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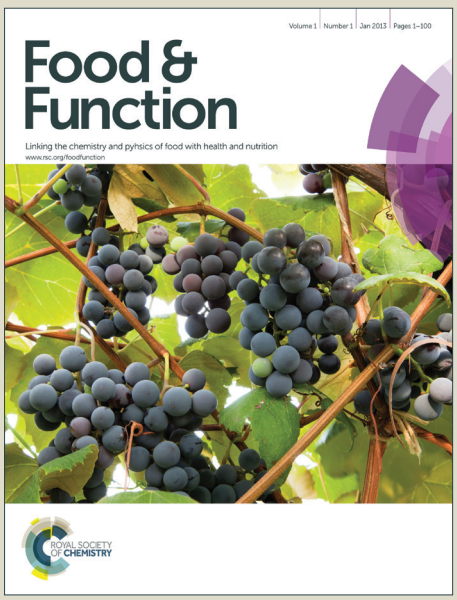

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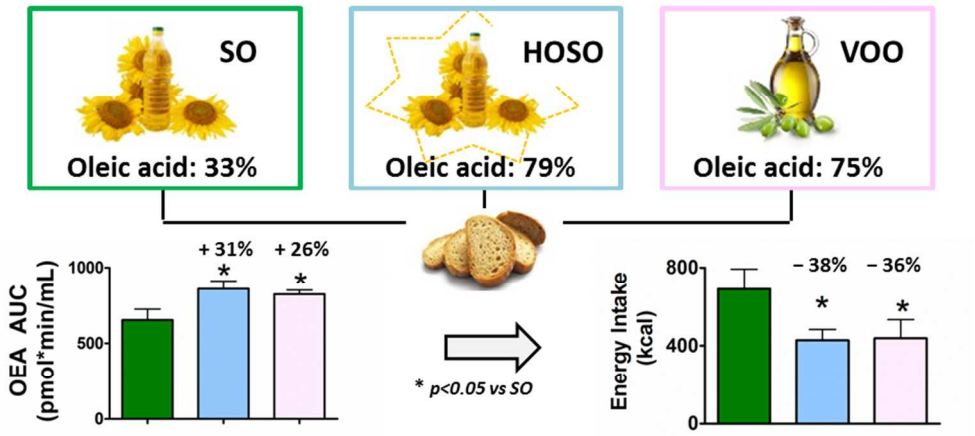

HOSO and VOO induce a postprandial higher response of plasma oleylethanolamide (OEA) and a concomitant reduction of energy intake at subsequent meal in humans

Graphical Abstract
254x142mm (96 x 96 DPI)

Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intakes in humans

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ABSTRACT

Animal data suggest that dietary fat composition may influence endocannabinoids (ECs) response and dietary behavior. This study tested the hypothesis that fatty acid composition of a meal can influence short-term response of ECs and subsequent energy intakes in humans. Fifteen volunteers in three occasions were randomly offered a meal containing 30g of bread and 30 mL of one of three selected oils: sunflower oil (SO), high oleic sunflower oil (HOSO) and virgin olive oil (VOO). Plasma ECs concentrations and appetite ratings over 2h and energy intakes over 24h following the experimental meal were measured. Results showed that after HOSO and VOO circulating oleoylethanolamide (OEA) was significantly higher than SO; a concomitant significant reduction of energy intake was found. For the first time oleic acid content of a meal was demonstrated to increase post-prandial response of circulating OEA and to reduce energy intakes at subsequent meal in humans.

Keywords: oleoylethanolamide, oleic acid, virgin olive oil, endocannabinoids, satiety

INTRODUCTION

Endocannabinoids (ECs) are a class of lipid mediators acting as endogenous ligands of the G protein-coupled cannabinoid receptors. In the early nineties, the two primary ECs were discovered: the arachidonoylethanolamide (AEA) and the 2-arachidonoylglycerol (2-AG)[1,2]. AEA together with palmitoylethanolamide (PEA), oleoylethanolamide (OEA), linoleoylethanolamide (LEA) belong to the chemical group of *N*-acylethanolamines (NAEs)[3-5]. All these compounds take part to a wide range of biological processes: pain, anxiety and depression, nausea, addiction and withdrawal[6], innate immunity[7]. Moreover, they are involved in feeding regulation by influencing metabolic and reward system[8]. In particular, AEA and 2-AG showed orexigenic properties in rodents as they dose-dependently increased food intake by central and peripheral administration[9,10] and were shown to be modulated by fasting and feeding states in brain[11]. In humans, a role of 2-AG in hedonic eating was demonstrated by Monteleone et al.[12] who found a significant increase of 2-AG concentration in plasma 2 h after consumption of a high palatable meal but not after consumption of non-palatable meal.

On the contrary oral or intraperitoneal administration of OEA, as well as its duodenal increase, determined a decrease of food intake in mice and rats[13-20] (for a review of the literature see Piomelli[21]); the mechanism underlying such effect being recently demonstrated to involve the histaminergic system.[22]

The chemical composition of the ingested food plays a primary role in the OEA formation: infusion into the duodenum of glucose or proteins did not show any effect, whereas among several fats, only oleic acid elicited OEA production in animals[23].

Interestingly, in humans Joosten and co-workers[24] found that fasting and non-fasting plasma concentrations of AEA, OEA, PEA and stearoylethanolamide (SEA) were positively associated with both serum total free fatty acids and their specific fatty acid precursors namely arachidonic, oleic, palmitic and stearic acid, respectively.

However, in humans the evidence of diet influence on ECs system is still scarce and limited on macronutrient ratios[25]. Moreover, to the best of our knowledge, the post-prandial ECs response was never associated to appetite cues and following energy intakes in humans.

The objective of this study was to test the hypothesis that fatty acid composition of a meal, and mainly its oleic acid content, can influence short-term response of ECs and subsequent energy intakes in humans. To this purpose three equicaloric meals with the same macronutrient composition but containing oils providing different amounts of oleic acid were offered to healthy and fasted volunteers. Blood drawings were performed over the following two hours and energy intakes at subsequent meal and over the following 24h were measured by self-recorded food diaries.

MATERIALS AND METHODS

Materials

AEA, LEA, OEA, PEA, 2-AG and d8-AEA were purchased by Cayman (Cayman Chemical, Ann Arbor, MI). Ethanol (EtOH), methanol (MeOH), chloroform, acetone, water, were from Merck (Darmstadt, Germany). Plastic vacutainer® serum tubes (16x100mm, 10ml) were purchased from Becton & Dickinson (1 Becton Drive, Franklin Lakes, NJ, USA). Polypropylene 1.5 ml tubes were from Eppendorf (Hamburg, Germany), 12 × 75 mm glass tubes from Corning (Corning S.r.l., Via Mercantini 5,Turin, Italy). Verex™ Vial, 9 mm, screw top, µVial i3 (Qsert) and PTFE/Silicone Cap were purchased from Phenomenex (Torrance, CA, USA). Sunflower seed oil, high oleic sunflower oil and virgin olive oil were provided by the Oleifici Mataluni (Montesarchio, Benevento, Italy).

Subjects

Healthy subjects were selected among students and staff of Department of Agriculture of "Federico II" University of Naples. Thirty five subjects were screened. Subjects taking any kind of drug, or

presenting endocrine, hepatic, renal, tumoral, autoimmune, cardiovascular, hematological, neurological or psychiatric diseases, sleep disorders, or allergies requiring treatment, as well as those who experimented variation of their body weight over the previous three months or who were on a restrictive diet, were excluded. The 51-items Three Factor Eating Questionnaire (TFEQ) was used to exclude restraint subjects (score in the restraint subscale F1>8)[26]. Fifteen subjects were eligible and they were enrolled to participate after signing an informed written consent. They were 7 Male and 8 Female, between 22 and 40 years old with a BMI between 18.1 and 25.0 kg/m². All experimental procedures were approved by the Ethics Committee of the University of Naples.

Meals

Three oils differing for their fatty acid compositions were used in this study (**Table 1**). They were sunflower seed oil (SO), high oleic sunflower oil (HOSO) and virgin olive oil (VOO).

Thirty milliliters of each oil together with 30 g white bread were offered to fasting subjects in 3 different occasions. Each meal provided an energy intake of 357 kcal, of which 75.9% came from lipids, 2.9% from proteins and 21.2% from carbohydrates. A higher content of lipids than a nutritionally balanced meal was used in order to exclude the potential confounding factors from other meal components on both short-term physiological response of ECs and appetite cues.

Study protocol

The study was conducted at the Department of Agriculture of the University of Naples. It was a randomized intervention trial with a cross-over design. Volunteers were invited to reach the nutrition laboratory at 8:00 a.m. in a fasting condition from 10 hours on three occasions with a 1-week wash-out period from each other. On the evening before each test volunteers were instructed to consume a standardized dinner and to refrain from eating and drinking alcoholic or energy-drinks from 22:00h. Once arrived to the laboratory participants had a 10 min rest and they were instructed to rate their hunger, fullness, satiety, thirst and desire to eat on 100 mm visual analogue scales (VAS)[27] anchored on the left as ‘‘not at all’’ and on the right as ‘‘extremely’’. The questionnaire comprised 3 main questions (How great is your desire to eat?, How full do you feel?, How satiated

do you feel?), and subjects were asked to answer indicating on the scale the point corresponding to their sensations. After the first blood drawing (baseline) each subject was asked to seat in a specific position isolated from the others, and was presented a tray containing the experimental meal including the type of oil he/she was randomized to consume in that occasion. Subjects were asked to consume the meal within 15 minutes and the compliance was evaluated by controlling that the glass and plate containing the foods were empty at the end of breakfast. At the following 30, 60, 120 minutes subjects rated their appetite sensations on VAS and underwent to blood drawings. After the last blood drawing, before participants left the laboratory, they were instructed to fill a 24h-food diary by recording the exact time, the types and amount (weight) of foods and beverages consumed from the moment they left the laboratory until the day after. On the next day volunteers had to return their 24h-food diary to the expert nutritionist of the research group and were submitted to a 24h diet recall interview in order to assess the compliance and to validate the 24h-food diary.

Biochemical analysis

Blood was collected in vacutainer® serum tubes and centrifuged at 2400 x g per 10 min at 4 °C. Serum was aliquoted (by 500 µL) and kept frozen at -80 °C until analysis [28]. Concentration of AEA, LEA, OEA, PEA, 2-AG were determined by isotopic dilution liquid chromatography-mass spectrometry as described previously by Cote and co-workers [29]. Five hundred microliters of each sample were added in polypropylene 1.5 mL tubes and protein precipitation was obtained by adding 3 volumes of acetone and centrifuging at 14000 x g per 10 min at 4°C. The supernatants were collected, transferred into 12 × 75 mm glass tubes and subjected to lipid extraction adding 1.5 mL of methanol/chloroform (1:2) containing 5 pmol of d8-anandamide as internal standard. The organic phase was then dried under nitrogen, the resulting residue re-suspended in 100 µL of acetonitrile:water (1:1) and centrifuged (4°C; 2400 g; 10 min).

Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Perkin-Elmer series 200 (Norwalk, CT, USA). A Synergi Max RP 80 column, 50x2.1

mm (Phenomenex, USA) was used and the flow rate was set to 0.2 mL/min. Injection volume was 10 µL. Mobile phase A consisted of H2O, 0.2% formic acid, while mobile phase B was CH3CN. The gradient program was as follows: 50-79 % B (10 min), 79-95 % B (1 min), constant at 95% B (2 min), finally returning to the initial conditions in 2 min. MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada). All the analyses were performed with a TurboIonSpray source with the following settings: drying gas (air) was heated to 300 °C, capillary voltage (IS) 5000 V. The declustering potential (DP) and the collision energy (CE) were optimized for each compound by directly infusion of standard solutions (10 µg/mL) into the mass spectrometry at a flow rate of 6 µl/min, using a Model 11 syringe pump (Harvard, Apparatus, Holliston, MA, USA). The acquisition was carried out in MRM (Multiple Reaction Monitoring) in positive ion mode for each compound.

Data acquisition and processing were performed using Analyst software v. 1.4. Acquisition parameters are summarized in **Table 2**.

Data analysis and statistics

The sample size needed to detect an effect of meal composition on primary outcome (post-prandial response of ECs) and secondary outcome (the effect of meal composition on subsequent energy intakes) was estimated on the basis of previous studies. A sample size of 13 participants was calculated to be adequate to find changes in ECs response significantly different using variation in accordance with Monteleone et al.[12] and Gatta-Cherifi et al. [30] , with an 80% power and an $\alpha = 0.05$. A sample size of 12 subjects was adequate to detect a 19% difference in energy intake with a power of 80% and $\alpha = 0.05$ using variation in accordance with findings of our previous studies [31,32].

Statistical analyses were performed using the statistical package SPSS for Windows (version13). The results of both appetite scores and of biochemical analyses were analyzed and expressed as the absolute changes from the baseline to reduce possible effects of inter-subject fasting variability. The subjective appetite sensations recorded after the consumption of the three types of oils and the ECs curves were compared and tested for the effect of treatment and of time as factors using ANOVA

151 for repeated measures. The total area under the curves (AUC) for hunger, fullness and satiety
152 ratings (from baseline over 2 h from breakfast consumption) as well as for ECs blood
153 concentrations were also estimated using the linear trapezoidal rule. Differences in the AUC values
154 were analyzed by one-way ANOVA and by Newman-Kleus multiple comparison test as post hoc.
155 Differences were considered significant at $p<0.05$.
156

RESULTS

Biochemical analysis

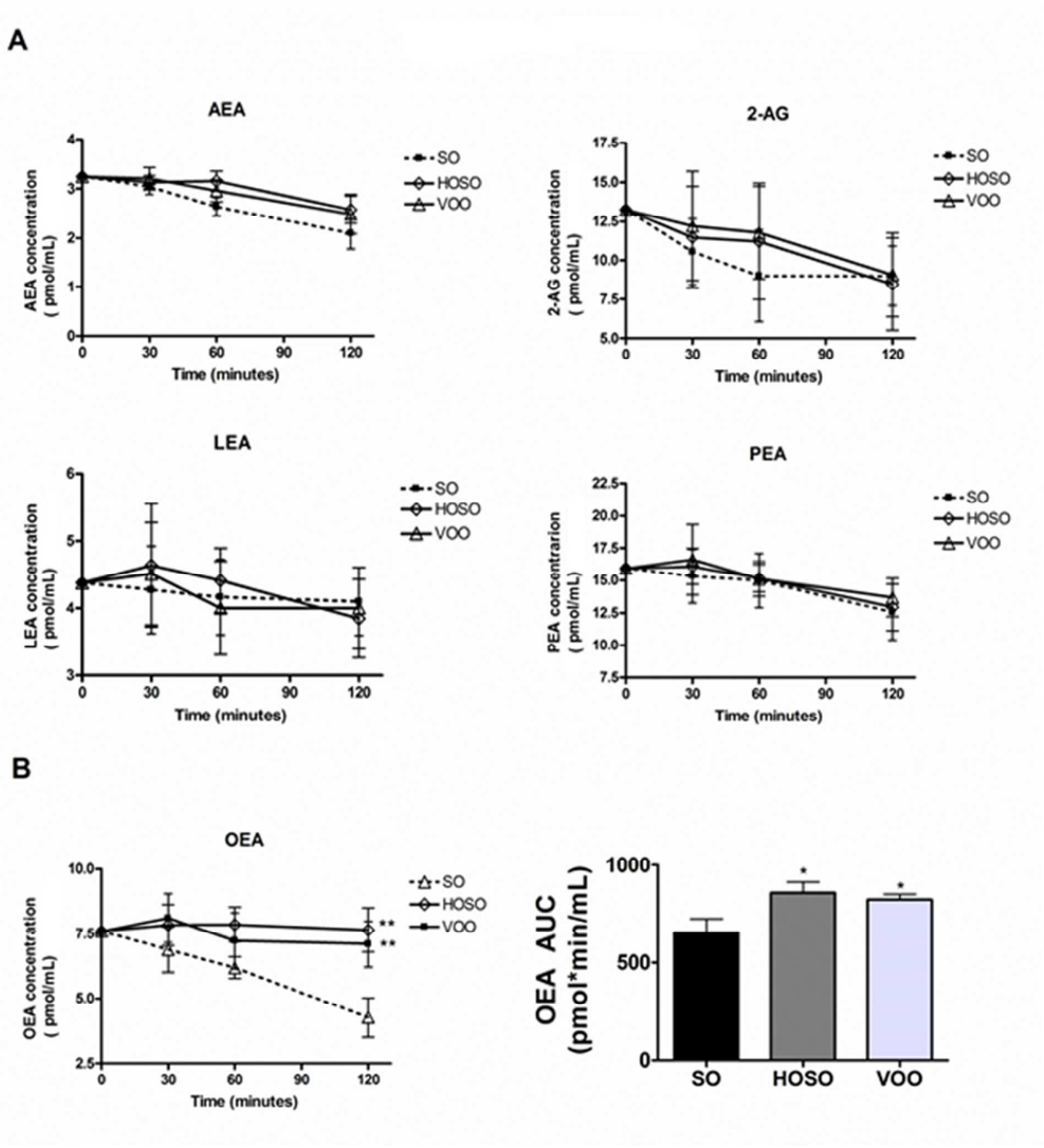

Figure 1: Post-prandial response of endocannabinoids - A) Concentration-time curves of AEA, 2-AG, LEA and PEA over 120 min following experimental meals; no significant difference of concentrations at baseline and following time points among experimental meals was found; B) Concentration-time curve and AUC of OEA over 120 min following experimental meals; no

significant difference of baseline concentrations among experimental meals was found. Values are expressed as means ± SEM. * $p<0.05$ vs SO; ** $p<0.001$ vs SO).

No significant difference of plasma concentrations of ECs at baseline among experimental meals was found. **Figure 1** (**panel A**) shows the variations of plasma concentration of AEA, 2-AG, LEA and PEA over 2 hours following the three meals. A tendency for reduced concentrations of AEA, 2-AG and PEA irrespective to the type of breakfast consumed was found. However, OEA concentrations following HOSO and VOO were 23.7% and 20.5% significantly higher than that following SO consumption, $AUC_{0\text{-}120}$ being 858±54 pmol•min/mL, and 823±28 pmol•min/mL vs 654±70 pmol•min/mL, respectively (**Figure 1**, **panel B**). LEA concentrations did not change over time upon the three meals.

Energy intakes at subsequent lunch and over 24h

All participants returned a well done 24-food diary and were submitted to 24-h diet recall interview. Data indicated that no difference in timing of subsequent lunch was present among participants following the three experimental meals. All subjects had their lunch always 3h after the experimental meal. However, subjects had a significant 261 kcal and 250 kcal energy reduced lunch after HOSO and VOO compared to SO, respectively (**Figure 2**).

No significant difference of energy intakes over the 24h was found (1787 ±602 kcal 1803±542 kcal and 1646 ±430 kcal following HOSO, VOO and SO, respectively).

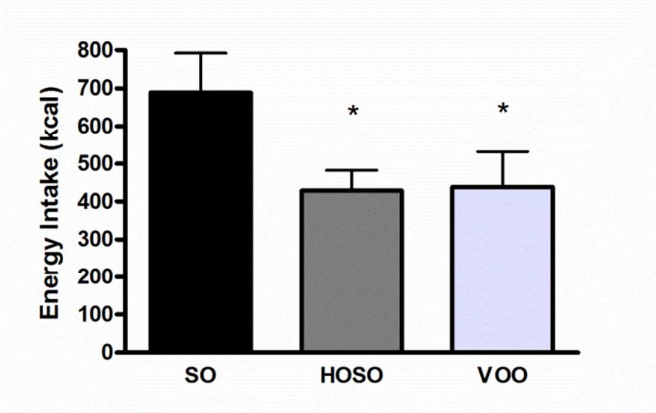

Figure 2: Energy intakes - Energy intakes (kcal) consumed during the lunch subsequent each experimental meal expressed as means ± standard deviation. * $p<0.05$ for VOO and HOSO vs SO.

Appetite ratings

No significant difference of sensations of hunger, fullness and satiety at baseline among experimental meals was found. **Figure 3** shows appetite ratings and AUC over the 2h following the consumption of breakfasts containing VOO, HOSO or SO. A trend of hunger reduction at 30 min and return to baseline value over the following 60 min after the three meals were recorded. Only after 120 min from SO consumption subjects perceived a hunger sensation higher than baseline and that perceived after HOSO and VOO consumption. Interestingly, increased fullness and satiety compared to baseline were found between 30 min and 60 min after meals containing HOSO and VOO, but not after SO. These perceptions were prolonged at 120 min only following VOO consumption. Looking at the appetite sensations over the 2h after the breakfasts ($AUC_{0\text{-}120}$), significant reductions of hunger and increase of fullness and satiety were found after VOO compared to SO consumption.

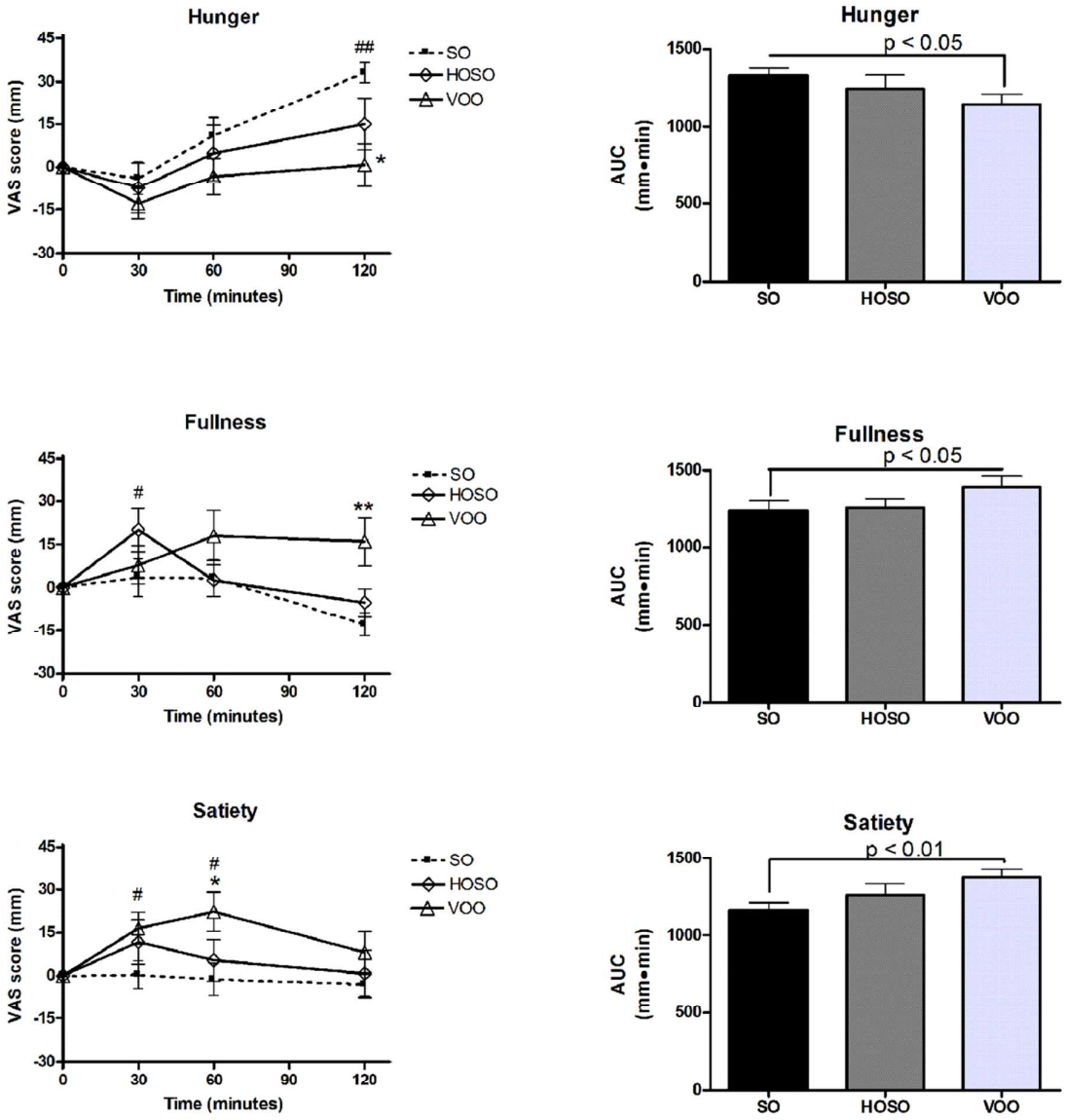

Figure 3: Appetite - Appetite rating-time curves and AUC of appetite sensations over 120 min following experimental meals. Values are expressed as means ± SEM. No significant difference of appetite sensations at baseline among experimental meals was found. At 120 min: *p<0.01 for hunger following VOO vs SO; **p<0.001 for fullness following VOO vs SO; # p<0.01 from baseline; ## p<0.001 from the baseline. AUCs of hunger, fullness and satiety after VOO are significantly different from SO.

DISCUSSION

The main finding of this study is that the content of oleic acid ingested at a meal influences post-prandial ECs response, appetite sensations and energy intake at subsequent meal in humans.

Few human studies investigated the response of ECs to meals with specific chemical composition. In this study a trend to reduced postprandial concentrations of all ECs except that of OEA after HOSO and VOO and of LEA after all experimental conditions were found.

The reduced post-prandial concentrations of ECs were in accordance with findings of previous studies[24,33,30]. A physiological reason to this response might be linked to the peripheral action of post-prandial insulin and to the direct influence of meal lipids on ECs biosynthesis/hydrolysis route in the upper intestine. In fact, Di Marzo and co-workers[27] suggested that insulin reduces ECs levels in a way inversely related to insulin resistance and it is known that dietary monounsaturated or polyunsaturated fatty acids can increase post-prandial insulin sensitivity in healthy subjects[34]. Thus, it is likely that the consumption of a meal rich in unsaturated fatty acids might have generally reduced ECs response through insulin.

On the other hand, the consumption of meals providing higher amount of oleic acid (such as that including HOSO and VOO vs SO) might sustain post-prandial concentration of OEA independently from insulin action. In fact, oleic acid may act as precursor of OEA formation in the intestine as previously demonstrated in animals[35-37] and/or trigger some physiological mechanisms modulating its selective spillover from the intestinal membrane phospholipids. This hypothesis is consisting with a previous study demonstrating that the consumption of virgin olive oil and high-oleic sunflower oil determined, over the following 2 hours, a significant increase of circulating oleic acid-rich phospholipids[38], which are known to be the precursors of intestinal biosynthesis of ECs at level of mucosa, epithelial cells and serosa[39]. In addition, a strict connection between circulating ECs and free fatty acids was recently suggested in humans by Joosten and co-workers[24].

231 Other factors than fatty acid composition of oils might have influenced post-prandial response of
232 LEA whose concentration did not change vs baseline after the three meals.
233 It could not be excluded in the present study that different cephalic responses triggered by oral taste
234 and/or different preference for the oils might contribute to influence the circulating pattern of ECs,
235 through their well-known interaction with the gut metabolism. That dietary fat (but not other
236 nutrients) can modify gut metabolism of ECs through oral sensing and selectively mobilize ECs in
237 the upper gut, also influencing dietary behavior, was demonstrated in rats[40]. On the other hand a
238 link between circulating 2-AG and food preference was found by Monteleone and co-workers[12]
239 who showed increased plasma 2-AG in humans after consumption of their preferred food but not
240 after the non-preferred one[12].
241 Further human studies should clarify the role of meal lipid composition on formation of different
242 ECs induced by cephalic response.
243 Strikingly, both the meals eliciting the highest post-prandial OEA response (VOO and HOSO vs
244 SO) were associated with the highest reductions of energy intakes at subsequent meal. These
245 findings were in disagreement with the animal study conducted by Gaetani and co-workers[15], where
246 OEA administration in free-feeding rats reduced only meal frequency without altering meal size,
247 whereas they were perfectly in line with Provensi and co-workers [22].
248 Social and cognitive cue could majorly influence the timing of eating in humans compared to
249 animals thus rendering insignificant the effect of OEA response on time of eating while evidencing
250 OEA effect on food intake at subsequent meal in our free-living participants. Several researchers
251 aimed at ranking the effect of lipid composition on satiety[41-43]. Alfenas and co-workers[44] proposed
252 that the satiety effect of fatty acids was linked to their oxidation rate: the higher is the number of
253 double bonds, the faster is the rate of oxidation, the higher is satiety. However other studies did not
254 confirm this suggestion[45,46]. Several differences among the studies, including the amount and source
255 of fats provided to the volunteers might cause such discrepancies rendering the debate still open.

Only appetite ratings after consumption of the meal containing VOO were coherent with the reduced energy intake compared to the meal with SO. This might be a matter of dietary habits and cognitive factors on appetite sensations. In fact, data from food frequencies questionnaire (not shown) indicated that all study participants were used to consume virgin olive oil as conditioning fat, while the consumption of seed oils was sporadic. Familiarity with a food and expected satiation are interrelated. More familiar foods are expected to be more filling[47] and measures of expected satiety are highly correlated with actual satiety[48]. From a mechanistic point of view it could not be excluded that non-fat components present in VOO (but not in SO or HOSO) such as several volatile compounds (attributing to VOO the characteristic aroma) and phenolic compounds might contribute to the effect of VOO on energy intake and appetite regulation as recently suggested in the elegant study by Frank and co-workers[49] or reviewed by Panickar[50], respectively.

CONCLUSION

In conclusion, in this study for the first time it was demonstrated that oleic acid content of a meal can increase post-prandial response of circulating OEA and it may reduce energy intakes at subsequent meal in humans. The present data offer a concept to design new food ingredients for energy intake control using edible oils rich in oleic acid. Further studies should evaluate whether these findings can be reproduced also in overweight/obese subjects and/or in the context of meals nutritionally balanced for macronutrients ratio.

Acknowledgements

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363 **Table 1:** Fatty acid composition and differences among sunflower seed oil (SO), high oleic
364 sunflower oil (HOSO) and virgin olive oil (VOO) used in this study.

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Fatty acid	composition (%)			difference (%)	
	SO	HOSO	VOO	HOSO - SO	VOO - SO
Myristic acid	0.07	0.05	0.01	-0.02	-0.06
Palmitic acid	6.42	4.42	12.08	-2.00	5.66
Palmitoleic acid	0.14	0.16	0.69	0.02	0.55
Heptadecanoic acid	0.04	0.03	0.04	-0.01	0.00
Heptadecenoic acid	0.02	0.04	0.06	0.02	0.04
Stearic acid	3.29	2.67	2.34	-0.62	-0.95
Oleic acid trans	0.04	0.04	0.00	0.00	-0.04
Oleic acid	33.20	79.04	75.26	45.84	42.06
Linoleic acid trans	0.45	0.09	0.00	-0.36	-0.45
Linoleic acid	55.02	11.99	8.25	-43.03	-46.77
Arachidic acid	0.24	0.24	0.28	0.00	0.04
Linolenic acid	0.07	0.04	0.67	-0.03	0.60
Eicosenoic acid	0.17	0.24	0.25	0.07	0.08
Behenic acid	0.64	0.73	0.04	0.09	-0.60
Lignoceric acid	0.21	0.23	0.01	0.02	-0.20

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369 **Table 2:** Acquisition parameters used for the LC/MS/MS analysis.

	Precursor Ion $[M+H]^+$	Product Ion $[M+H]^+$	DP	CE
AEA	348.0	62	40	35
OEA	326.0	62	40	35
LEA	324.0	62	60	30
PEA	300.0	62	60	30
2-AG	396.5	379.5	35	11
		287.3		14
		268.9		18
AEA-d8	356.5	63.2	50	31
		209.3		18

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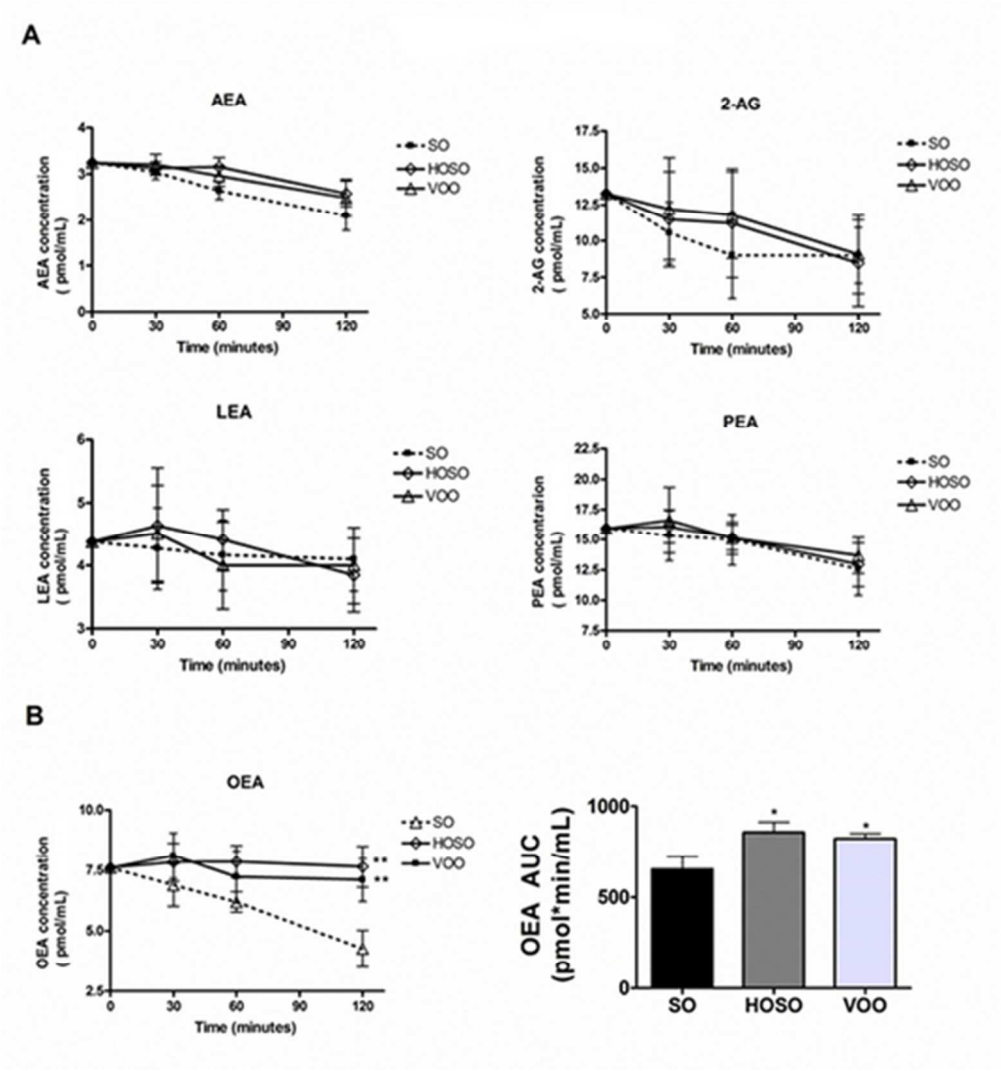

Figure 1: Post-prandial response of endocannabinoids - A) Concentration-time curves of AEA, 2-AG, LEA and PEA over 120 min following experimental meals; no significant difference of concentrations at baseline and following time points among experimental meals was found; B) Concentration-time curve and AUC of OEA over 120 min following experimental meals; no significant difference of baseline concentrations among experimental meals was found. Values are expressed as means ± SEM. * $p<0.05$ vs SO; ** $p<0.001$ vs SO).
45x48mm (300 x 300 DPI)

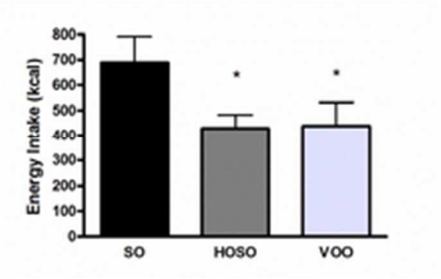

Figure 2: Energy intakes - Energy intakes (kcal) consumed during the lunch subsequent each experimental meal expressed as means ± standard deviation. * $p<0.05$ for VOO and HOSO vs SO.
18x11mm (300 x 300 DPI)

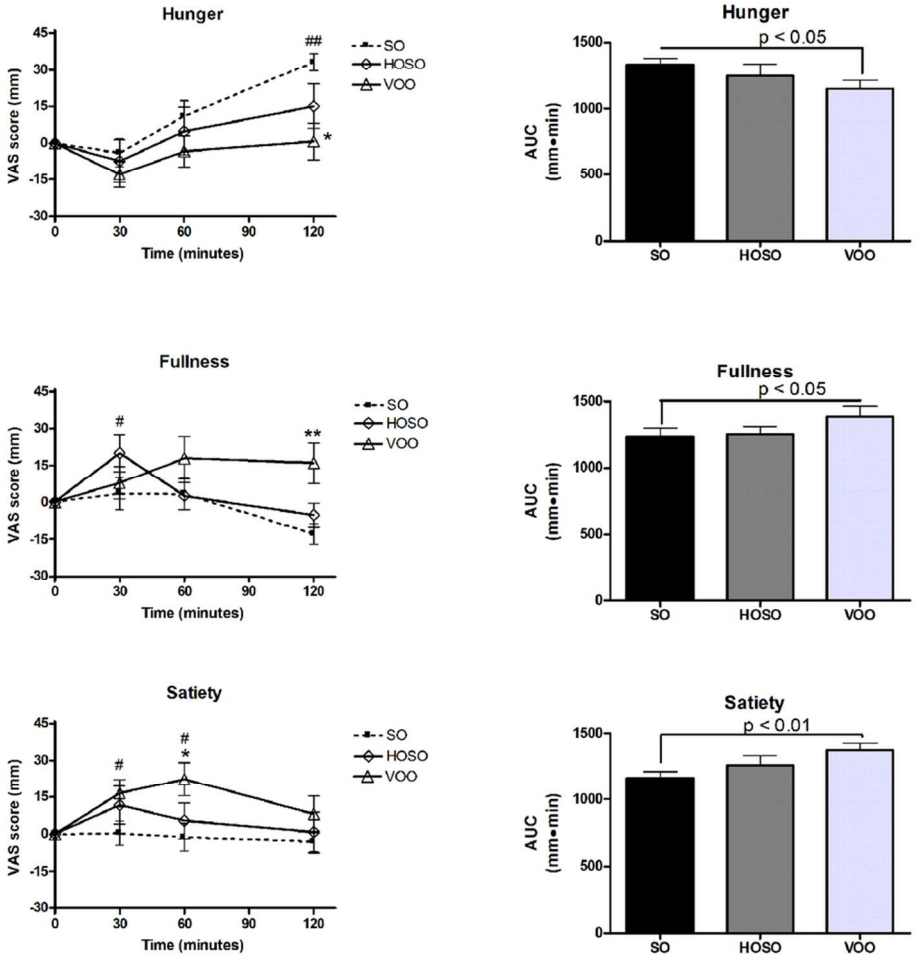

Figure 3: Appetite - Appetite rating-time curves and AUC of appetite sensations over 120 min following experimental meals. Values are expressed as means ± SEM. No significant difference of appetite sensations at baseline among experimental meals was found. At 120 min: *p<0.01 for hunger following VOO vs SO; **p<0.001 for fullness following VOO vs SO; # p<0.01 from baseline; ## p<0.001 from the baseline. AUCs of hunger, fullness and satiety after VOO are significantly different from SO.
90x93mm (300 x 300 DPI)