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RESEARCH ARTICLE

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An extra virgin olive oil-enriched chocolate spread positively modulates insulin-resistance markers compared with a palm oil-enriched one in healthy young adults: A double-blind, cross-over, randomised controlled trial

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Abstract

Aims: To investigate if extra virgin olive oil (EVOO) or palm oil enriched chocolate spreads consumption leads to different results in terms of plasma ceramides concentration, glucose and lipid metabolism, inflammatory markers and appetite regulation in young healthy subjects.

Methods: In a 2-week, double-blind, cross-over, randomised controlled trial, 20 healthy, normal-weight subjects with a mean age of 24.2 years (SD: 1.2), consumed chocolate spread snacks (73% of energy [%E] from fat, 20% from carbohydrates and 7% from proteins), providing 570 Kcal/day added to an isocaloric diet. The chocolate spreads were identical, except for the type of fat: EVOO oil, rich in monounsaturated fatty acids (MUFAs), or palm oil, rich in Saturated Fatty Acids (SFAs).

Results: EVOO-enriched chocolate spread consumption led to better circulating sphingolipids and glucose profile, with reduced plasma ceramide C16:0, ceramide C16:0/ceramide C22:0-ceramide C24:0 ratio and sphingomyelin C18:0 ($P = 0.030$, $P = 0.032$ and $P = 0.042$, respectively) compared to the palm oil-enriched chocolate spread diet. HOMA-IR and plasma insulin were lower, while the Quicki and the McAuley Index were higher after the EVOO diet compared to the palm oil diet ($P = 0.046$, $P = 0.045$, $P = 0.018$ and $P = 0.039$ respectively). Subjects maintained a stable weight throughout the study. No major significant changes in total cholesterol, triglycerides, HDL, inflammatory markers, and appetite-regulating hormones/visual analogue scale were observed between the groups.

Conclusions: Partially replacing SFAs with MUFAs in a chocolate-based snack as part of a short-term isocaloric diet in healthy individuals may limit SFAs detrimental effects on insulin sensitivity and decrease circulating harmful sphingolipids in young adults.

KEYWORDS
ceramides, chocolate, diet, extra virgin olive oil, insulin resistance, palm oil

1 | INTRODUCTION

Western diets are characterised by high fat intake and are associated with an increased risk of metabolic derangements.[1,2] The two most common fatty acids consumed in this kind of diet are oleic acid (OA; C18:1) and palmitic acid (PA; C16:0), present in similar quantities. Despite dietary fat intake in Mediterranean countries being similar to that of other western countries, epidemiological studies have shown better overall metabolic health,[1,2] and the higher consumption of olive oil, rich in OA and low in PA, has been suggested as an important protective factor.[3-6]

Several studies have previously reported that palm oil, rich in PA, is deleterious to metabolic health,[7,8] leading to a state of lipotoxicity for the accumulation of fats that are toxic to the body, such as ceramides,[9] a group of sphingolipids. Sphingolipids are a family of ubiquitous lipid elements of cell membranes but also shown to be bioactive compounds implicated in cell growth regulation and metabolism control.[10] Ceramides are synthesised either de novo from saturated fatty acids or from other sphingolipids including sphingomyelin (SM). An increased intake of PA is expected to boost the de novo synthesis pathway. An elegant study conducted by Kien and collaborators using palm oil as main source of PA demonstrated that the PA/OA ratio correlated with insulin resistance and increased ceramide levels in muscle and plasma of healthy subjects.[9] In fact, an excess of ceramides can lead to insulin resistance through intracellular mechanisms, which, protracted over time, can in turn cause a state of chronic inflammation, possibly leading to diabetes and cardiovascular disease.[11,12] In particular, recent studies have highlighted the role of ceramide C16:0 as the primary mediator of insulin resistance related to obesity.[8,13] In addition to the mechanisms related to insulin resistance, preclinical evidence suggests new pathogenetic mechanisms of ceramides in the induction of cardiovascular disease. Ceramides can promote the aggregation and subendothelial sequestration of lipoproteins in arterial vessels.[14] Similar to cholesterol, ceramides also accumulate in atherosclerotic lesions[14,15] and can be modulated by the diet[16,17] since they are synthesised in all tissues through pathways that mainly use saturated fatty acids.[18]

These data support the increasingly widespread evidence that PA enhances ceramides synthesis, leading to higher cardiometabolic risk. On the contrary, consumption of OA, abundant in the Mediterranean diet staple EVOO, is associated with an improvement in terms of insulin resistance and chronic inflammation,[19,20] and is therefore protective against metabolic disease.[6,21] In an intervention trial that compared the effect of overfeeding diets with different composition (i.e., high in carbohydrates vs. saturated vs. unsaturated fat intake) the diet enriched with saturated fat led to the highest hepatic fat accumulation while the subjects consuming more olive oil showed the lowest increase in liver fat.[22] Moreover, when added to PA in vitro, OA appears to attenuate the toxic effect of PA.[23] These data suggest that a diet rich in olive oil may balance the harmful effects of excessive PA consumption. A large study, the PREDIMED trial, showed indeed a strong association between circulating ceramides and the risk of cardiovascular disease, mitigated by the consumption of an EVOO-rich Mediterranean diet.[20]

It was previously reported that more than 27% of children's energy intake comes from snacks,[24] and snack frequency is associated with a higher risk of developing abdominal obesity and overweight in children.[25] Chocolate-based snacks are widely consumed throughout the world.[26] Many types are available, and the primary components are usually cocoa, hazelnuts and added oils. The main consumers of these are children and adolescents, categories susceptible to the development of fatty liver and related metabolic derangements.[27] With the prevalence of obesity rapidly increasing throughout the world, both in adults and children,[28,29] and that of NAFLD following the same pace,[30,31] it is important to determine whether the components of widely consumed foods such as chocolate-based snacks might play a role in the development of metabolic derangements.

We, therefore, aimed to evaluate the impact of two different oils added to the same chocolate spread consumed for 14 days in terms of (1) plasma ceramides as the primary outcome, (2) glucose metabolism markers (HOMA-IR, plasma insulin and glucose) and cholesterol levels (Plasma Low Density Lipoprotein, Triglycerides, Total Cholesterol) (3) inflammation markers (Plasma C-reactive protein, Interleukin-6, Tumour Necrosis Factor-alpha), (4) evaluation of satiety levels through the appetite-regulating hormones measurement (Plasma Glucagon Like Peptide-1, Oxyntomodulin, Ghrelin, leptin, adiponectin, and Peptide YY) and the visual analogue scale analysis as secondary outcomes.

2 | MATERIALS AND METHODS

2.1 | Study design and subjects

Twenty-four subjects were screened (Figure S1) and 20 normal-weight subjects (10 males and 10 females) were enrolled, with a mean age of 24.2 (SD: 1.18) years (Table 1). The subjects were randomised (1:1) in a 2-week, double-blind, controlled, cross-over study to the daily consumption of 100 g chocolate spread enriched with either Extra Virgin Olive Oil (EVOO) or palm oil as part of an

TABLE 1 Anthropometric parameters before and after 14 days of EVOO and Palm oil based chocolate spread

	Olive oil				Palm oil				
Parameter	**Visit 1**		**Visit 3**		**Visit 1**		**Visit 3**		***p***
N	19				19				
Female (%)	47.4				47.4				
Age (years)	24.21	(1.18)			24.21	(1.18)			
BMI (Kg/m^2)	22.56	(2.10)	22.40	(2.04)	22.42	(2.08)	22.22	(2.00)	0.290
Weight (Kg)	68.53	(9.68)	68.06	(9.47)	68.08	(9.61)	67.48	(9.35)	0.245
WC (cm)	74.13	(7.07)	73.71	(6.65)	74.01	(6.60)	74.21	(6.85)	0.279
FM (Kg)	14.88	(5.45)	15.00	(5.86)	14.88	(5.37)	14.41	(4.90)	0.329
FFM (Kg)	53.61	(9.36)	52.39	(8.39)	53.20	(9.07)	53.06	(8.59)	0.376

Note: Data shown as means (standard deviation) of the mean. The p value shown in the right column is from a general linear mixed model analysis, with random intercepts, in which the dependent variable was the value at day 14 from the start of the consumption of the product with olive oil or palm oil. The variables of treatment, sequence and baseline values (when available) were included in the model as fixed effects.

Abbreviations: BMI, body mass index; EVOO, extra virgin olive oil; FFM, free fat mass; FM, fat mass; WC, waist circumference.

isocaloric diet. If the subjects received the EVOO chocolate spread in the first phase, they received the palm oil chocolate spread in the second and vice versa. A random numeric sequence blocked for gender was used for the randomisation process performed by an independent researcher. The subjects were recruited at the Campus Bio-Medico University Hospital (UCBM) and university residences. This study was approved by the Campus Bio-Medico University of Rome's ethics committee (approval number 73/18 PAR ComEt CBM), and the subjects signed an informed consent prior to any study procedure. The subjects were overall healthy and the study inclusion and exclusion criteria are listed in the supporting information.

Briefly, the subjects underwent a screening visit (Visit 0) at the UCBM Hospital, during which blood sampling, medical history, physical examination and bioimpedance analysis were performed (Figure S2). Subsequently, the eligible subjects were put on an isocaloric run-in diet before the randomisation visit (Visit 1), where the same procedures of visit 0 were performed. The subjects were then randomised to the 2-week-long EVOO or palm oil chocolate spread consumption as part of an isocaloric diet. On the seventh day, the subjects underwent a compliance visit (Visit 2) to verify the products' consumption (collection of empty chocolate jars and dispensation of new jars); anthropometric measures were also taken for weight and body composition monitoring. On the fourteenth day, the subjects were asked to consume a 100 g chocolate spread jar (challenge meal) and serial blood samples were drawn at times 0', 30', 60', 90', 120' and 180'. At the same time points, subjects were required to complete a visual analogue scale to investigate hunger and satiety. Also, the same procedures of visit 1 were performed. Subsequently, the subjects underwent another week of run-in diet, identical to the previous run-in diet, to be then allocated to another two weeks of the opposite treatment to the previous one, with the same described methods (Figure S2). All meals were prepared according to the study protocol and provided to the study subjects, who were asked not to consume other food and not to change the intensity of their physical activity. Daily food records and the international physical activity questionnaire (IPAQ) were used to monitor for dietary and physical activity compliance.

2.2 | Diets and study chocolate spread

The isocaloric diets were designed by registered dieticians based on subjects' gender, age, weight, height and physical activity level recorded at the screening using established formulae.[32,33] Independently from the energy requirement established for each subject, the macronutrient compositions were identical between subjects (percentage of energy [%E], on the total daily calorie intake, from carbohydrates, protein, fat, PA and OA). Each subject was assigned a dietician for the entire duration of the study. All meals were prepared by the university canteens and consumed on-site or collected daily in individual meal boxes. In addition, subjects were required to fill out a daily food record to assess their compliance with the established nutritional plans and send photographic records of food leftovers that were considered for analysis. These food records were collected at the V1 visits, representing the run-in phases, at V2 representing the first week of intervention and V3 representing the second week of intervention for both phases. The dosage and preparation of the EVOO samples, served raw in specific disposable cups with the meals, were carried out at the metabolic laboratory of Endocrinology of the UCBM and then portioned depending on the quantity of oil provided by the reference diet, weighed with a precision balance.

The subjects could consume 100 g of the chocolate spread during breakfast or as mid-morning and/or mid-afternoon snack. The dieticians elaborated the subjects' food records using the Metadieta software (METEDA Srl), taking into account food leftovers. Moreover, the chocolate leftovers were weighed with a precision balance.

During the run-in periods, the subjects were asked to adhere to the isocaloric diet, which was developed to make their metabolic/nutritional status homogeneous, before being allocated into the two nutritional treatment phases. The subjects ate a diet low in fat and PA with approximately the following composition of macronutrients in percentage of energy [%E] on the total daily calorie intake (carbohydrates, 53.48% Kcal; fat, 32.11% Kcal; protein, 15.45% Kcal; PA, 2.82% Kcal; and OA, 11.61% Kcal).

For the intervention diets, the macronutrient compositions were identical ([%E] from carbohydrates, protein and fat), whereas the fatty acid compositions differed (Table S1). The same calories of the run-in diets were maintained, reducing the calories from the food and replacing them with those of the chocolate spread to keep the same subjects' energy requirement and weight. The lipid content of the experimental product provided about 21.47% Kcal of the total daily calorie intake [%E]. The energy [%E] from PA in the diet was 3.46% Kcal in the EVOO group and 6.59% Kcal in the palm oil group. The energy [%E] from OA in the diet was 18.54% Kcal in the EVOO group and 15.48% Kcal in the palm oil group (Table S1).

The 100 grams of study chocolate spread contained ~570 Kcal distributed in % of energy on the product's total calories: 73% Kcal from fats, 20% Kcal from carbohydrates and 7% Kcal from protein (Table S2). The carbohydrates were mostly refined sugars. The EVOO chocolate spread contained in percentage of energy on the product's total calories: SFAs, 16% Kcal; MUFAs, 51% Kcal; Polyunsaturated Fatty Acids (PUFA), 5.89% Kcal; PA 0.99% Kcal; OA, 5.63% Kcal. The palm oil chocolate spread contained in % of energy on the product's total calories: SFAs, 27% Kcal; , MUFAs 40% Kcal; PUFAs 5.51% Kcal; PA, 2.2% Kcal; and OA 4.45% Kcal (Table S2). More details on the chocolate spread ingredients are reported in Table S3.

Visual analogue scale questionnaire analysis to evaluate the hunger, satiety, cravings and the potential amount of food to eat is described in the supporting information.

2.3 | Biochemical analysis

Venous blood samples were obtained from all study participants. UCBM central laboratory assessed serum levels of glucose, total cholesterol, High Density Lipoprotein (HDL), Triglycerides, Low Density Lipoprotein (LDL), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Gamma-Glutamyl Transferase (GGT), total bilirubin, direct bilirubin, complete blood count, creatinine, Na, K and Uric Acid. Insulin, Adiponectin, IL-6, Leptin and TNF-alpha were measured using the automated ELISA technique (Protein Simple 3001 Orchard Parkway San Jose). ELISA assays were used also to measure serum levels of Glucagon-Like-Peptide-1 (GLP-1), Ghrelin (Novus Biological 614 McKinley Place NE Minneapolis), oxyntomodulin (Ansh Labs 445 Medical Centre Blvd|Webster, TX 77598) and Peptide YY (PYY) (Merck, Darmstadt, Germany and AnshLabs). The blood processing procedure of hormones and other molecules is described in the supporting information. An LC-MS/MS assay technique was used for the target analysis of sphingolipids and the analysis of plasma fatty acids as previously reported[34,35] (Analytical Chemistry Lab, Fondazione Istituto Italiano di Tecnologia). Plasma Antioxidant Test (PAT) and the Plasma Reactive Oxygen Metabolites (D-roms) were measured as previously shown[36,37] (H&D srl Parma-Italy). Chocolate spread analysis was performed using Gas Chromatography with flame-ionisation detection (FID) (Eurofins Chemical Control Srl). The following formulas have been used to calculate the HOMA-IR = ([fasting insulin in mU/mL x fasting glucose in mg/dL]/405), the QUICKI index = 1/[log (Fastin insulin in μU/ml) + log (Fasting Glucose in mg/dL)] and the Mc-Auley index = exp [2.63–0.28 ln (fasting insulin in μU/ml)−0.31 ln (fasting triglycerides in mmol/l)].

2.4 | Statistical analysis

The R software version 4 was used for the statistical analysis (R Foundation for Statistical Computing). The continuous variables were expressed as means (standard deviation) when the variables were normally distributed and as medians (IQR) when the variables were not normally distributed. The Wilcoxon signed-rank test and the paired *t*-test were used for the analysis of diet adherence. For the inferential statistics, a logarithmic transformation was performed to obtain an approximately Gaussian distribution. For the analysis of the outcomes, mixed generalised linear models were used, with random intercepts, in which the dependent variable was the value at day 14 from the start of the consumption of the product with EVOO or palm oil. Treatment, sequence and baseline values (when available) have been entered as fixed effects.

3 | RESULTS

3.1 | Adherence to the diet and consumption of the chocolate spread

Changes in plasma fatty acid composition reflected the allocated dietary oil, denoting compliance, plasma OA increased most in the group that consumed the EVOO product, and PA increased most in the group that consumed the product with palm oil; however, these changes did not reach a significant difference (Table S4). Product intake adherence monitored at visit 2 and 3 showed high compliance. Subjects consumed 98% of the chocolate samples in the EVOO group and 97% in the palm oil group with no difference between the two groups ($P = 0.51$). However, one subject had to be excluded from the analysis, as required by the protocol, for not consuming the product for more than three days because of reported lack of willingness in eating the entire daily amount of the product. The dietary records showed high compliance and excluding the nutritional data derived from the product consumption, there were no significant variations in Kcal, macronutrients, and fatty acids consumed, together with no difference in physical activity between the groups (Table S5). In

addition, all subjects maintained stable weight, waist circumference and body composition throughout the study (Table 1). No adverse events were reported (Table S6).

3.2 | Effect of diets on ceramides and lipids

EVOO and palm oil products induced different effects on circulating ceramides (CERs). Plasma concentration analysis at the end of 14 days of nutritional tre2`atment showed no difference in terms of total ceramides between the two groups. However, plasma ceramide C16:0 was significantly higher in the group consuming the palm oil-enriched spread compared to the group consuming the EVOO-enriched spread ($P = 0.030$; Table 2). The plasma C16:0/C22:0 + C24:0 ceramides ratio (calculated as the ratio between C16:0 concentration divided by the sum of C22:0 and C24:0 concentrations) was also significantly lower in the EVOO diet compared to the palm oil diet ($P = 0.032$; Table 2). Plasma C18:0 sphingomyelin was higher in the palm oil group compared to the EVOO group ($P = 0.042$; Table 2). No other significant differences were found in other sphingolipids (Table 2). No differences in total cholesterol, LDL, HDL and triglycerides were observed between the two groups (Table 3).

3.3 | Glucose metabolism

The products with the two different edible oils differentially impacted insu3lin resistance markers. Fasting plasma glucose concentration showed a tendency to be reduced in the EVOO group compared to the palm oil group ($P = 0.079$; Table 3), fasting plasma insulin concentration was significantly lower in the EVOO group than the palm oil one ($P = 0.045$; Table 3). Pointing in the same direction, the insulin resistance marker HOMA-IR was significantly lower in the EVOO compared to the palm oil group ($P = 0.046$; Table 3), and the insulin sensitivity markers Quicki Index and Mc-Auley Index were significantly higher in the EVOO than in the palm oil group ($P = 0.018$ and $P = 0.039$ respectively, Table 3). Interestingly, the differential effect of the two diets on HOMA-IR decreased when the model was corrected for ceramide C16:0 ($P = 0.132$).

No significant differences were found in the glucose and insulin excursion (0–180' area under the curve) and in the Gutt-ISI and Matsuda indices calculated at visit 3 during the product consumption (Table 3).

3.4 | Inflammation

The oxidative stress marker measured by plasma D-roms showed a tendency to be higher in the palm oil group than in the EVOO group ($P = 0.06$; Table 3). No significant differences in plasma values of the Plasma Antioxidant Test (PAT), interleukin 6 (IL-6), C-reactive protein (CRP) and tumour necrosis factor-alpha (TNF-alpha) were observed between the two groups (Table 3).

3.5 | Appetite regulating hormones and visual analogue scale

No difference was observed in the postprandial excursion after the consumption of the product based on EVOO or palm oil (0–180' area under the curve) on the fourteenth day in terms of plasma values of ghrelin, adiponectin, leptin, GLP-1, PYY and oxyntomodulin (Table 4) and visual analogue scale (Table 4).

4 | DISCUSSION

Herein we show that the short-term consumption of an EVOO-enriched chocolate spread in healthy subjects was associated with lower C16:0-ceramide levels and higher insulin sensitivity indices compared with a palm oil-enriched chocolate spread which led to higher circulating C16:0-ceramide and C18:0 sphingomyelin plasma levels, coupled with higher insulin resistance indices although within normal ranges.

As previously shown, ceramides may be playing a mechanistic role in the development of insulin resistance and they have been shown to increase the cardiovascular risk.[38] They vary in acyl-chain lengths from C14:0 to C30:0, each manifesting specific biologic properties. Some studies suggest that, among the ceramides, the C16:0-ceramide is the most involved in mediating the key pathophysiology of insulin resistance,[39,40] also inhibiting mitochondrial complex IV activity with subsequent increased oxidative stress.[41] Along the same lines, inhibition of ceramide synthase 6, involved in the synthesis of C16:0-ceramide, leads to insulin sensitisation, improvement in hyperglycemia and reduced body weight in animal studies.[42] Similarly, whole body or tissue specific (liver and/or adipose tissue) deletion of dihydroceramide desaturase 1, which converts dihydroceramides in ceramides, resolved insulin resistance.[43] Conversely, the inhibition of ceramide synthase 2, which synthesises the very-long-chain ceramides C22-C24, leads to severe and progressive liver disease in rodent models[41] and compensatory increases in C16-ceramides,[40] suggesting a protective role of these ceramides' subclasses with longer chain length.

Herein, we also show that the ratio C16:0/C22:0 + C24:0 ceramides was higher in the palm oil group, further supporting the effects of palm oil in the synthesis of detrimental ceramides, such as C16:0, associated with insulin resistance onset. Besides, when the analysis of the insulin resistance marker levels after 14 days was corrected by the C16:0-ceramide plasma levels, the effect size of the result was reduced and not significant, supporting preclinical data suggesting that C16:0-ceramide plays a causal role in the insulin resistance pathogenesis. These effects go in the same direction of those as Iggman et al., reporting that lean and young individuals consuming palm oil-rich muffins increased insulin levels compared with those consuming sunflower oil-rich muffins as part of a high-fat diet leading to modest weight gain.[44]

In addition, we observed significantly higher circulating levels of SM C18:0 in the palm oil group compared to the EVOO group. SM is

TABLE 2 Plasma lipidomics analysis before and after 14 days of EVOO and Palm oil based chocolate spread

	EVOO				Palm oil				
Parameter	**Visit 1**		**Visit 3**		**Visit 1**		**Visit 3**		***p***
Ceramides									
Cer_Total	4553	(1010)	4686	(2061)	4793	(1390)	4768	(2248)	0.803
cer_12:0 (nmol)	91.0	(27.0)	78.5	(29.4)	88.1	(30.5)	80.5	(26.6)	0.554
cer_16:0 (nmol)	8.03	(2.95)	6.46	(2.34)	7.46	(2.89)	8.36	(2.27)	**0.030**
cer_18:0 (nmol)	27.6	(9.71)	32.3	(10.84)	31.8	(11.8)	38.0	(20.6)	0.463
cer_20:0 (nmol)	86.2	(43.2)	110.0	(32.3)	89.4	(24.0)	98.0	(40.5)	0.850
cer_22:0 (nmol)	935	(276)	1025	(416)	978	(318)	1081	(546)	0.969
cer_24:0 (nmol)	1961	(504)	2146	(881)	2081	(726)	2217	(1111)	0.867
cer_24_1 (nmol)	1406	(350)	1572	(497)	1540	(590)	1514	(499)	0.876
C16:0/C22:0 + C24:0 (nmol)	0.00245	(0.00107)	0.00210	(0.00114)	0.00257	(0.00114)	0.00244	(0.00089)	**0.032**
Dihydroceramides									
dh_c_totali (nmol)	419	(300)	414	(183)	401	(270)	516	(283)	0.439
dh_c_16:0 (nmol)	3.72	(1.87)	3.78	(1.82)	3.33	(1.44)	4.50	(2.27)	0.155
dh_c_18:0 (nmol)	10.9	(3.46)	11.2	(6.62)	10.4	(6.45)	17.3	(12.80)	0.228
dh_c_24:0 (nmol)	225	(139)	213	(112)	200	(141)	285	(163)	0.577
dh_c_24:1 (nmol)	178	(139.2)	177	(54.5)	187	(86.4)	232	(126.6)	0.332
Glucosylceramides									
gc_totali (nmol)	343	(77.3)	438	(143.1)	344	(75.3)	456	(186.7)	0.460
gc_16:0 (nmol)	3.67	(1.35)	5.70	(2.28)	4.05	(0.963)	5.09	(4.232)	0.254
gc_18:0 (nmol)	1.42	(0.37)	1.98	(0.85)	1.32	(0.304)	1.93	(1.450)	0.268
gc_18:1 (nmol)	16.2	(3.99)	20.8	(3.62)	17.0	(5.48)	19.7	(8.62)	0.806
gc_24:1 (nmol)	319	(68.4)	409	(134.5)	318	(67.1)	431	(169.9)	0.433
Sphingomyelins									
sm_tot (nmol)	34,172	(3194)	34,849	(3640)	33,878	(3487)	35,177	(2798)	0.818
sm_16:0 (nmol)	9172	(717)	9447	(330)	9423	(542)	9589	(479)	0.552
sm_18:0 (nmol)	1847	(324)	2104	(495)	1885	(384)	2176	(401)	**0.042**
sm_24:0 (nmol)	12,718	(2900)	11,558	(2496)	11,627	(2401)	12,227	(2887)	0.674
sm_24:1 (nmol)	10,084	(1176)	10,863	(1126)	10,552	(1309)	10,850	(1538)	0.472
Sphinganine (nmol)	8.29	(2.87)	13.53	(20.07)	7.6	(5.08)	11.5	(12.56)	0.790
Sphingosine (nmol)	16.5	(15.5)	39.8	(58.8)	19.1	(16.1)	27.4	(38.3)	0.278
Sphingosine-1-Phosphate (nmol)	346	(169)	562	(232)	407	(148)	501	(173)	0.747
Sphinganine-1-Phosphate (nmol)	40.5	(19.9)	61.2	(24.9)	44.4	(30.7)	53.1	(17.7)	0.905

Note: Data shown as median and Inter Quartile Range (IQR). The *p* value shown in the right column is from a general linear mixed model analysis, with random intercepts, in which the dependent variable was the value at day 14 from the start of the consumption of the product with EVOO or palm oil. The variables of treatment, sequence and baseline values (when available) were included in the model as fixed effects. These variables have been analysed in the logarithmic scale. *P* values <0.05 are highlighted in bold.

Abbreviations: Cer, Ceramides; C16:0/C22:0 + C24:0, Ceramide C:16/Ceramide C22:0 + Ceramide C24:0; dh, Dihydroceramides; EVOO, Extra Virgin Olive Oil; gc, Glucosylceramides; sm, Sphingomyelins.

the most abundant sphingolipid in mammals and is involved in one of the ceramide-generating pathways.[45] SM correlates with cardiovascular disease.[46,47] In particular, high levels of SM species containing saturated acyl chains, such as C18:0, but not high total SM levels, are associated with obesity and insulin resistance.[48]

Although there still is no clear association between SFA intake and cardiovascular disease when it comes to clinical endpoints,[49] data suggests that replacing SFAs with MUFAs or carbohydrates, but not with PUFAs, prevents coronary heart disease.[50] In the current study, we could not show a significant difference in LDL cholesterol

TABLE 3 Glucose, lipid and inflammatory markers before and after 14 days of EVOO and Palm oil based chocolate spread

	Olive oil				Palm oil				
Parameter	**Visit 1**		**Visit 3**		**Visit 1**		**Visit 3**		***p***
Glucose metabolism									
Glucose (mg/dL)	83	(10.5)	82	(5.5)	81	(6)	82	(6.75)	0.079
Insulin (μU/mL)	7.92	(3.02)	6.84	(2.73)	6.21	(1.75)	7.50	(2.38)	**0.045**
HOMA-IR	1.65	(0.678)	1.41	(0.586)	1.29	(0.369)	1.60	(0.568)	**0.046**
McAuley index	9.45	(1.60)	9.95	(1.72)	10.00	(1.73)	9.61	(1.29)	**0.039**
Quicki index	0.349	(0.034)	0.364	(0.040)	0.367	(0.024)	0.362	(0.020)	**0.018**
AUC glucose (mg/dL*min)	NA		15,088	(1291)	NA		14,628	(1437)	0.284
AUC insulin (pmol/L*min)	NA		2568	(850)	NA		2748	(850)	0.380
GUTT ISI index	NA		7.76	(3.33)	NA		7.76	(3.08)	0.681
Matsuda index	NA		13.6	(8.81)	NA		13.9	(7.28)	0.994
Regular lipids									
HDL (mg/dL)	49	(11.5)	51	(15.0)	47	(12.5)	47	(19.5)	0.756
LDL (mg/dL)	80.7	(20.9)	74.4	(21.2)	81.8	(23.2)	76.1	(20.1)	0.874
Total cholesterol (mg/dL)	143	(26.0)	141	(29.6)	142	(27.8)	143	(28.2)	0.468
Tryglicerides (mg/dL)	47	(14)	49	(13)	46	(14)	52	(22)	0.785
Inflammation									
IL-6 (pg/mL)	1.12	(0.384)	1.26	(0.532)	1.17	(0.481)	1.26	(0.527)	0.636
PCR (mg/dL)	0.0713	(0.0422)	0.0944	(0.0906)	0.1012	(0.0703)	0.0827	(0.0524)	0.199
TNF alpha (pg/mL)	11.2	(2.68)	10.4	(2.29)	10.9	(3.13)	10.5	(2.92)	0.677
Oxidative stress									
D roms (U. Carr)	388	(67.1)	394	(66.8)	371	(59.1)	416	(83.2)	0.060
PAT (U. cor.)	3505	(307)	3731	(435)	3462	(378)	3790	(325)	0.520

Note: Data shown as mean and standard deviation (SD) of the means or median and inter quartile range (IQR) according to their distribution. The *p* value shown in the right column is from a general linear mixed model analysis, with random intercepts,in which the dependent variable was the value at day 14 from the start of the consumption of the product with extra virgin olive oil or palm oil. The variables of treatment, sequence and baseline values (when available) were included in the model as fixed effects. GUTT ISI index, Matsuda index, HDL, triglycerides have been analysed in logarithmic scale. *P* values <0.05 are highlighted in bold.

Abbreviations: CRP, C-reactive protein; EVOO, extra virgin olive oil; HOMA-IR, homeostatic model assessment for insulin resistance, HDL, high density lipoprotein; IL-6, Interleukin-6; LDL, low density lipoprotein; NA, not applicable. TNF alpha, tumor necrosis factor alpha; D roms, reactive oxygen metabolites; PAT, plasma antioxidant test.

between the two groups likely due to the very short treatment duration.

As previous studies have reported inflammatory derangements upon palm oil consumption also in young adults,[9] we explored whether the short-term palm oil/EVOO-enriched chocolate spread consumption would have any differential effect on inflammation markers, but no significant difference was recorded.

As other authors reported a role of different types of fat sources or dietary patterns on appetite and satiety,[51-53] we investigated whether chocolate spread enriched in EVOO would lead to increased satiety compared to the same chocolate spread enriched in palm oil. However, no difference was observed in the appetite-regulating hormones and in the visual analogue scale assessment.

Of note, in our study, all of the observed modifications regarding insulin resistance were within normal ranges, but it should be noted that the length of the study was short, the subjects were all healthy and young, and an isocaloric diet was delivered ensuring weight stability. Therefore, it is not possible to exclude that a more extended administration, or enrolling unhealthy or older subjects, would have led to pathological changes, as previously shown in other studies.[8,44]

Our study has several limitations. The study duration was short, possibly limiting the amplitude of the effects. However, the chocolate spread portion herein used may not represent the daily usual intake, possibly exceeding it. Overall, the short timing and the amount of daily chocolate spread consumption may have counterbalanced the effects. Moreover, no significant difference was observed in fasting PA and OA circulating levels across groups. This result is likely because the chocolate spread base, with hazelnuts, was rich in OA, and the addition of palm or EVOO still left the ratio of the two acids in favour of OA in both spreads. Although this limits the

TABLE 4 Appetite regulating hormones and visual analogue scale (AUCs) on the fourteen day of the intervention phases during a challenge meal with the EEVO and palm oil based chocolate spread

	EVOO			Palm oil			
Parameter	**Visit 1**	**Visit 3**		**Visit 1**	**Visit 3**		***p***
Appetite regulating hormones (AUCs)							
Adiponectin (pg/mL*min)	NA	387	(535)	NA	338	(586)	0.879
Ghrelin (pg/mL*min)	NA	46,702	(23791)	NA	46,999	(22265)	0.926
GLP-1 (pg/mL*min)	NA	256,305	(241229)	NA	214,919	(259298)	0.424
Leptin (pg/mL*min)	NA	650	(1843)	NA	873	(1751)	0.953
Oxyntomodulin (pg/mL*min)	NA	98,932	(31190)	NA	124,001	(82727)	0.069
PYY (pg/mL*min)	NA	16,757	(6424)	NA	15,860	(10716)	0.428
Visual analogue scale (AUCs)							
Full (mm*min)	NA	6655	(3686)	NA	6340	(3924)	0.298
Hungry (mm*min)	NA	9494	(5760)	NA	7094	(5871)	0.956
Nausea (mm*min)	NA	405	(2853)	NA	1589	(3449)	0.976
Pleasure to eat (mm*min)	NA	9253	(4012)	NA	9226	(3813)	0.970
Quantity (mm*min)	NA	6518	(1812)	NA	6164	(1697)	0.427

Note: Data shown as mean and standard deviation (SD) of the means or median and inter quartile range (IQR) according to their distribution. The *p* value shown in the right column is from a general linear mixed model analysis, with random intercepts, in which the dependent variable was the value at day 14 from the start of the consumption of the product with extra virgin olive oil or palm oil. The variables of treatment, sequence were included in the model as fixed effects. Adiponectin, GLP-1, leptin, oxyntomodulin, PYY have been analysed in logarithmic scale.

Abbreviations: EVOO, extra virgin olive oil; GLP-1, glucagon like Peptide 1, PYY, peptide YY, NA, not applicable.

interpretation of data, where the effects of PA and OA are not isolated, in our opinion, it better replicates a real-life fatty acid proportion in the diet. It was previously reported that OA could limit the detrimental effects of PA.[20,23] Our report possibly goes in the same direction, where palm oil addition within food in a setting where OA might be limiting the damage still proves signs of the worse metabolic profile in our cohort of healthy subjects.

Our study also features some strengths. The study dieticians closely monitored the diet to maintain subjects' body weight and composition stable throughout the study. Adherence to the diet was maximised thanks to meals being prepared by trained cooks and directly delivered to the subjects, who consumed the meals on-site or collected them daily in individual meal boxes. Moreover, study compliance was closely monitored through food records, chocolate spread and food leftovers checking and physical activity questionnaire. In addition, participants did not report a taste difference between the two chocolate spreads, which allowed for blinding, which is seldomly possible in dietary interventions that include foods rather than capsules or supplements. Maintaining a setting similar to real life in terms of PA-OA proportions allowed to provide a more physiologic picture, paving the way for other mechanistic studies. The crossover design allowed for the patients to be control of themselves, limiting possible selection bias. All patients were healthy, normal weight and young, with a very narrow age range, bringing the heterogeneity of the cohort to a minimum.

Our study gives novel knowledge by showing a better insulin sensitivity profile when the calories are proportionally higher in MUFA than SFA using a chocolate-based snack as part of an isocaloric diet in healthy individuals. This indicates the importance of choosing the source of fats in the snacks. However, it is important to underline that we used an isocaloric diet to keep the weight stable across the study, and that the consumption of these high-calorie density snacks (~570 kcal) in an ad libitum free diet could have led to weight gain and more evident metabolic disorders, as suggested by Iggman et al.[44]

In conclusion, we report that the short-term consumption of an EVOO-enriched chocolate spread has a better impact on sphingolipids composition and markers of glucose metabolism compared to the palm oil comparator. The co-administration of high quantities of OA might have limited the metabolic derangements in the palm oil group. In line with the recommendations for CVD prevention,[54,55] and considering the effect of snacking on health,[24,25] our study suggests that partially replacing the SFAs with MUFAs in the snacks may limit their detrimental effects on insulin sensitivity and decrease circulating harmful sphingolipids in young adults.

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CONFLICT OF INTERESTS

Authors have no competing interests to declare.

ETHICS STATEMENT

The study was approved by the local IRB, conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice. Written informed consent was obtained from all study participants before enrolment.

AUTHOR CONTRIBUTIONS

Dario Tuccinardi, Silvia Manfrini, Paolo Pozzilli, Antonio Di Mauro and Yeganeh Manon Khazrai contributed to the conception and design of the work. Dario Tuccinardi, Silvia Manfrini, Antonio Di Mauro, Greta Lattanzi, Yeganeh Manon Khazrai, Shadi Kyanvash and Andreea Soare conducted the study. Antonio Di Mauro, Greta Lattanzi, Lavinia Monte, Giovanni Rossini, Ivan Beato, Chiara Spiezia, Maria Bravo, Shadi Kyanvash and Andreea Soare acquired the data. Claudio Pedone conducted the statistical analysis. Antonio Di Mauro, Andrea Armirotti, Sine Mandrup Bertozzi, Dario Tuccinardi, Mikiko Watanabe conducted the biochemical assays. Dario Tuccinardi, Silvia Manfrini and Paolo Pozzilli wrote the article and all authors Yeganeh Manon Khazrai, Antonio Di Mauro, Greta Lattanzi, Lavinia Monte, Giovanni Rossini, Ivan Beato, Chiara Spiezia, Maria Bravo, Shadi Kyanvash, Andreea Soare, Andrea Armirotti, Sine Mandrup Bertozzi and Amalia Gastaldelli provided substantial scientific input in interpreting the results, drafting and or reviewing the manuscript. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. We thank the subjects who participated in this trial and Miss Milena Rosati r. n. for her precious help.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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PEER REVIEW

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SUPPORTING INFORMATION

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