2-OHOA supplementation reduced adiposity and improved cardiometabolic risk to a greater extent than n-3 PUFA in obese mice

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Abstract

Objective: We aimed to assess whether 2-hydroxyoleic acid (2-OHOA) and n-3 polyunsaturated fatty acids (PUFA) could counteract changes on adipokine secretion and cardiometabolic risk biomarkers associated with high-fat diet-induced obesity in mice.

Methods: Female ICR/CD1 mice (8 weeks old) were divided into four groups receiving different diets (n=8/group): 1) standard chow (control) for 18 weeks; 2) 22% fat for 4 weeks + 60% fat for 14 weeks (obesogenic diet, OD); 3) OD + 2-OHOA (1500 $mg \cdot kg^{-1}$ diet) for the last 6 weeks (OD-HO); and 4) OD + n-3 PUFA (eicosapentaenoic + docosahexaenoic acids, 1500+1500 $mg \cdot kg^{-1}$ diet) for the last 6 weeks (OD-N3). After 18 weeks, body weight, periovarian visceral fat, heart and liver weights were measured, as well as cardiometabolic parameters (systolic and diastolic blood pressure, blood glucose, insulin, HOMA index, triglycerides, total cholesterol, apolipoproteins A1 and E), plasma adipokines and inflammatory proteins (leptin, adiponectin, plasminogen activator inhibitor 1 [PAI1], soluble E-selectin [sE-selectin], matrix metalloproteinase-9 [MMP-9], fibrinogen, soluble intercellular adhesion molecule [sICAM] and soluble vascular adhesion molecule [sVCAM]), and secretion of pro-inflamatory cytokines and inflammatory biomarkers from periovarian adipocytes.

Results: OD mice had greater body and heart weights, and plasma leptin, and lower adiponectin and resistin secretion from adipocytes. Supplementation with 2-OHOA reduced body and heart weights, blood pressure, triglycerides and leptin, and restored adiponectin and resistin secretion, while n-3 PUFA only reduced triglyceride levels (all $P<0.05$).

Conclusion: 2-OHOA supplementation was more effective in reducing adiposity, modulating adipokine secretion and ameliorating cardiometabolic risk than n-3 PUFA.

Keywords: 2-hydroxioleic acid; n-3 polyunsaturated fatty acids; high-fat diet; obese mice; cardiometabolic risk.

Abbreviations

2-OHOA; 2-hydroxioleic acid

Apo; apolipoprotein

BSA; bovine serum albumin.

cAMP; cyclic adenosine monophosphate

CVR; cardiovascular risk

DBP; diastolic blood pressure

DHA; docosahexanoic acid

DMEM; Dulbecco's modified Eagle's medium

EPA; eicosapentanoic acid

HDL-c; high density cholesterol

IL; interleuquin

LDL-c; low density cholesterol

LPS; lipopolysaccharide

MMP-9; matrix metalloproteinase-9

MUFA; monounsaturated fatty acids

OD; obesogenic diet

PAI-1; plasminogen activator inhibitor-1

PUFA; n-3 fatty acids

SBP; systolic blood pressure

SD; standard deviation

sE-selectin; soluble E-selectin

sICAM; soluble intercellular adhesion molecule

sVCAM; soluble vascular adhesion molecule

TNF-α; tumor necrosis factor alpha

1. Introduction

Eating habits worldwide are leaning towards an ever more Westernized dietary pattern, characterized by high refined fat and sugar intakes [1], a change coincident with a rise of overweight and obesity prevalence [2]. Dietary fats have been argued to constitute a principal factor in the development of obesity and its comorbidities [3]. Excess adiposity present in obesity is accompanied by low-grade inflammation, characterized by high levels of pro-inflamatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), as well as dysregulation of adipokine secretion from adipose tissue. In particular, monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inibitor (PAI)-1, leptin and adiponectin produced by adypocytes seem to play key roles in insulin resistance development [4], which in turn might lead to dyslipemia and hypertension, well-known cardiovascular risk factors which constitute typical features of the metabolic syndrome [3].

Modification of dietary fat quality of, rather than quantity, represents a key strategy for the prevention of obesity and its associated complications [5]. Long chain n-3 polyunsaturated fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), and the monounsaturated fatty acid (MUFA) oleic acid have received special attention. Both rodent and human studies have reported that n-3 PUFA intake can be beneficial for improving cardiometabolic risk factors, such as the lipid profile [6, 7], although results are still inconclusive in part due to differences in the dose and duration of treatments [7]. Regarding MUFA, there is wider consensus on their beneficial effects on adiposity, blood lipids profile, glucose levels and blood pressure in the context of a Mediterranean diet [8], or following dietary supplementations with olive oil and/or oleic acid [9]. In addition, a new synthetic derivative of oleic acid, the 2-hydroxyoleic acid (2-OHOA), can reduce body weight and blood pressure in lean rats. The 2-OHOA, also known as 2-hydroxy-D9-cis-octadecenoic acid, presents a hydroxyl group in the α-position, which may potentiate its effects on metabolism due to the fatty

acid undergoing a different catabolic route than naturally occurring fatty acids [10]. These findings suggest a role for 2-OHOA in obesity and associated comorbidities in animal models.

Our study aimed to investigate the effects of a progressive high-fat diet on markers of cardiometabolic risk (*i.e.*, blood pressure, lipids, glucose, insulin and inflammatory proteins), circulating adipokines, and adipokine secretion from visceral adipocytes in ICR-CD1® mice, and to assess whether supplements of 2-OHOA and n-3 PUFA could revert any changes observed. We hypothesized that dietary supplementation with unsaturated fatty acids would ameliorate the detrimental effects of feeding an obesogenic diet in mice.

2. Materials and methods

2.1. Animal housing and handling

Eight week-old female ICR-CD1® mice were purchased from Interfauna Harlan Iberica (Italy). The animals were maintained in polyurethane cages (4 animals per cage, 32 animals in total) and housed under constant conditions (12:12 h light/darkness, lights on at 8:00 pm, relative humidity at 50-60%, temperature of 22 ± 1 ºC and adequate ventilation) with free access to food and water. Animals were kept in these conditions in the animal facilities of the Department of Genetics, Physiology and Microbiology (University Complutense of Madrid). Sample size was calculated taking final body weight as the outcome variable, according to Vogler *et al.* 2008 [10], with an alpha error of 0.05 and 90% power, and estimating a 20% of experimental losses, resulting in 7 animals per group.

2.2. Dietary supplementations

All mice received standard chow for 5 days (acclimation period). Then they were split into four groups (n=8 animals/group): the control group received standard chow for 18 weeks; the other three groups were fed a transition diet with moderate fat content (22% energy from fat) for 4 weeks, and then an obesogenic diet (60% energy from fat) for 8 weeks (all mice were 21 weeks old). The OD group stayed on this diet for 6 more weeks; the OD-HO group received the obesogenic diet supplemented with 2-OHOA in the last 6 weeks; and the OD-N3 group received the obesogenic diet supplemented with a combination of EPA and DHA in the last 6 weeks. The experiment lasted 18 weeks (until mice were 27 weeks old) (figure 1).

The obesogenic diet contained lard (310.0 $g \cdot kg^{-1}$), casein (265.0 $g \cdot kg^{-1}$), maltodextrin (160.0 $g \cdot kg^{-1}$), sucrose (90.0 $g \cdot kg^{-1}$), cellulose (65.5 $g \cdot kg^{-1}$), mineral mix AIN-93G-MX (94046, 48.0 $g \cdot kg^{-1}$), soybean oil (30.0 $g \cdot kg^{-1}$), vitamin mix AIN-93-VX (94047, 21.0 $g \cdot kg^{-1}$), L-cystine (4.0 $g \cdot kg^{-1}$), calcium phosphate dibasic (3.4 $g \cdot kg^{-1}$), and choline bitartrate (3.0 $g \cdot kg^{-1}$). The list of ingredients for the control and transition diets cannot be disclosed due to manufacturer's restrictions, but detailed nutritional composition of all diets used in the study is present in Supplementary table 1. Diets were provided by Harlan Interfauna Iberica. BTSA-Biotecnologías Aplicadas, S.L. (Spain) manufactured and supplied the fatty acids; the 2-OHOA is a synthetic derivative of the oleic acid, and the n-3 PUFA were extracted from fish (anchovy). For supplementation, the obesogenic diet was mixed with 2-OHOA (1500 $mg \cdot kg^{-1}$ diet), or n-3 PUFA mixture (1500+1500 $mg \cdot kg^{-1}$ diet), in the facilities of the Department of Genetics, Physiology and Microbiology of the University Complutense, pelleted to match the texture of the control diet, and administered directly in the appropriate cage compartments.

All experimental procedures were approved by the Committee for Animal Experimentation of the University Complutense of Madrid (ref. CEA-UCM 06/2012), and conducted in accordance with the guidelines and protocols of the European Community Council

Directives (86/6091 EEC) and the Spanish Royal Decree 1201/2005 regarding the care and use of laboratory animals for experimental procedures. The ARRIVE guidelines were also considered.

2.3. Body and organ weight measurements

Body weight of the animals was recorded twice a week throughout the whole study, using a standard scale (BOECO Germany, max 610 g, d = 0.01 g); these measurements took place in the Department of Genetics, Physiology and Microbiology (University Complutense). At the end of the 18-week period, all mice were euthanized by decapitation early in the morning (between 8:00 and 9:00 a.m.) within a week. Blood was collected; periovarian fat depots were excised, weighed and kept in Dulbecco's modified Eagle's medium (DMEM) at 37 °C; livers and hearts were also collected and weighed.

2.4. Blood measurements

Systolic (SBP) and diastolic (DBP) blood pressure was measured on the tail; measures were taken 3 times to obtain the average thereof. This was conducted at the end of the study in a stress-free environment using a non-invasive pressure gauge (Panlab Non-Invasive Blood Pressure System for Rodents, Harvard, USA), in the Department of Genetics, Physiology and Microbiology (University Complutense).

Glucose, cholesterol and triglyceride levels were quantified in blood drops obtained from the tail vein using reactive strips via an automatic meter (Accutrend Roche, Mannheim, Germany). Concentrations of insulin, leptin, adiponectin, fibrinogen, PAI1, apolipoprotein (Apo) A1, ApoE, soluble E-selectin (sE-selectin), matrix metalloproteinase-9 (MMP-9), soluble intercellular adhesion molecule (sICAM) and soluble vascular adhesion molecule (sVCAM) were determined in plasma from heparinized blood samples obtained after euthanasia of the mice, and centrifuged at 1000 *g* during 15 min. Analyses were performed in the Institute of Food Science, Technology and Nutrition (ICTAN-CSIC,

Madrid) with multiplex assay kits (Mouse Cytokine MPXMCYTO-70K, Millipore Corporation Billerica, Massachusetts, USA) and the Luminex-100 IS reader (Integrated System: Luminex Corporation, Austin, TX, USA). Acquired fluorescence data were analyzed by the Luminex 2.3 version software. All analyses were performed according to the manufacturer's protocols. Insulin resistance was estimated as the HOMA index, calculated by the formula:

$$\text{HOMA index} = (\text{Insulin } [\mu U \cdot ml^{-1}] \times \text{Glucose } [mg \cdot dl^{-1}])/405.$$

2.5. Secretion of adipokines and inflammation-related proteins from adipocytes

The periovarian adipose depot was obtained immediately after the euthanasia of the mice. Adipocytes were isolated and incubated in 6-well plates, according to the protocol by Moreno-Aliaga and co-workers [11]. After 50 min incubation, 1 µg/ml of lipopolysaccharide (LPS) was added to half of the wells, and plates were incubated at 37 °C in 5% CO_2 for 48 hours. Culture media were then collected and kept at −80 °C for further assays. Leptin, adiponectin, resistin, PAI1, TNF-α, IL-6 and MCP1 were determined using multiplex assay kits as described above. The concentration of adipokines was normalized by the total protein concentration in each well measured by a colorimetric method using bovine serum albumin (BSA) as the reference protein. This work was carried out in the ICTAN-CSIC.

2.6. Statistical analysis

Statistical tests were performed using IBM SPSS Statistic 23, setting a significance level of $P<0.05$. Normality was checked by the Shapiro-Wilk test. For variables following a normal distribution, data are presented as mean and standard deviation (SD), and for those non-normally distributed, data are presented as median and quartiles 1 and 3 (Q1-Q3). For normally distributed variables, the homogeneity of variances was evaluated with the Levene test, and groups were compared by one-way ANOVA with Bonferroni and Tamhane *post-hoc* tests; for non-normally distributed variables, comparisons were conducted by the Kruskal-Wallis test, with pairwise *post-hoc* correction. Associations

between variables were evaluated with the Spearman rank correlation test. Partial correlations adjusted by dietary treatment were subsequently calculated.

3. Results

3.1. Effects of dietary supplementations on body weight, fat and organs weight

Body weight at the beginning of the study (9 weeks of age) was similar among groups (P=0.100). At the end of the study (27 weeks of age), the OD mice showed the greatest body and heart weights, particularly compared to control and OD-HO mice (P=0.042 and P=0.009 for body weight; P=0.026 and P=0.011 for heart weight, respectively; table 1). The OD-HO mice presented the lowest body weight of all the groups, and lower heart weight than the OD group; the OD-N3 group showed values similar to OD mice, and the heaviest periovarian fat depots of all groups (P=0.001), significantly heavier than the control group (P=0.039). No differences were observed in liver weight among groups (table 1).

3.2. Effects of dietary supplementations on cardiometabolic risk and inflammatory biomarkers

At the end of the study, the OD-HO mice showed significantly lower values of SBP and DBP than the OD group (P=0.011 and P=0.007 for SBP and DBP, respectively); the control and the OD-N3 groups presented intermediate values (figure 2). There were no significant differences in glucose and insulin levels among groups, although there was a trend for the HOMA index to be highest in the OD-N3 group, and lowest in the OD-OH group (P=0.057). The OD-HO and OD-N3 groups had lower triglyceride levels compared to OD mice (P=0.045 and P=0.035, respectively); total cholesterol, ApoA1 and ApoE were similar among groups (table 1). The OD and OD-N3 groups showed higher plasma leptin concentrations than the control group (P=0.002 and P=0.008, respectively), and the OD-

HO group showed lower levels compared to OD group (P=0.009). There were no differences among groups for plasma adiponectin or any of the inflammatory molecules measured (table 2).

3.3. Effects of dietary supplementations on adipocyte secretion of adipokines and inflammation-related proteins

Adiponectin secretion was reduced in adipocytes from obese mice, the difference being statistically significant for LPS-stimulated secretion (P=0.002; table 3). Both 2-OHOA and n-3 PUFA partially restored adiponectin secretion, with values between control and obese mice. A similar pattern was observed for resistin, with lower levels in the OD group compared to the control group (P=0.034 and P=0.005, respectively, for basal and LPS-stimulated secretion), and partial restoration by 2-OHOA supplementation. On the contrary, OD-N3 supplementation did not restore resistin secretion, showing lower values than the control, in particular for LPS-stimulated resistin. Secretions of leptin, PAI1, TNF-α, IL-6 or MCP1 did not differ among groups (table 3).

3.4. Associations between adipokines and cardiometabolic risk biomarkers

After controlling for dietary treatment, plasma leptin levels were positively correlated with heart weight, DBP, triglycerides, and ApoE (figure 3). The latter was also positively correlated with fibrinogen (r_s=0.880, P<0.001).

Periovarian fat depot weight was positively correlated with plasma leptin (r_s=0.842, P<0.001), and negatively with LPS-stimulated secretion of resistin (r_s=−0.519, P=0.006) and adiponectin (r_s=−0.442, P=0.024). In turn, LPS-stimulated adiponectin was negatively correlated with liver weight (r_s=−0.417, P=0.034).

4. Discussion

The amount of dietary fat has traditionally been associated with obesity and the metabolic syndrome, main factors for increased cardiovascular risk (CVR) [3]. Our results showed that progressive adaptation to an obesogenic diet increased body weight, heart weight, and plasma leptin levels, and reduced adiponectin and resistin secretion from adipose tissue, features all related to the pathogenesis of metabolic syndrome and higher CVR [12]. Supplementation with 2-OHOA and n-3 PUFA reverted some of these changes, in agreement with previous studies showing that the quality of dietary fat is a key factor that modulates obesity and CVR [13]. In our study, 2-OHOA and n-3 PUFA improved triglyceride levels, but only the 2-OHOA reduced body and heart weights, leptin levels and blood pressure values, and restored adipokine secretion from adipocytes to levels closer to the control group.

There is controversy regarding the potential anti-obesity effects of MUFA and n-3 PUFA. Some evidence in mice supports that consumption of a high-fat diet enriched in olive oil can decrease body weight and fat gain [14], whereas other studies showed increased body weight after olive oil intake [15]. Evidence is scarce regarding the effects of the synthetic 2-OHOA on obesity, but one study in lean rats showed that the intake of 2-OHOA (600 $mg \cdot kg^{-1}$ body weight every 12 hours, for 7 days) led to reduced body weight, adipose fat mass and leptin levels, in agreement with our findings [10]. The mechanisms for the effects of 2-OHOA could be related to an increase in energy expenditure through activation of the expression of uncoupling proteins in white adipose tissue [10], suggesting potential “beiging” of white adipocytes [16]. In addition, a different catabolic route has been hypothesized for 2-OHOA, through an alternative α-oxidation pathway (instead of β-oxidation, as with naturally occurring fatty acids), implying higher accumulation of this fatty acid in adipose tissue, and thus increased metabolic use as energy source [10]. Since our mice were already obese before beginning the 2-OHOA supplementation, our findings suggest that 2-OHOA could have a positive role in obesity management.

In contrast, mice supplemented with n-3 PUFA showed similar weight gain, visceral fat accumulation and plasma adipokine levels to the obese mice. Other studies in mice have reported positive effects of EPA and/or DHA feeding on body fat reduction [17, 18], although the effect on body weight could be limited in obese animals [18], as observed in our study. The duration of supplementation and the individual contributions of EPA and DHA to n-3 PUFA-based supplements seem to account for their effects on body weight and fat mass, and beneficial effects of these fatty acids on obesity and metabolic syndrome have been reported in studies using higher doses, different EPA:DHA ratios, or longer durations [15, 19].

Our obese mice presented higher circulating leptin levels; however, its secretion by adipocytes did not change significantly, so the elevated circulating levels likely resulted from greater fat mass, as could be expected. In contrast, we observed significant reductions in adiponectin and resistin secretion. Adipose tissue dysfunction and altered adipokine levels in obesity have been related to the pathogenesis of insulin resistance and cardiovascular disease [20]. Leptin is considered a good marker of CVR [21], and in our study, circulating leptin was positively correlated with blood pressure, heart weight, triglycerides and ApoE, independently of the diet administered. Decreased adiponectin levels represent another key feature of obesity [3], and have also been related to insulin resistance [22]. Insulin resistance may develop when the capacity of adipose tissue for storing energy is exceeded, as can occur under overfeeding, resulting in lipid accumulation in non-adipose tissues such as liver and muscle [3]. Our mice fed the obesogenic diet showed a trend toward greater liver weights compared to controls, and we observed a negative correlation between LPS-stimulated adiponectin secretion and liver weight, suggesting a link between these two events. However, we did not find significant changes in glucose or insulin levels in the obese mice, despite the reduced adiponectin secretion. Previous research has linked high resistin levels to insulin resistance in animal models [23], and we observed a reduction in resistin secretion, but

whether this reduction influenced our results remains to be elucidated. Actually, the down-regulation of resistin in our obese mice was unexpected and deserves further research. The literature presents conflicting results regarding resistin and obesity, with both increased and decreased levels having been reported [24]. Absence of changes on glucose and insulin levels despite altered lipid metabolism and adipokine secretion has been previously reported in another model of high-fat diet-induced obesity [25]. It is worth noting that experimental high-fat diets are usually more saturated than our obesogenic diet, which had a higher proportion of monounsaturated than saturated fat. In addition, some studies suggest that high-carbohydrate diets are more detrimental to glucose tolerance than high-fat diets [26]; our diet had a higher fat proportion at the expense of the carbohydrate content, compared to the control diet. Therefore, the different contribution of fat and carbohydrates in our experimental diet could contribute to the absence of changes in glucose and insulin levels.Classical markers of CVR, *i.e.*, triglyceride levels and blood pressure, were significantly higher in our obese mice, and associated with circulating leptin, as mentioned above. Hyperleptinemia has been related to triglyceride accumulation and increased lipogenesis [27], and to the development of an anti-natriuretic response and oxidative stress in the kidneys, which can eventually lead to hypertension [28]. Our results showed that blood pressure, heart weight and triglyceride levels were also reduced by 2-OHOA, in agreement with other studies linking MUFA with lower CVR [29]. Previous work had shown that 2-OHOA could lower SBP in hypertensive rats, an event mediated through increased cyclic adenosine monophosphate (cAMP) and G-proteins expression in cardiovascular tissues [30]; our findings suggest that 2-OHOA can be a useful hypotensive agent in obesity as well. In contrast, n-3 PUFA supplementation in our study did not reduce blood pressure to the same extent as 2-OHOA, only triglyceride levels, which is a well-known effect of PUFA intake [31]. Total cholesterol and apolipoproteins A and E were similar among groups. LDL-c and HDL-c fractions were not analyzed separately, and thus differential effects of 2-OHOA and n-3 PUFA on cholesterol subfractions need to be studied further. Similarly, we did not observe

any differences in the inflammatory markers of cardiovascular risk (*i.e.*, acute phase proteins and adhesion molecules in plasma, and cytokines in adipocytes) between groups.

We must address limitations in our study. Firstly, the experiment was conducted in female mice, due to methodological requirements associated with functional essays conducted on markers of immune function and oxidative stress [32], as aggressiveness displayed by males caged in groups might impact the interpretation of results. It is thus important to keep in mind that males and females may express distinctive responses regarding body fat accumulation, hormonal regulation and cardiometabolic risk. Another limitation is the lack of significant changes on circulating and adipocyte-secreted cytokines, chemokines and adhesion molecules. Obesity is defined as a chronic low-grade inflammatory state characterized by high levels of pro-inflammatory cytokines [3], and both leptin and adiponectin have a role in initiating the inflammatory response [20]. In our study, altered adipokine levels were not paralleled by higher concentrations of inflammatory proteins. Two possible explanations should be considered; on the one hand, circulating levels of cytokines and other inflammatory markers show great variability, limiting the power of the statistical analysis of the data; on the other hand, the sequence of events that link obesity, adipose tissue dysfunction and systemic inflammation is not fully understood yet, and the time required for the inflammatory condition to be established may depend on the nature of the stimulus, its duration, or the inter-individual genetic variability, among others factors. The use of a transition diet (22% fat) for the first 4 weeks could have delayed or ameliorated the onset of the inflammatory response. We have previously published that our obese mice presented immunological alterations and increased oxidative stress [32], as well as intestinal dysbiosis [33]. We could argue that changes in the balance of the bacterial groups, accompanied by changes in adipose tissue and immune system homeostasis, may precede the systemic, chronic low-grade inflammation associated with obesity. Indeed, high fat intake has been related to increased intestinal inflammation and

bacterial dysbiosis before the onset of obesity [34], suggesting that strategies focused on dietary fat modification should be considered when addressing obesity and their comorbidities. Supplementation with 2-OHOA in our study was associated with cardioprotective effects, including partial restoration of adiponectin and resistin secretion from adipocytes, which suggests a beneficial effect on adipose tissue functionality. These changes, however, could also be a consequence of body weight and fat loss induced by the supplementation; we need further research to elucidate the mechanisms by which 2-OHOA reduces body weight, and its specific effects on the adipocyte biology.

In conclusion, a progressive high-fat diet induced obesity and increased cardiovascular risk in our mice. The 2-OHOA showed both anti-obesity and cardioprotective actions, reducing blood pressure, triglyceride and leptin levels, and partially restoring adiponectin and resistin secretion from adipocytes, whereas n-3 PUFA were effective in improving triglycerides values only. Further research is needed to elucidate the mechanisms of 2-OHOA action on adipocyte function.

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Declarations of interest

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Experimental design: all animals were fed standard chow during the acclimation period (5 days). The control group received standard chow for the entire duration of the experiment (18 weeks); the OD group received the transition diet (TD, 22% fat) for 4 weeks, and then the obesogenic diet (OD, 60% fat) for 14 weeks; the OD-HO group were on the TD for 4 weeks, the OD for 8 weeks, and the OD diet supplemented with 2-OHOA in the last 6 weeks; the OD-N3 group followed the same pattern as the OD-HO, but with the diet supplemented with n-3 PUFA.

Figure 2. Systolic (A) and diastolic (B) blood pressure values in the four experimental groups. C: control (n=7 both); OD: obesogenic diet (n=7 both); OD-HO: obesogenic diet + 2-hydroxyoleic acid (n=7 for systolic and n=6 for diastolic); OD-N3: obesogenic diet + n-3 polyunsaturated fatty acids (n=7). Differences between groups were analyzed by one way ANOVA, with pairwise *post-hoc* correction. Different superscript letters indicate significant differences at $P<0.05$.

Figure 3. Associations between circulating leptin and: (A) heart weight (n=29), (B) diastolic blood pressure (n=28), (C) triglycerides (n=25) and (D) apolipoprotein E (n=20). Treatment-adjusted Spearman's rank correlations were used to assess the strength of the associations.

Figure 1

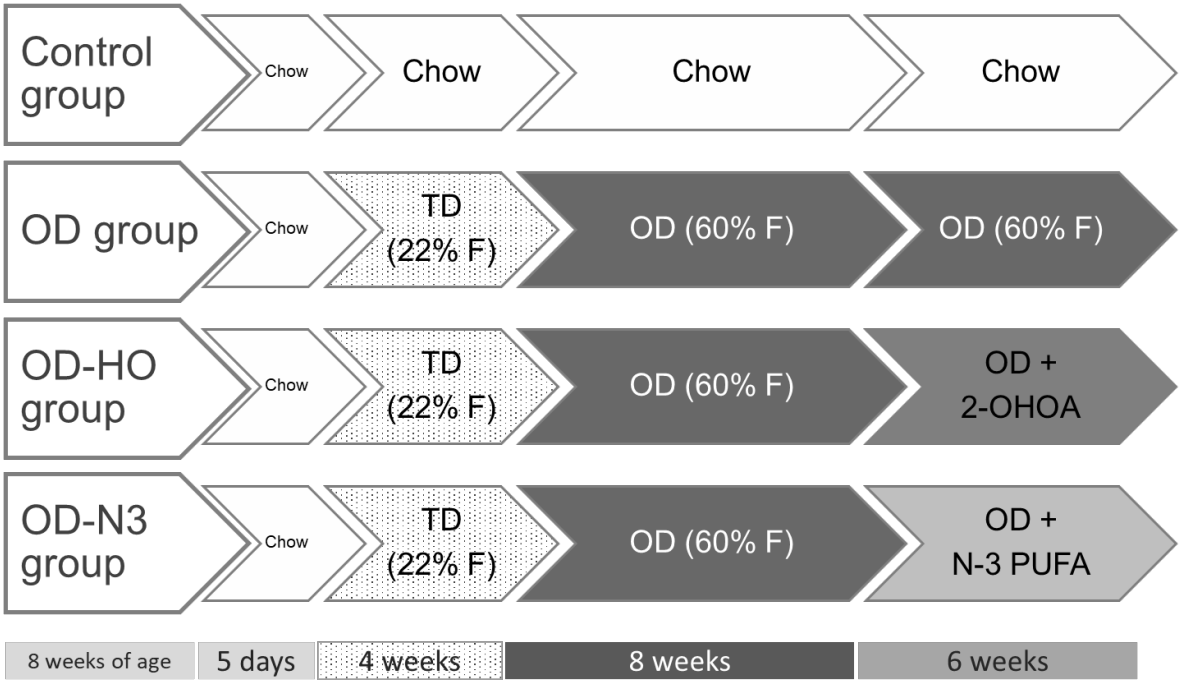

Control group
Chow
Chow
Chow
Chow
OD group
Chow
TD (22% F)
OD (60% F)
OD (60% F)
OD-HO group
Chow
TD (22% F)
OD (60% F)
OD + 2-OHOA
OD-N3 group
Chow
TD (22% F)
OD (60% F)
OD + N-3 PUFA
8 weeks of age
5 days
4 weeks
8 weeks
6 weeks

Figure 2

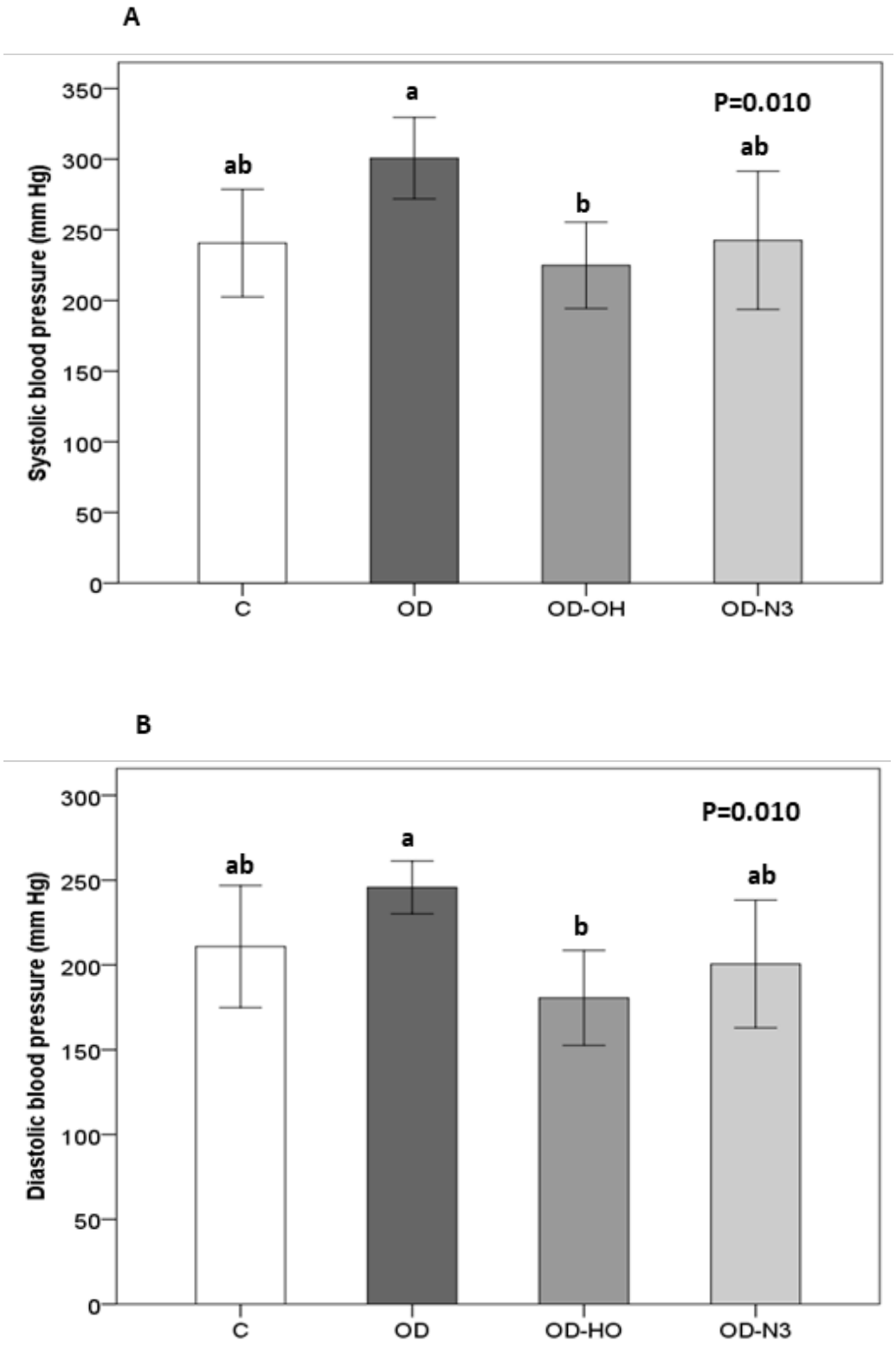
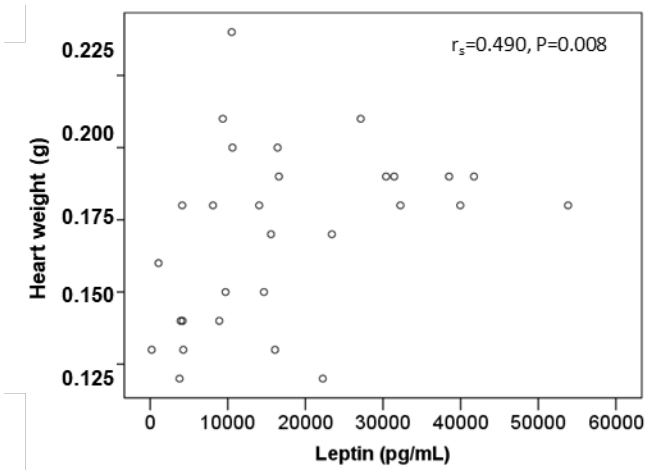
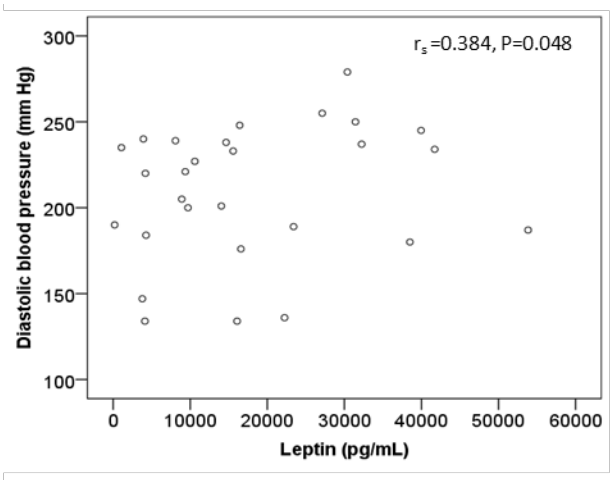
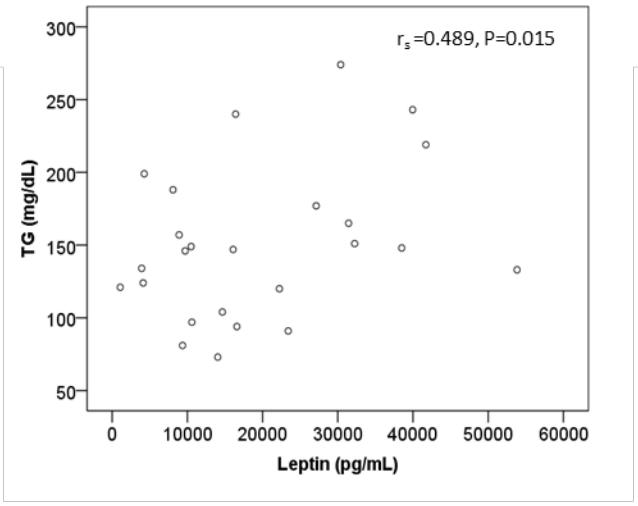

A
Systolic blood pressure (mm Hg)
350
300
250
200
150
100
50
0
ab
a
b
ab
P=0.010
C
OD
OD-OH
OD-N3
B
Diastolic blood pressure (mm Hg)
300
250
200
150
100
50
0
ab
a
b
ab
P=0.010
C
OD
OD-HO
OD-N3

Figure 3


r_s=0.490, P=0.008
Heart weight (g)
0.225
0.200
0.175
0.150
0.125
0
10000
20000
30000
40000
50000
60000
Leptin (pg/mL)


r_s=0.384, P=0.048
Diastolic blood pressure (mm Hg)
300
250
200
150
100
0
10000
20000
30000
40000
50000
60000
Leptin (pg/mL)


r_s=0.489, P=0.015
TG (mg/dL)
300
250
200
150
100
50
0
10000
20000
30000
40000
50000
60000
Leptin (pg/mL)

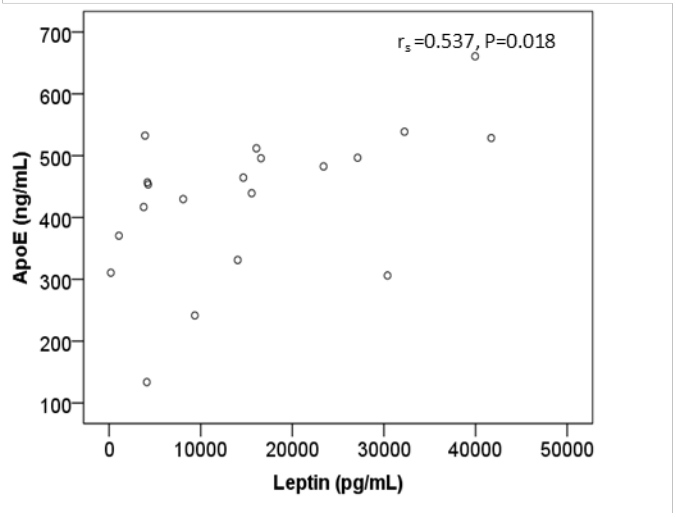

r_s=0.537, P=0.018
ApoE (ng/mL)
700
600
500
400
300
200
100
0
10000
20000
30000
40000
50000
Leptin (pg/mL)

Table 1. Cardiovascular risk biomarkers in all groups at the end of the supplementations.

	n	C	n	OD	n	OD-HO	n	OD-N3	P*
Initial body weight (g)	8	26.8 (1.4)	8	28.1 (1.6)	8	26.2 (2.0)	8	26.4 (1.4)	NS
Final body weight (g)	8	34.1 (3.4)^{b}	7	50.8 (11.3)^{a}	7	28.4 (3.5)^{c}	7	48.2 (11.7)^{abc}	<0.001
Periovarian fat (g)	8	0.62 (0.38)^{a}	7	3.46 (2.59)^{ab}	7	1.17 (0.69)^{ab}	7	4.20 (2.35)^{b}	0.001
Liver weight (g)	8	1.44 (1.29-1.67)	8	1.83 (1.70-1.90)	8	1.72 (1.28-2.23)	8	1.85 (1.62-2.38)	*0.057*
Heart weight (g)	7	0.17^{a} (0.14-0.18)	7	1.19^{b} (0.18-0.20)	7	0.14^{a} (0.12-0.18)	7	0.18^{ab} (0.15-0.21)	0.027
Glucose (mg/dl)	8	136.9 (27.1)	7	151.3 (27.6)	7	131.7 (31.0)	7	144.7 (24.0)	NS
Insulin (ng/ml)	8	1.04 (0.91)	7	0.82 (0.43)	7	0.67 (0.42)	7	1.52 (0.70)	NS
HOMA index	8	9.2 (7.3)	7	8.9 (5.1)	6	6.0 (4.7)	7	15.3 (6.2)	*0.057*
Triglycerides (mg/ml)	6	145.5 (25.1)^{ab}	7	198.4 (62.6)^{a}	6	125.5 (43.9)^{b}	7	125.9 (34.9)^{b}	0.019
Cholesterol (mg/dl)	8	152.8 (3.1)	7	155.9 (2.5)	7	153.4 (1.8)	7	154.9 (2.1)	NS
ApoA1 (µg/ml)	6	234.5 (50.7)	5	333.5 (109.6)	5	271.9 (30.1)	5	280.5 (89.5)	NS
ApoE (ng/ml)	6	369.4 (137.2)	4	508.6 (147.7)	5	430.9 (62.3)	5	439.4 (112.0)	NS

Data presented as mean (SD) or median (Q1-Q3), according to the distribution of the data. C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-hydroxyoleic acid; OD-N3: obesogenic diet + n-3 polyunsaturated fatty acids. *Differences between groups analyzed by one-way ANOVA with Tamhane or Bonferroni *post-hoc* tests, or by Kruskal-Wallis test. Different superscript letters indicate significant differences at P<0.05.

Table 2. Inflammation-related biomarkers in all groups at the end of the supplementations.

	n	C	n	OD	n	OD-HO	n	OD-N3	P*
Leptin (pg/ml)	8	6,500 (5,183)[a]	7	28,963 (11,525)[b]	7	10,684 (7,207)[ac]	7	26,137 (15,508)[bc]	0.001
Adiponectin (µg/ml)	4	6.97 (2.13)	4	5.94 (3.39)	4	8.11 (2.49)	5	5.96 (3.18)	NS
PAI1 (ng/ml)	8	4.43 (2.95)	7	6.02 (3.92)	7	5.46 (2.13)	6	4.64 (2.50)	NS
MMP9 (ng/ml)	8	240.6 (123.2)	7	384.2 (150.3)	6	364.5 (312.4)	7	206.4 (169.3)	NS
Fibrinogen (µg/ml)	6	21.1 (14.3)	4	69.8 (75.0)	5	74.3 (66.4)	5	93.1 (56.0)	NS
sE-Selectin (ng/ml)	7	20.4 (9.3)	6	39.5 (5.8)	7	39.9 (22.4)	7	31.1 (24.3)	NS
sICAM1 (ng/ml)	8	37.3 (9.9)	7	39.3 (11.7)	6	42.7 (15.0)	6	39.0 (12.9)	NS
sVCAM (ng/ml)	8	500.5 (215.4)	7	663.5 (341.6)	6	879.3 (525.4)	6	708.7 (488.2)	NS

Data presented as mean (SD). C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-hydroxyoleic acid; OD-N3: obesogenic diet + n-3 polyunsaturated fatty acids.

*Differences between groups analyzed by one-way ANOVA with Tamhane or Bonferroni *post-hoc* tests. Different superscript letters indicate significant differences at $P<0.05$.

Table 3. Basal and LPS-stimulated secretion of adipokines and inflammation-related proteins from culture periovarian adipocytes.

	Basal secretion (pg/µg total protein)					LPS-stimulated secretion (pg/µg total protein)				
	C	OD	OD-HO	OD-N3	P*	C	OD	OD-HO	OD-N3	P*
Leptin	1.27 (1.21)	0.62 (0.34)	0.91 (0.82)	0.98 (0.67)	NS	1.06 (0.97)	0.45 (0.24)	0.64 (0.85)	0.78 (0.80)	NS
Adiponectin	22.4 (12.4)	9.7 (6.5)	16.6 (11.3)	17.7 (18.4)	NS	18.6 (8.8) [a]	5.3 (1.8) [b]	11.7 (5.7) [ab]	8.3 (4.8) [ab]	0.002
Resistin	2.80 [a]	0.89 [b]	3.33 [ab]	1.37 [ab]	0.034	3.25 [a]	0.59 [c]	1.36 [ab]	0.99 [bc]	0.005
	(2.49-4.88)	(0.57-1.95)	(0.80-4.37)	(1.04-3.00)		(1.96-5.80)	(0.42-1.26)	(1.16-3.51)	(0.58-1.83)	
PAI1	16.3 (13.1)	16.6 (12.1)	18.9 (13.9)	5.5 (6.2)	NS	11.7	4.9	25.12	4.7	NS
						(3.2-16.7)	(4.5-6.9)	(7.1-37.5)	(1.9-8.5)	
MCP1	4.68 (4.07)	2.79 (2.92)	7.92 (5.00)	1.64 (2.46)	NS	9.43 (10.13)	2.34 (1.68)	7.29 (5.25)	1.54 (1.68)	NS
TNF-α	0.012	0.033	0.021	0.009	NS	0.012	0.008	0.025	0.008	NS
	(0.006-0.015)	(0.012-0.057)	(0.010-0.047)	(0.005-0.020)		(0.005-0.020)	(0.003-0.034)	(0.012-0.060)	(0.004-0.017)	
IL-6	9.5 (10.4)	12.0 (10.5)	16.5 (13.5)	5.7 (5.3)	NS	14.6 (12.1)	11.3 (12.5)	17.6 (9.4)	7.5 (6.2)	NS

Data presented as mean (SD) or median (Q1–Q3), according to the distribution of the data. C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-hydroxyoleic acid; OD-N3: obesogenic diet + n-3 polyunsaturated fatty acids. *Differences analyzed by one-way ANOVA with Tamhane or Bonferroni *post-hoc* tests, or by Kruskal-Wallis with pairwise *post-hoc* correction, according to the distribution of the data; different superscript letters indicate significant differences at $P<0.05$. N = 7 for all analyses, except for PAI1 (n=6, n=7, n=4 and n=6, respectively), MCP1 in OD-HO (n=6), basal TNF-α (n=6, n=4, n=4, and n=7), LPS-TNF-α (n=6 for C, OD and OD-HO), and basal IL-6 (n=6 for OD, OD-HO and OD-N3).

Supplementary Table 1. Dietary composition of the experimental diets.

Components	Control diet	Transition diet	Obesogenic diet
Energy (kcal/g)	2.9	3.3	5.1
% Total energy			
Carbohydrates	67.0	55.0	21.4
Protein	19.7	23.0	18.4
Fat	13.1	22.0	60.5
Content (g) per 100 g diet			
Carbohydrates	48.0	44.9	27.3
Fiber*	18.0	12.1	6.55
Protein	14.3	19.0	23.5
Fat	4.21	9.0	34.3
Saturated	0.60	1.20	12.48
Monounsaturated	0.70	1.70	16.05
Polyunsaturated	2.10	4.40	5.40
Fatty acid composition			
C14:0 Myristic	0.00	0.00	0.47
C16:0 Palmitic	0.50	0.90	8.20
C18:0 Stearic	0.10	0.20	3.90
C18:1 n-9 Oleic	0.70	1.70	14.68
C18:2 n-6 Linoleic	2.00	3.90	4.70
C18:3 n-3 α-Linolenic	0.10	0.40	0.55

Values calculated according to manufacturer data. *Detergent neutral fiber for control and transition diet; cellulose for the obesogenic diet. Control diet: Teklad Global 14% Protein Rodent Maintenance Diet 2014; Transition diet: Teklad Global 19% Protein Extruded Rodent Diet 2019; Obesogenic diet: Harlan TD.06414.