Products & Allergies, 2011). It is important to note that oils meeting this definition can have diverse phenolic profiles (Servili et al., 2014). Recent epidemiological evidence has indicated the potential utility of EVOO intake for the primary prevention of cardiovascular disease (Buckland & Gonzalez, 2015). Notably, intake of phenol-rich EVOO for close to five years reduced primary cardiovascular disease events in an elderly Spanish population at risk for cardiovascular disease (Estruch et al., 2013). The underlying mechanism of this cardioprotective effect is as yet unknown, however intervention trials with virgin and extra virgin olive oil have demonstrated anti-platelet and anti-inflammatory effects (Covas et al., 2015).

Atherosclerotic cardiovascular disease is a chronic inflammatory disease, initiated by endothelial damage and promoted by a number of cell types to include platelets (May, Seizer, & Gawaz, 2008). Therapeutic reduction of cardiovascular disease progression and events has focused on limiting platelet activation through inhibition of cyclooxygenase (COX), phosphodiesterase, adenosine diphosphate (ADP) receptors, and platelet-platelet interactions through glycoprotein IIb/IIIa (Yousuf & Bhatt, 2011). Major lipid remodelling occurs upon platelet activation that includes shape change, degranulation and generation of a number of bioactive species that amplify activation while promoting clot stabilization and inflammation (O'Donnell, Murphy, & Watson, 2014). Oxylipins are a superclass of bioactive lipids produced from polyunsaturated fatty acid oxidation. The best known of these associated with clot formation is thromboxane, whose production is targeted through the inhibition of COX with aspirin. In addition, oxylipins produced from lipoxygenase (LOX) and cytochrome P450 may be also have roles in platelet activation and inflammatory modulation (Tourdot, Ahmed, & Holinstat, 2013).

While clear mechanisms have yet to be demonstrated *in vivo*, structural specificity has been demonstrated for EVOO-derived phenolic impacts on platelet function and inflammatory processes *in vitro*. For example, hydroxytyrosol appears to be a more potent inhibitor of ADP- or collagen- induced platelet activation than other EVOO-derived phenolics including oleuropein (Petroni et al., 1995), while oleocanthal (the dialdehydic form of decarboxymethyl ligstroside aglycon) has received considerable interest as a COX inhibitor (Beauchamp et al., 2005), and oleacein (the dialdehydic form of decarboxymethyl oleuropein aglycon) has been reported as a 5- LOX inhibitor (Vougogiannopoulou et al., 2014). Since the characterization of specific phenolic profiles in EVOO interventions remains rare, it remains difficult to discriminate *in vivo* EVOO-associated effects of specific phenolics on the biochemical networks associated with cardiovascular disease initiation and progression.

Therefore, while substantial evidence exists to support the hypothesis that phytochemicals within EVOO are beneficial to health, it is still unclear if the total phenolic content or the specific phenolic profile is the more important aspect. In this pilot study, we seek to directly address this issue by comparing the impact of the acute intake of oleocanthal-rich and oleocanthal-poor EVOO, each containing an equivalent total phenolic content, on platelet reactivity in healthy adults. We hypothesize that the intake of oleocanthal-rich EVOO will reduce platelet aggregation to a greater extent than olive oils containing a lesser amount of oleocanthal, and that these effects will be related to changes in COX-dependent oxygenated lipids but not the 5-LOX-dependent or auto-oxidative polyunsaturated fatty acid metabolites.

2. Materials and methods

2.1. Study design

A double-blind, randomized controlled crossover study tested the acute effects of ingesting three unique EVOOs on platelet aggregation. Randomization was performed following a plan formulated via a random number generator and all participants were randomized in one block. All EVOO were provided in coded bottles of similar size, shape and color. Therefore, the investigators responsible for the conduct of the intervention trial and the study participants were blinded as to the specific EVOO provided for any given study day. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the institutional review board of the University of California, Davis. Written informed consent was obtained from all subjects before inclusion in the study. This study is also registered with *ClinicalTrials.gov* as NCT02902913.

Participants were asked to participate in four study days, separated by at least one week (7.4 ± 1.6 days; one of 27 subject visits involving EVOO consumption occurred 14 days after the proceeding visit), at the University of California Davis, Department of Nutrition, Ragle Human Nutrition Center. For the first three study visits, the volunteers were asked to consume 40 mL of their assigned EVOO within 5 min. On the fourth and final study visit, participants consumed one 400 mg dose of ibuprofen by mouth. Participants were asked to refrain from taking drugs that are known or may affect platelet function (e.g. acetaminophen, aspirin and non-steroidal anti-inflammatory drugs) or consuming olive oil for at least one week prior to their first study day visit, and throughout the study period. Subjects were also asked to refrain from consumption of foods previously described to affect platelet function for at least 24 h prior to each study visit. A list of these foods was provided to the subjects during recruitment, and included phenolic- and polyphenol-rich foods, such as, cocoa products, coffee, tea, wine, other grape products and other colorful fruits and vegetables (Holt, Heiss, Kelm, & Keen, 2012; Miller, Rice, Garrett, & Stein, 2014). Dietary compliance was confirmed via questioning of the individual participants upon their arrival to the facility. Prior to each study day, volunteers were asked to participate in an overnight fast (no food or water 12 h prior to their scheduled visit). See Fig. A.1 for a diagrammatic representation of the study design.

2.2. Participants

Healthy men aged 20–50 years and willing to drink 40 mL of EVOO were recruited by public announcement, and assessed by telephone interview and clinical screening prior to enrolment. Exclusion criteria included current supplement and/or prescription drug use; the daily use of aspirin or other non-steroidal anti-inflammatory drugs; a history of cardiovascular disease; vegetarian, vegan, or non-traditional diets; diets that promoted weight loss or that deviated significantly from the average diet of the general population; elevated lipids beyond levels recommended for

lifestyle intervention; any self-reported bleeding or coagulation disorder.

2.3. Olive oil selection and characterization

Olives (*Olea europaea* L.) harvested between November 2014 and January 2015 were used to generate EVOOs discussed in this study. In preparation for this study, 300 commercial samples were screened from Greece and California for chemical composition analysis. Oils were first selected to include extra virgin grades with similar total phenolic content as determined by the Folin-Ciocalteu method (Garcia, Coelho, Costa, Pinto, & Paiva-Martins, 2013), similar lipid profiles (International Olive Oil Council, 2001), and similar peroxide values (AOAC International, 2013). The subset of oils meeting these criteria underwent complete phenolic characterization, with final selection of test oils based on their specific phenolic profile using a recently proposed EVOO bioactive phenolic index (Karkoula, Skantzari, Melliou, & Magiatis, 2012). This "D" index (D = University of California, Davis) provides the sum of oleocanthal and oleacein (D1 index, $D1_i$), and the oleacein/oleocanthal ratio (D2 index, $D2_i$). The D2 index was inconsistently defined by Karkoula et al. in their original report (Karkoula et al., 2012), but was calculated correctly in the manuscript and has been consistently defined since (Karkoula, Skantzari, Melliou, & Magiatis, 2014). Characteristics of the test oils are shown in Table 1. While all three test oils had a similar total phenolic content, two of the test oils were similar in their $D1_i$, but inversed in the $D2_i$, with both oils containing trace or no detectable levels of other secoiridoid conjugated phenolics. The third oil was chosen as a control and had $D1_i$ of zero. All oils were stored at 4 °C immediately after their analysis and during the study period to minimize possible alterations in chemical composition.

2.4. EVOO qNMR analysis

All commercial oils were analyzed by quantitative ^{1}H NMR (qNMR) to determine their oleocanthal, oleacein and total phenolic content. Sample preparation for qNMR analysis consisted of homogenizing 5 g of EVOO with 20 mL cyclohexane and 25 mL acetonitrile for 30 s and then centrifuging the homogenate for 5 min at 4000 × *g*. The acetonitrile phase was subsequently mixed with 1 mL of a syringaldehyde (4-hydroxy-3,5-dimethoxybenzalde hyde) solution (0.5 mg/mL in acetonitrile) and evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland). Samples were reconstituted in 750 μL of deuterated chloroform ($CDCl_3$) and 550 μL of the reconstituted solution was transferred to a 5 mm NMR tube.

^{1}H NMR spectra were recorded at 600 MHz using an Avance600 spectrometer (Bruker, Billerica, MA). Typically, 50 scans were collected into 32 K data points over a spectral width of 0–16 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s. Prior to Fourier transformation, an exponential weighting factor corresponding to a line broadening of 0.3 Hz was applied. The spectra were phased corrected and integrated automatically using Bruker TopSpin software. Manual integrations were performed to enhance accuracy peaks of interest when necessary. Quantitation of oleocanthal and oleacein was performed using calibration curves as previously described (Karkoula et al., 2012).

2.5. EVOO UPLC-DAD analysis

The oils selected above were further subjected to semi-quantitative phenolic profiling using ultra performance liquid chromatography coupled to a diode array detector (UPLC-DAD). Phenolic compounds were extracted from oils using modifications of published solid phase extraction protocols (Gutierrez-Rosales, Rios, & Gomez-Rey, 2003). The pre-weighed oil sample (2.5 ± 0.001 g) and 0.5 mL of an internal standard solution consisting of 67.5 μg/mL *p*-hydroxyphenyl-acetic acid (Sigma Aldrich, St Louis, MO) was dissolved in 6 mL of hexane. A 1 g-6 mL diol-bonded phase cartridge (Thermo Scientific, Waltham, MA, USA) was conditioned with 6 mL of methanol and 6 mL of hexane consecutively, by vacuum elution. Cartridges were then loaded with oil, washed twice with 6 mL of hexane, once with 6 mL of hexane/ethyl acetate (90:10, v/v), and eluted with 10 mL of methanol. The eluent was reduced to dryness by rotatory evaporation at room temperature and the residue was reconstituted in 1 mL of methanol/water (1:1, v/v).

The UPLC-DAD protocol was adapted from a previously published method (Daskalaki, Kefi, Kotsiou, & Tasioula-Margari, 2009). A 20 μL aliquot of the extract was injected onto a 4.6 × 250 mm, 5 μm C18 column (Agilent Technologies, Santa Clara, CA, USA) and separated by gradient elution at a flow rate of 1 mL/min using mobile phases of 98:2, water/acetic acid (v/v) (Solvent A) and 1:1, methanol/acetonitrile (v/v) (Solvent B). The mobile phase gradient was as follows: 0 min - 5% B; 25 min - 30% B; 50 min - 35% B; 65 min - 70% B; 70 min - 100% B; 5 min re-equilibration. Absorbance was recorded at 280 nm and 340 nm. Analytes were quantified by relative response to the internal standard.

2.6. Blood collection

Blood was collected at 0 and 2 h after test product ingestion from an antecubital vein in Vacutainer® tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) containing sodium citrate for platelet aggregometry and post-stimulation oxylipin analysis or potassium EDTA for metabolomics analysis. All collection procedures were performed between 08:00 and 13:00 to avoid circadian effects.

2.7. Platelet aggregometry measurements

Optical platelet aggregometry was performed in citrated blood using a modified version of the method by Born and Cross (1963). Whole blood impedance lumi-aggregometry was measured in duplicate using a 2-channel Chrono-Log 700 (Havertown, PA). Fifteen minutes after the blood draw, platelet rich plasma (PRP) was separated from whole blood by centrifugation (200*g*, 10 min at 25 °C). After the top 75% of the PRP layer was collected into a separate tube, platelet counts were manually obtained using a haemocytometer. The final platelet count was adjusted to 250,000 cells/μL with autologous platelet poor plasma obtained by re-centrifugation of the whole blood tubes at 1500*g* for 10 min at 25 °C. After resting the PRP for a minimum of 15 min, platelet aggregation testing commenced. PRP was incubated at 37 °C for a minimum of 2 min prior to stimulation with collagen at a final concentration of either 1 or 3 μg/mL in duplicate at a stirring speed of 1200 rpm and showed intra-assay mean and standard error of 10 ± 2%. After 8 min of data collection, stimulated and unstimulated PRP samples were immediately centrifuged for 3 min at 1500*g*, and frozen at −80 °C for the oxylipin analysis.

2.8. Oxylipin analysis

Oxylipins derived from COX, LOX, and cytochrome P450 dependent metabolism of arachidonic acid (AA) were quantified using UPLC with tandem quadrupole mass spectrometry in 100 μL of stimulated or unstimulated PRP plasma collected at 0 and 2 h post-EVOO or ibuprofen ingestion. Sample preparation generally followed a previously published protocol (La Frano et al., 2017). Briefly, the plasma was enriched with a suite of deuterated surrogates and extracted with 300 μL acetonitrile with 1% formic acid

Table 1
Experimental EVOO characteristics. Where shown averages are means ± SD.

	Units	Assay	$D2_i0$	$D2_i2$	$D2_i0.5$
D1 index	mg/kg	^{1}H NMR	0	484	460
D2 index	–	^{1}H NMR	0	1.8	0.48
Oleocanthal	mg/kg	^{1}H NMR	<10	172 ± 8	310 ± 15
Oleacein	mg/kg	^{1}H NMR	<10	312 ± 15	150 ± 8
Tyrosol	mg/kg	^{1}H NMR	189 ± 10	<10	<10
DAFOA	mg/kg	^{1}H NMR	<10	<10	<10
DAFLA	mg/kg	^{1}H NMR	<10	<10	<10
AFOA	mg/kg	^{1}H NMR	25 ± 2	5 ± 1	23 ± 2
AFLA	mg/kg	^{1}H NMR	21 ± 2	5 ± 1	10 ± 1
Peroxide values	meq/kg	PV-AcA-Iso	10.2	7.78	4.63
Total phenols	mg caffeic /kg	F-C	212	295	214
K232 index	–	HPLC-DAD	<2.5	<2.5	<2.5
K270 index	–	HPLC-DAD	<0.22	<0.22	<0.22
Tyrosol[a]	% UV detected	HPLC-DAD	19	1	3
Hydroxytyrosol[a]	% UV detected	HPLC-DAD	13	3	5
Vanillic acid[a]	% UV detected	HPLC-DAD	0	3	1
p-Coumaric acid[a]	% UV detected	HPLC-DAD	1	5	0
Ferullic acid[a]	% UV detected	HPLC-DAD	0	0	0
1-Acetoxypinoresinol[a]	% UV detected	HPLC-DAD	5	22	8
Luteolin[a]	% UV detected	HPLC-DAD	2	4	2
Apigenin[a]	% UV detected	HPLC-DAD	1	1	1
C14:0	mol%	GC-FID	<0.1	<0.1	<0.1
C16:0	mol%	GC-FID	12.2	14.5	15.5
C16:1	mol%	GC-FID	0.76	1.12	1.36
C17:0	mol%	GC-FID	<0.3	<0.3	<0.3
C17:1	mol%	GC-FID	<0.3	<0.3	<0.3
C18:0	mol%	GC-FID	2.65	2.48	1.97
C18:1	mol%	GC-FID	73.9	72.6	69.7
C18:2	mol%	GC-FID	9.26	6.63	7.53
C18:3	mol%	GC-FID	0.13	0.18	0.19
C20:0	mol%	GC-FID	0.46	0.43	0.4
C20:1	mol%	GC-FID	0.33	0.28	0.3
C22:0	mol%	GC-FID	<0.2	<0.2	<0.2
C24:0	mol%	GC-FID	0.15	0.12	0.11

Abbreviations: AFLA, Aldehydic Form of Ligstroside Aglycone; AFOA, Aldehydic Form of Oleuropein Aglycone; D1 Index, oleocanthal + oleacein; D2 Index, oleocanthal/oleacein ratio; $D2_i0$, undetectable oleocanthal and oleacein; $D2_i0.5$, 1:2 oleacein/oleocanthal; $D2_i2$, 2:1 oleacein/oleocanthal; DAFLA, Dialdehydic Form of Ligstroside Aglycone;. DAFOA, Dialdehydic Form of Oleuropein Aglycone; F-C, Folin-Ciocalteu assay; PV-AcA-Iso, peroxide value acetic acid-isooctane.

[a] The internal standard normalized absorbance of the individual compound divided by the sum of normalized phenolics in all three oils (i.e. sum of % UV detected across all oils = 100).

using an Ostro™ Pass-through Sample Preparation Plate (Waters Corp; Milford, MA). Extracts were concentrated and reconstituted in internal standard solution prior to analysis. Analytical targets were separated on a 2.1 × 150 mm, 1.7 μm BEH C18 column (Waters; Milford, MA) and detected by negative mode electrospray ionization and tandem mass spectrometry on a 4000 QTRAP® (Sciex; Framingham, MA) as previously described (Agrawal et al., 2017). Calibrants and internal standards were purchased from Cayman Chemical (Ann Arbor, MI), Avanti Polar Lipids Inc. (Alabaster, AL), and Larodan Fine Lipids (Malmo, Sweden). Data was processed utilizing MultiQuant™ v. 3.0.2 (Sciex). Inter- and intra-assay variability was less than 20% for all reported analytes.

2.9. Metabolomics analysis

Small molecules were semi-quantitatively determined in EDTA plasma collected at baseline and 2 h post-EVOO ingestion by gas chromatography time-of-flight mass spectrometry as previously described (Fahrmann et al., 2015). Briefly, 30 μL aliquots of plasma, were thawed on ice, extracted with methyl tert-butyl ether, derivatized by methoximation/silylation, and analyzed. Data, reported as quantitative ion peak heights, were normalized by the sum intensity of all annotated metabolites and used for further statistical analysis. Compounds were identified by processing acquired spectra using the BinBase database (Fiehn, Wohlgemuth, & Scholz, 2005; Scholz & Fiehn, 2007). All analyses were performed in a single run. Inter- and intra-assay variability in normalized metabolomics data is routinely <25% using the described methods.

2.10. Statistical analysis

All changes (Δ) in maximum platelet aggregation (Pmax) and oxylipin concentrations were assessed by repeated measures ANOVA with a threshold of $P < 0.05$ using GraphPad Prism 6 (La Jolla, CA) considering all subjects. Due to high inter-individual variability, Pmax and oxylipin concentration results were range-scaled to each subject's minimum and maximum responses across all four treatments. This practice promotes the identification of biological effects (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006) and by reducing the influence of intra-individual variability (Krishnan, Newman, Hembrooke, & Keim, 2012).

To identify the main component(s) of the EVOOs that best predicted the observed changes in Pmax or oxylipin concentration, multiple linear regression analyses were conducted using JMP Pro v12 (Cary, NC) considering all subjects. Regression models were built using either Pmax or the sum of selected oxylipin concentrations as the dependent variable, and the mg/kg of body weight dose of various individual EVOO phenolics, total EVOO phenolic content or total EVOO peroxide content as the independent variable. Treatment and subject orders were considered and had no effect on regression results. Oxylipins used in the regression model have been associated with platelet function (Tourdot

et al., 2013), and included the sum of the COX products thromboxane B2 (TXB2), prostaglandin E2 (PGE2) and 11-hydroxyeicosatetraenoic acid (11-HETE), and the LOX products 12-HETE, 15-HETE (collectively referred to as Δ[COX + LOX]). Individual phenolics used in the regression model were oleocanthal, oleacein, and tyrosol as the mg/kg of body weight dose of these phenolics were > 0.1 mg/kg for at least one EVOO consumed. Model selection was made using the corrected Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC) scores, with differences in AICc or BIC scores > 2 considered statistically different (Chatterjee & Hadi, 2015; Ward, 2008).

In addition to the statistical analysis described above sources of variability in the subjects' platelet aggregation response were explored. Specifically, plasma metabolomics profiling pre- and post-EVOO intake was evaluated to determine if these profiles would predictably vary with platelet aggregation responsiveness and may reflect factors not controlled by our study design (e.g. habitual diet, fitness, metabolic variance). Metabolomics data were assessed by partial least squares-discriminant analysis (PLS-DA), with group classification based on ingested oils and a Pmax decrease of > or < 25% in following D2i0.5 or $D2_i2$ consumption. These multivariate analyses were performed in the R-statistical environment (R Foundation for Statistical Computing, Vienna, Austria) using imDEV v1.42, a Microsoft Excel (Microsoft Corporation, Redmond, Washington) Add-In (Grapov & Newman, 2012). Prior to PLS-DA, data were curated such that analytes with < 70% completeness of data were removed from consideration. Curated data were screened for outliers using the Grubb's test (Grubbs, 1950), and missing data were imputed by a two-component probabilistic principle components analysis with univariate scaling (Wang & Wang, 2006). Following normalization of data according to the procedures of Box and Cox (Box & Cox, 1964), PLS-DA was conducted using the orthogonal scores algorithm with univariate scaling and leave-one-out cross-validation. Variables were clustered by Spearman correlation coefficients using the Minkowski distance and Ward agglomeration.

Sample size was estimated for the primary outcome measure from previous data that observed flavonoid-induced changes in collagen-induced platelet aggregation (Hubbard, Wolffram, Lovegrove, & Gibbins, 2004). For a mean difference of 40% and an expected standard deviation of residuals of 25% with a two-tailed α set at 0.05, a minimum of $n = 9$ provides sufficient power at 0.8 to detect a significant difference between three groups with ANOVA.

3. Results

3.1. Subject recruitment and retention

Recruitment and interventions occurred between January and September of 2015 and continued until 10 subjects qualified for enrolment. Out of 11 subjects screened for this study, 10 qualified for enrolment and 9 were included in and completed the study. One subject chose not to participate, citing difficulty in giving up olive oil. Baseline physiological characteristics of the enrolled subjects are listed in Table A.1. The average age of the study participants was 26 ± 4 yr with a body mass index of 25.5 ± 4.1 kg/m^2. In general, the subject population was healthy, with plasma lipids and glucose within the normal reference range, and a mean blood pressure of 124 ± 9 mmHg systolic and 77 ± 4 mmHg diastolic.

3.2. Olive oil composition

Of the 300 extra virgin olive oils screened and analyzed, three oils were selected: a commercial oil of Mediterranean origin obtained from a California supermarket with undetectable oleocanthal and oleacein ($D2_i0$); an Arbequina monovarietal oil provided by Corto Olive Co. (Lodi CA) harvested in November 2014 in California with 2:1 oleacein/oleocanthal ($D2_i2$); a Koroneiki monovarietal blend of oils harvested in November 2014 from the Akritohori, Leukohora and Lambaina regions in Kalamata, Greece with 1:2 oleacein/oleocanthal ($D2_i0.5$). Each could be classified as good quality oil, having high phenolic content, as well as low peroxide values and free fatty acid content, K232 values < 2.5, and K270 values < 0.22 (Table 1). Concentrations of oleuropein- and ligstroside-derived phenolics quantified by ^{1}H NMR demonstrate their differences in tyrosol, oleocanthal and oleacein content, while their UPLC-DAD profiles suggest levels of other phenolics were all low and quite similar except for apparently high levels of 1-acetoxypinoresorsinol in the Arbequina oil ($D2_i2$). The $D2_i0$ oil contained < 10 mg/kg of both oleocanthal and oleacein, but was high in tyrosol compared to the other two oils, while both the $D2_i2$ and $D2_i0.5$ oils contained < 10 mg/kg of tyrosol with a D1 index of 460 and 484 mg/kg, respectively. The phenolic content of $D2_i0.5$ and $D2_i2$ was close to the average values reported for Messenia, the main olive oil producing region of Greece (Karkoula et al., 2014). Oil total phenolics and oleacein content increased together and were highest in $D2_i2$, whereas oil peroxide values and oleocanthal were inversely related (Table 1). Data for the complete characterization of all screened oil are available upon request.

3.3. Effects on platelet aggregation

Between day differences in basal platelet aggregation responses were not observed (Table 2). Ibuprofen treatment (400 mg) served as an assessment of individual platelet response to a known platelet inhibitor. Ibuprofen treatment reduced Pmax by 57.5 ± 32.9% with 3 μg/mL collagen-stimulation, and by 71.8 ± 14.3% with 1 μg/mL collagen-stimulation in all subjects. While EVOO intake did not affect 2 h 3 μg/mL collagen-stimulated ΔPmax, 1 μg/mL collagen-stimulated ΔPmax was decreased with a large effect size ($\eta^2 = 0.2$) by both $D2_i0.5$ (−35 ± 39%) and $D2_i2$ (−13 ± 36%) intake compared to $D2_i0$ (7 ± 24%) as seen in Fig. 1A.

Regression analyses were performed to attempt to predict the ΔPmax in terms of the estimated amount per subject body weight of oleocanthal, oleacein, tyrosol, total EVOO phenolics, or total EVOO peroxides consumed by each subject. Oleocanthal provided the strongest individual ΔPmax prediction (R = 0.563, $P = 0.002$), though a two-factor model of oleocanthal and peroxides (R = 0.640, $P = 0.002$), appeared slightly better with an AICc improvement of 1.2. The model was not significantly improved by the addition of tyrosol, oleacein or total EVOO phenolics as factors (Table 3).

3.4. Effects on oxylipins

This study measured the non-esterified pool of oxylipins produced from the collagen-induced platelet aggregate. Basal oxylipin levels were determined from unstimulated PRP. While a less sensitive measure of lipid autooxidation than inspection of the esterified lipid pool, the presence of high levels of non-esterified 9-HETE and or F2-isoprostanes are still viable measures of autooxidation, if observed. The relative abundance of the measured AA mid-chain alcohols (5-, 8-, 9-, 11- and 15-HETE) were not altered by EVOO consumption, and F2-isoprostanes were not observed. Ibuprofen treatment decreased 1 μg/mL collagen-stimulated oxylipin concentrations compared to the three EVOOs in all subjects (Fig. 1B). EVOO intake did not change either the 1 μg/mL collagen-stimulated individual (Table A.2) or Δ[COX + LOX] pro-

Table 2
Baseline platelet aggregation (mean ± SD) in collagen-stimulated plasma by study visit.

Study visit	n	1 μg/mL collagen	3 μg/mL collagen	P[a]
1	9	76.4 ± 31.5	91.1 ± 11.7	0.22
2	9	69.2 ± 23.9	88.8 ± 7.7	0.04
3	9	65.9 ± 32.5	85.9 ± 12.9	0.12
4	9	72.0 ± 21.7	85.5 ± 7.7	0.11
P[b]		0.35	0.49	
Inter-day variance[c]		6.31%	3.01%	

[a] *P* value determined by two-tailed heteroscedastic Student's *t*-test.
[b] *P* value determined by repeated measures ANOVA with Tukey's post hoc HSD.
[c] Variance determined using the average of daily average platelet aggregation measures.

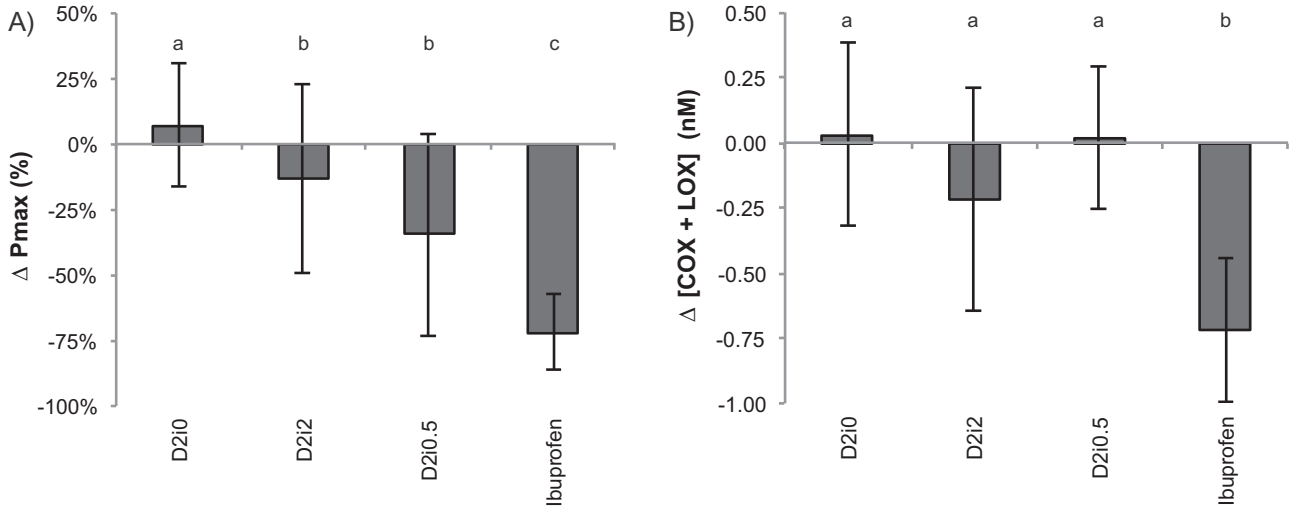

Fig. 1. Effects of tested EVOOs on (A) maximum platelet aggregation and (B) oxylipins associated with platelet function in healthy male subjects. D2$_i$2, D2$_i$0.5 and Ibuprofen all decreased maximum platelet aggregation compared to D2$_i$0, and Ibuprofen decreased oxylipin concentrations compared to all oils. Data shown are presented as means (n = 9) with their standard deviations. Data points with unlike letters were significantly different at $P < 0.05$ (repeated measures ANOVA).

Table 3
Phenolic-dependent ΔPmax inhibition regression models. Models were built iteratively using all EVOO treatments; n = 9 participants × 3 treatments = 27. The regression equation takes the form ΔPmax: $y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5$. All values reported as mean ± SE.

b_0 (intercept)	b_1 (oleocanthal)	b_2 (peroxides)	b_3 (tyrosol)	b_4 (oleacein)	b_5 (total phenols)	R	AICc	BIC	P
0.13 ± 0.10	−3.41 ± 1.00	–	–	–	–	0.57	18.1	20.9	0.002
0.82 ± 0.37	−5.64 ± 1.49	−148 ± 76	–	–	–	0.64	16.9	20.3	0.002
0.85 ± 0.38	−6.21 ± 1.93	−130 ± 84.8	−0.79 ± 1.64	–	–	0.65	19.7	23.3	0.006
1.21 ± 0.42	−9.47 ± 2.61	224 ± 217	−13.4 ± 7.4	−8.94 ± 5.07	–	0.70	19.5	23.1	0.004
1.25 ± 0.43	−40.3 ± 42.9	197 ± 223	−74.3 ± 84.8	−85.9 ± 107	102 ± 141	0.71	22.5	25.7	0.008

Abbreviations: AICc, Corrected Akaike information criterion score; BIC, Bayesian information criterion score.

duct formation (Fig. 1B). Changes in the other detected COX1 product, 6-keto-PGF1a were also not seen between EVOOs (Table A.2).

As changes in eicosanoid production was observed, regression analyses were performed assess the ability of other factors to predict the Δ[COX + LOX]. Specifically, the estimated amount per subject body weight of oleocanthal, oleacein, tyrosol, total EVOO phenolics, or total EVOO peroxides consumed by each subject were evaluated. Total EVOO phenolic intake provided the strongest prediction of oxylipin production (R = 0.593, P = 0.001) with the model not significantly improved by the addition of tyrosol, oleacein, oleocanthal, or total EVOO peroxides as factors (Table 4).

3.5. Identification of a responsive platelet aggregation phenotype

Exploration of individual variability in platelet aggregation response showed five of the nine subjects exhibited >25% reduction in 1 μg/mL collagen-stimulated ΔPmax with D2$_i$0.5 or D2$_i$2 EVOO intake. This 25% reduction in ΔPmax was used to define a responsive phenotype. Within this group of individuals, 1 μg/mL collagen-stimulated production of TXB2, 11-HETE and 15-HETE was reduced after D2$_i$2 intake compared to non-responders (Table 5).

Metabolomics analysis of compounds derived from primary metabolism conducted the pre- and post-EVOO intake plasma showed a total of 311 metabolites of which 107 were identified using the BinBase database. Using this data, PLS-DA discriminated EVOO responsive phenotypes (Fig. 2). In general, subjects that "responded" to EVOO intake showed increased plasma concentrations of carbohydrates such as glucose, xylose and pinitol, as well as sugar acids such as glycolic acid, gluconic acid and threonic acid, both prior to, and after EVOO intake. On the other hand, subjects that did not "respond" to EVOO intake had increased fasting plasma non-esterified fatty acid concentrations (particularly oleic acid) that remained elevated relative to "responders" after EVOO

Table 4
Phenolic-dependent Δ[COX + LOX] inhibition regression models. Models were built iteratively using all EVOO treatments; $n = 9$ participants × 3 treatments = 27. The regression equation takes the form Δ[COX + LOX]: $y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5$. All values reported as mean ± SE.

b_0 (intercept)	b_1 (total phenols)	b_2 (oleacein)	b_3 (peroxides)	b_4 (tyrosol)	b_5 (oleocanthal)	R	AICc	BIC	*P*
0.93 ± 0.27	−8.71 ± 2.4	–	–	–	–	0.59	15.8	18.6	0.001
1.08 ± 0.36	−10.7 ± 3.9	1.00 ± 1.56	–	–	–	0.60	18.1	21.5	0.005
1.44 ± 0.42	−22.6 ± 8.7	5.91 ± 3.57	179 ± 118	–	–	0.65	18.6	22.2	0.005
1.39 ± 0.43	−23.7 ± 8.8	3.03 ± 4.74	355 ± 224	−4.36 ± 4.72	–	0.66	20.9	24.5	0.009
1.31 ± 0.43	−169 ± 143	113 ± 108	386 ± 226	82.8 ± 86.0	44.1 ± 43.5	0.69	23.3	26.5	0.015

Abbreviations: AICc, Corrected Akaike information criterion score; BIC, Bayesian information criterion score.

Table 5
Effect of EVOOs on COX and LOX-derived oxylipins associated with platelet function. Responders and non-responders demonstrated differential effects on oxylipin formation following consumption of EVOOs, with TXB2, 11-HETE and 15-HETE formation significantly changing following EVOO consumption in the responders. Data points with unlike letters were significantly different at $P < 0.05$ (repeated measures ANOVA). All values reported as mean ± SD.

Oxylipin	Responders ($n = 5$)				Non-responders ($n = 4$)			
	D2i0	D2i2	D2i0.5	*P*[a]	D2i0	D2i2	D2i0.5	*P*[a]
ΔTXB2	−0.04 ± 0.21^{A}	−0.37 ± 0.15^{B}	−0.14 ± 0.22^{A,B}	0.02	0.10 ± 0.41	0.08 ± 0.40	0.22 ± 0.08	0.77
ΔPGE2	−0.02 ± 0.24	−0.32 ± 0.26	−0.10 ± 0.22	0.09	0.05 ± 0.31	0.08 ± 0.40	0.28 ± 0.09	0.51
ΔPGF2a	0.04 ± 0.19	−0.31 ± 0.45	0.13 ± 0.54	0.18	0.05 ± 0.21	−0.19 ± 0.51	0.12 ± 0.25	0.31
Δ11-HETE	−0.02 ± 0.20^{A}	−0.40 ± 0.15^{B}	−0.11 ± 0.22^{A,B}	0.02	0.11 ± 0.38	0.13 ± 0.39	0.25 ± 0.10	0.65
Δ15-HETE	−0.02 ± 0.23^{A}	−0.40 ± 0.19^{B}	−0.10 ± 0.23^{A}	0.01	0.05 ± 0.44	0.11 ± 0.34	0.23 ± 0.06	0.67
Δ12-HETE	0.01 ± 0.23	−0.47 ± 0.41	−0.20 ± 0.18	0.15	0.09 ± 0.58	0.02 ± 0.50	0.25 ± 0.05	0.68

Abbreviations: D2$_i$0, EVOO with undetectable oleocanthal and oleacein; D2$_i$2, EVOO with 2:1 oleacein/oleocanthal; D2$_i$0.5, EVOO with 1:2 oleacein/oleocanthal.
[a] Calculated using repeated measures ANOVA with Tukey's post hoc HSD.

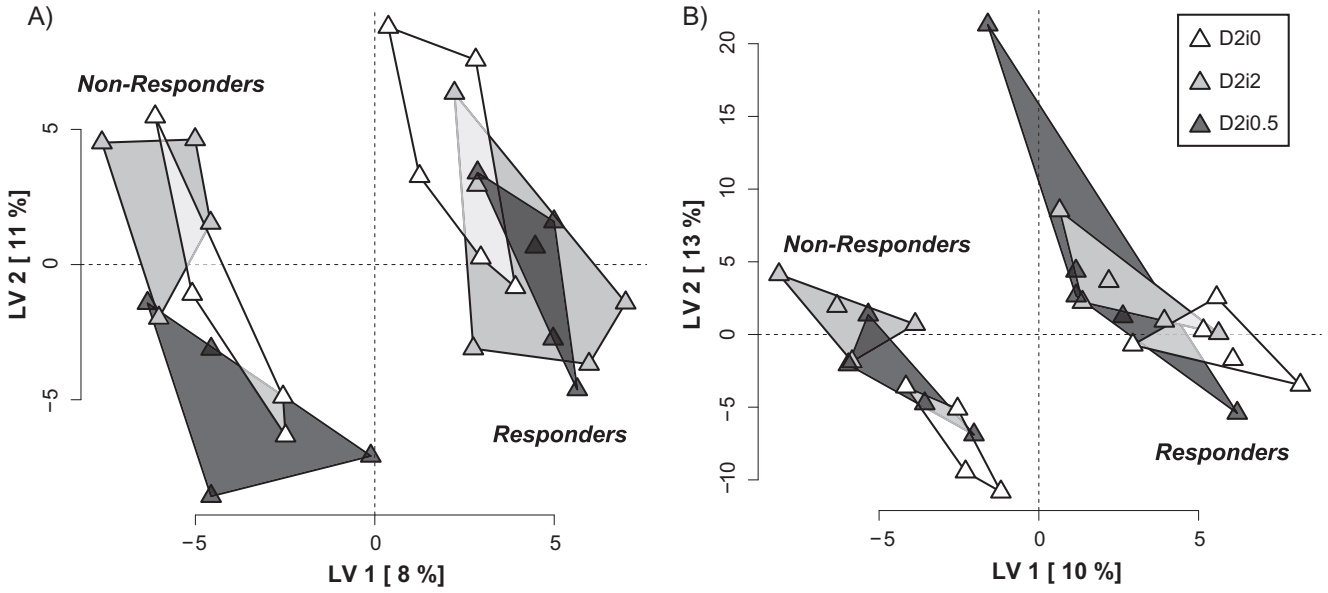

Fig. 2. Partial Least Squares Discriminant Analysis (PLS-DA) Scores Plots showing discrimination between "responders" and "non-responders" at (A) baseline and (B) two-hours post-EVOO consumption. "Responders" are defined at subjects exhibiting > 25% reduction in 1 μg/mL collagen-stimulated ΔPmax with D2i0.5 or D2i2 EVOO intake.

intake, as well as increased levels of the citric acid cycle metabolites malic acid, isocitric acid and citric acid. See Fig. A.2 for a partial list of identified metabolites, their metabolic relationships and their impact on the separation between "responders" and "non-responders".

4. Discussion

Data from epidemiological studies and the "Prevención con Dieta Mediterránea" or PREDIMED trial have demonstrated a reduced risk of cardiovascular disease development with increased intakes of olive oil (Buckland & Gonzalez, 2015; Estruch et al., 2013; Mozaffarian, 2016). Moreover, this trial suggested that increasing "phenolic-rich" virgin olive oil intake might be of particular benefit (Estruch et al., 2013). However, if specific phenolics are important factors in this process uniformity in metabolic and physiological responses to the intake of a specific food type should not be expected, since the content of any plant food can vary by agricultural variety, practices and manufacturing processes. Variation in phytochemical content is a prevalent issue in olive oil products, as the specific phenolics delivered within any oil are dependent on oil grade, olive variety and agricultural region grown, time of harvest, milling practices, and storage conditions (Caporaso et al., 2015; Karkoula et al., 2012). In particular, oleocanthal and oleacein concentrations within an EVOO are affected by olive tree varietal

and inversely correlated to time of harvest (Karkoula et al., 2014). A number of studies have observed benefit of virgin or extra virgin olive oils that are "high" in total phenols compared to low phenol oil (Covas et al., 2015), including a reduction in COX derived thromboxane and LOX derived LTB4 production (Bogani, Galli, Villa, & Visioli, 2007). To our knowledge, this is the first human dietary intervention to study the effects of the consumption of natural EVOOs, well characterized for their phenolic content and composition, which deliver a similar total phenol load upon intake. Most importantly, all tested oils qualified under the current European Food Safety Authority guideline for a health claim related to low density lipoprotein protection from oxidative damage (EFSA Panel on Dietetic Products, Nutrition & Allergies, 2011), yet for the outcomes measured in this trial (i.e. platelet aggregation) distinct biological differences in a healthy population were detected with acute intake of EVOOs that deliver different levels of tyrosol and hydroxytyrosol derivatives.

Platelets are the first responders of vascular injury during the early stages of endothelial disruption, after plaque rupture, and the final manifestation of disease; all the while, promoting the inflammatory processes that progress atherosclerotic disease (Nording, Seizer, & Langer, 2015; Rondina, Weyrich, & Zimmerman, 2013). Low-dose aspirin regimens and other antiplatelet strategies have been employed for both the primary and secondary prevention of acute events of occlusive arterial disease (Patrono & Baigent, 2014). The intake of a number of foods also positively modulate platelet reactivity and may be the basis for the reduced cardiovascular risk reported for individuals with dietary patterns that increase plant food intake, such as the Mediterranean diet. Of particular interest to the current trial are observations that acute and short-term intake of EVOOs (Sánchez-Muniz, Oubiña, Benedí, Ródenas, & Cuesta, 1998) and other phenol-rich foods, such as cocoa/chocolate, tea, and grape products inhibit platelet function (Holt et al., 2012; Hubbard, Wolffram, Lovegrove, & Gibbins, 2003; Miller et al., 2014). By testing EVOOs similar in lipid and total phenolic content, but differing in the specific phenols delivered, we were able to detect a reduction in maximal platelet aggregation after the acute (2 h) intake of EVOOs rich in oleocanthal, and the effect was best predicted by oleocanthal intake. These findings are consistent with studies in rats provided oils with low or high concentrations of "minor polar compounds", where inhibition of platelet aggregation increased with minor polar compound content (Priora et al., 2008). Interestingly, poor correlations between platelet aggregation and oxylipin production blockade by the two oleocanthal containing oils suggests differential effects on platelet activation signalling, and total EVOO phenols were the best predictor of ex vivo collagen stimulated oxylipin production.

Collagen activation induces thromboxane production and ADP release from platelet dense granules. Both agonists are part of a diverse array of cross talking intracellular signalling pathways that amplify, control, and sustain an appropriate level of haemostasis (Li, Delaney, O'Brien, & Du, 2010). Oleocanthal has received considerable interest as a potential platelet inhibitor of COX *in vitro* (Beauchamp et al., 2005). However, we have found that reduction in COX derived oxylipins was greatest after $D2_i2$ EVOO intake, which provided only half as much oleocanthal as the $D2_i0.5$ oil. Interestingly, EVOO peroxide values, a measure of poor oil quality, increased as oleocanthal content decreased, likely due to the anti-oxidant nature of oleocanthal (Smith, Han, Breslin, & Beauchamp, 2005). While our regression models suggest that peroxide value could also predict ΔPmax inhibition, its impact on a combined regression model with oleocanthal argues that the association is through similar, not independent mechanisms. Despite peroxide production being associated with collagen-induced platelet activation (Pignatelli, Pulcinelli, Lenti, Gazzaniga, & Violi, 1998), it is more likely that the oil peroxide index is simply a marker of EVOO quality and that higher quality EVOOs exhibit greater health benefits. However, this claim would need further verification.

Platelets are metabolically active, utilizing both anaerobic and oxidative phosphorylation for energy production, with the later predominant (Kramer, Ravi, Chacko, Johnson, & Darley-Usmar, 2014). While the current study did not access platelet specific metabolites, analysis of primary metabolism demonstrated that differences that subjects with different plasma metabolomics profiles had distinct platelet response to EVOO intake. Whether these effects were primarily due to dietary or metabolic factors is as of yet unknown. However, "responders" tended to have increased plasma glucose concentrations, along with increased levels of specific carbohydrates and sugar acids suggesting a diet rich in fruits and vegetables. Interestingly, the identification of pinitol as a significant discriminating variable of response regardless of time point suggests that responsive individuals had a higher habitual intake of soy products (Davis et al., 2000). In contrast, the "non-responsive" participants had increased circulating non-esterified fatty acids. In addition, non-responders had greater fasting levels of oleic acid, the primary fatty acid found in olive oil. Since high plasma oleic acid is a reported marker of habitual olive oil consumption that correlates with stroke prevention (Samieri et al., 2011) and EVOO effects on platelet function persist with continuous exposure (Sánchez-Muniz et al., 1998), it is possible that the EVOO effects are saturable and the non-responders in this study were already protected. That being said, the significance of these findings cannot be determined from the current study design, but warrant further exploration.

Platelet aggregation is regulated by a number of mediators, including COX-derived TXB2, platelet 12-LOX-derived 12-HETE, and 15-LOX-derived 15-HETE (Tourdot et al., 2013). While no changes in these, and other COX and LOX-derived mediators were observed in all subjects following EVOO consumption, "responders" did demonstrate a reduction in plasma TXB2, 11-HETE and 15-HETE concentrations following consumption of both $D2_i0.5$ and $D2_i2$, compared to $D2_i0$. As each of these mediators can be derived from COX-1 metabolism of arachidonic acid (Caughey, Cleland, Penglis, Gamble, & James, 2001; Tejera, Boeglin, Suzuki, & Schneider, 2012), the anti-platelet effects of the oleocanthal-containing EVOOs may in fact have a COX-1-dependent component. However, further experimentation would be needed to verify the specific mechanism by which oleocanthal affects platelet aggregation.

As platelet activation promotes the inflammatory process associated with atherosclerotic cardiovascular disease, the *in vivo* anti-inflammatory effects and mechanisms of oleocanthal and total phenolics should be explored in future studies. If true, both generally phenolic-rich oils and specifically oleocanthal-rich oils would be predicted to have positive impacts on both platelet aggregation and inflammation, and thus have benefits in cardiovascular risk reduction, as has been previously suggested in a 28-day intervention of post-menopausal women (Sánchez-Muniz et al., 1998). While the above associations are intriguing, care must be taken in their final interpretation as phenols and polyphenols are rapidly and extensively metabolized, with circulating metabolites detected in the plasma in significant levels within minutes and lasting for several hours post intake (Ottaviani et al., 2016; Zamora-Ros et al., 2016). Moreover, some studies evaluating dietary flavonoids on plasma flavanols and ex vivo platelet function have shown increases in plasma flavonoids with no effects on platelet function (Hubbard et al., 2003). Therefore, whether the effect observed here is the consequence of direct phenolic exposure or a chemical or metabolically responsive covariate of this exposure remains to be seen.

Limitations of the current study include a small sample size, complicated by population stratification (i.e. presence of responders and non-responders), which increased variance in the data. Notably, considerable individual variability in platelet response and oxylipin production was observed with acute EVOO intake, consistent with variability in other platelet responsive factors (e.g. "aspirin resistance") which have been postulated to result from differences in metabolism, sex, drug interactions and compliance failure (Fitzgerald & Fitzgerald, 2013; Rocca & Patrono, 2005). Dietary factors may also have played a role, and habitual diet assessments may have aided interpretation of this study. However, food frequency questionnaires are designed to provide diet assessment for study populations, not individuals, and our study design limited platelet interacting foods, obviating the need for 24 h recalls. However, including habitual high olive oil intake as an exclusion criterion may have strengthened the study. The lack of sensitive measures for reactive oxygen species (ROS) generation (i.e. esterified F2-isoprostanes) prevented us from unequivocally ruling out potential post-prandial changes in platelet derived ROS production, such as via NADPH oxidase (Carnevale et al., 2014). The single postprandial time point of two hours post-intake was chosen based on general knowledge on polyphenol pharmacokinetics, and not specifically for oleocanthal and oleacein, which have yet to be determined, and we may have missed the maximum inhibition time, which could differ by oil. Moreover, the anti-platelet effects of the oils may become more apparent for all subjects following short-term rather than acute intake, as has been noted for aspirin.

In conclusion, this study provides evidence that the specific phenolic content within EVOO can influence platelet aggregation responses in healthy male adults. Additionally, findings suggest that the extent of the response may be influenced by individual metabolism and/or diet. Future work will be needed to determine the time course of acute effects, as well as whether or not the observed response changes with prolonged intake periods.

Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.06.046.

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