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### Article

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

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1       **Abstract**

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

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

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

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

25  
26       **Keywords:** 2-hydroxyoleic acid; n-3 polyunsaturated fatty acids; high-fat diet; obese mice;  
27 cardiometabolic risk.

28	<b>Abbreviations</b>
29	2-OHOA; 2-hydroxioleic acid
30	Apo; apolipoprotein
31	BSA; bovine serum albumin.
32	cAMP; cyclic adenosine monophosphate
33	CVR; cardiovascular risk
34	DBP; diastolic blood pressure
35	DHA; docosahexanoic acid
36	DMEM; Dulbecco's modified Eagle's medium
37	EPA; eicosapentanoic acid
38	HDL-c; high density cholesterol
39	IL; interleuquin
40	LDL-c; low density cholesterol
41	LPS; lipopolysaccharide
42	MMP-9; matrix metalloproteinase-9
43	MUFA; monounsaturated fatty acids
44	OD; obesogenic diet
45	PAI-1; plasminogen activator inhibitor-1
46	PUFA; n-3 fatty acids
47	SBP; systolic blood pressure
48	SD; standard deviation
49	sE-selectin; soluble E-selectin
50	sICAM; soluble intercellular adhesion molecule
51	sVCAM; soluble vascular adhesion molecule
52	TNF- $\alpha$ ; tumor necrosis factor alpha
53	
54	

55 **1. Introduction**

56 Eating habits worldwide are leaning towards an ever more Westernized dietary pattern,  
57 characterized by high refined fat and sugar intakes [1], a change coincident with a rise of  
58 overweight and obesity prevalence [2]. Dietary fats have been argued to constitute a  
59 principal factor in the development of obesity and its comorbidities [3]. Excess adiposity  
60 present in obesity is accompanied by low-grade inflammation, characterized by high levels  
61 of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-  
62 6 (IL-6), as well as dysregulation of adipokine secretion from adipose tissue. In particular,  
63 monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor (PAI)-1,  
64 leptin and adiponectin produced by adipocytes seem to play key roles in insulin  
65 resistance development [4], which in turn might lead to dyslipemia and hypertension, well-  
66 known cardiovascular risk factors which constitute typical features of the metabolic  
67 syndrome [3].

68  
69 Modification of dietary fat quality of, rather than quantity, represents a key strategy for the  
70 prevention of obesity and its associated complications [5]. Long chain n-3 polyunsaturated  
71 fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA, C20:5 n-3) and  
72 docosahexaenoic acid (DHA, C22:6 n-3), and the monounsaturated fatty acid (MUFA)  
73 oleic acid have received special attention. Both rodent and human studies have reported  
74 that n-3 PUFA intake can be beneficial for improving cardiometabolic risk factors, such as  
75 the lipid profile [6, 7], although results are still inconclusive in part due to differences in  
76 the dose and duration of treatments [7]. Regarding MUFA, there is wider consensus on  
77 their beneficial effects on adiposity, blood lipids profile, glucose levels and blood pressure  
78 in the context of a Mediterranean diet [8], or following dietary supplementations with olive  
79 oil and/or oleic acid [9]. In addition, a new synthetic derivative of oleic acid, the 2-  
80 hydroxyoleic acid (2-OHOA), can reduce body weight and blood pressure in lean rats.  
81 The 2-OHOA, also known as 2-hydroxy-D9-cis-octadecenoic acid, presents a hydroxyl  
82 group in the  $\alpha$ -position, which may potentiate its effects on metabolism due to the fatty

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

86

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

93

94

## 95 **2. Materials and methods**

96

### 97 2.1. Animal housing and handling

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

108

### 109 2.2. Dietary supplementations

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

119  
120 The obesogenic diet contained lard (310.0 g·kg<sup>-1</sup>), casein (265.0 g·kg<sup>-1</sup>), maltodextrin  
121 (160.0 g·kg<sup>-1</sup>), sucrose (90.0 g·kg<sup>-1</sup>), cellulose (65.5 g·kg<sup>-1</sup>), mineral mix AIN-93G-MX  
122 (94046, 48.0 g·kg<sup>-1</sup>), soybean oil (30.0 g·kg<sup>-1</sup>), vitamin mix AIN-93-VX (94047, 21.0  
123 g·kg<sup>-1</sup>), L-cystine (4.0 g·kg<sup>-1</sup>), calcium phosphate dibasic (3.4 g·kg<sup>-1</sup>), and choline  
124 bitartrate (3.0 g·kg<sup>-1</sup>). The list of ingredients for the control and transition diets cannot be  
125 disclosed due to manufacturer's restrictions, but detailed nutritional composition of all  
126 diets used in the study is present in Supplementary table 1. Diets were provided by Harlan  
127 Interfauna Iberica. BTSA-Biotecnologías Aplicadas, S.L. (Spain) manufactured and  
128 supplied the fatty acids; the 2-OHOA is a synthetic derivative of the oleic acid, and the n-  
129 3 PUFA were extracted from fish (anchovy). For supplementation, the obesogenic diet  
130 was mixed with 2-OHOA (1500 mg·kg<sup>-1</sup> diet), or n-3 PUFA mixture (1500+1500 mg·kg<sup>-1</sup>  
131 diet), in the facilities of the Department of Genetics, Physiology and Microbiology of the  
132 University Complutense, pelleted to match the texture of the control diet, and administered  
133 directly in the appropriate cage compartments.

134  
135 All experimental procedures were approved by the Committee for Animal Experimentation  
136 of the University Complutense of Madrid (ref. CEA-UCM 06/2012), and conducted in  
137 accordance with the guidelines and protocols of the European Community Council

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

141

### 142 2.3. Body and organ weight measurements

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

150

### 151 2.4. Blood measurements

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

157

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



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

$$172 \text{ HOMA index} = (\text{Insulin } [\mu\text{U}\cdot\text{ml}^{-1}] \times \text{Glucose } [\text{mg}\cdot\text{dl}^{-1}]) / 405.$$

173

#### 174 2.5. Secretion of adipokines and inflammation-related proteins from adipocytes

175 The periovarian adipose depot was obtained immediately after the euthanasia of the mice.  
176 Adipocytes were isolated and incubated in 6-well plates, according to the protocol by  
177 Moreno-Aliaga and co-workers [11]. After 50 min incubation, 1  $\mu\text{g}/\text{ml}$  of lipopolysaccharide  
178 (LPS) was added to half of the wells, and plates were incubated at 37 °C in 5% CO<sub>2</sub> for  
179 48 hours. Culture media were then collected and kept at -80 °C for further assays. Leptin,  
180 adiponectin, resistin, PAI1, TNF- $\alpha$ , IL-6 and MCP1 were determined using multiplex assay  
181 kits as described above. The concentration of adipokines was normalized by the total  
182 protein concentration in each well measured by a colorimetric method using bovine serum  
183 albumin (BSA) as the reference protein. This work was carried out in the ICTAN-CSIC.

184

#### 185 2.6. Statistical analysis

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

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

196

197

### 198 **3. Results**

199

#### 200 3.1. Effects of dietary supplementations on body weight, fat and organs weight

201 Body weight at the beginning of the study (9 weeks of age) was similar among groups  
202 ( $P=0.100$ ). At the end of the study (27 weeks of age), the OD mice showed the greatest  
203 body and heart weights, particularly compared to control and OD-HO mice ( $P=0.042$  and  
204  $P=0.009$  for body weight;  $P=0.026$  and  $P=0.011$  for heart weight, respectively; table 1).

205 The OD-HO mice presented the lowest body weight of all the groups, and lower heart  
206 weight than the OD group; the OD-N3 group showed values similar to OD mice, and the  
207 heaviest periovarian fat depots of all groups ( $P=0.001$ ), significantly heavier than the  
208 control group ( $P=0.039$ ). No differences were observed in liver weight among groups  
209 (table 1).

210

#### 211 3.2. Effects of dietary supplementations on cardiometabolic risk and inflammatory 212 biomarkers

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

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

225

226 3.3. Effects of dietary supplementations on adipocyte secretion of adipokines and  
227 inflammation-related proteins

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

237

238 3.4. Associations between adipokines and cardiometabolic risk biomarkers

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

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

246

247

248 **4. Discussion**

249 The amount of dietary fat has traditionally been associated with obesity and the metabolic  
250 syndrome, main factors for increased cardiovascular risk (CVR) [3]. Our results showed  
251 that progressive adaptation to an obesogenic diet increased body weight, heart weight,  
252 and plasma leptin levels, and reduced adiponectin and resistin secretion from adipose  
253 tissue, features all related to the pathogenesis of metabolic syndrome and higher CVR  
254 [12]. Supplementation with 2-OHOA and n-3 PUFA reverted some of these changes, in  
255 agreement with previous studies showing that the quality of dietary fat is a key factor that  
256 modulates obesity and CVR [13]. In our study, 2-OHOA and n-3 PUFA improved  
257 triglyceride levels, but only the 2-OHOA reduced body and heart weights, leptin levels and  
258 blood pressure values, and restored adipokine secretion from adipocytes to levels closer  
259 to the control group.

260

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

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

285

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

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

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

335

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

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

368

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

375

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380

### 381 **Declarations of interest**

382 The authors have no conflict of interest to declare.

383

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394

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- 495

## 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

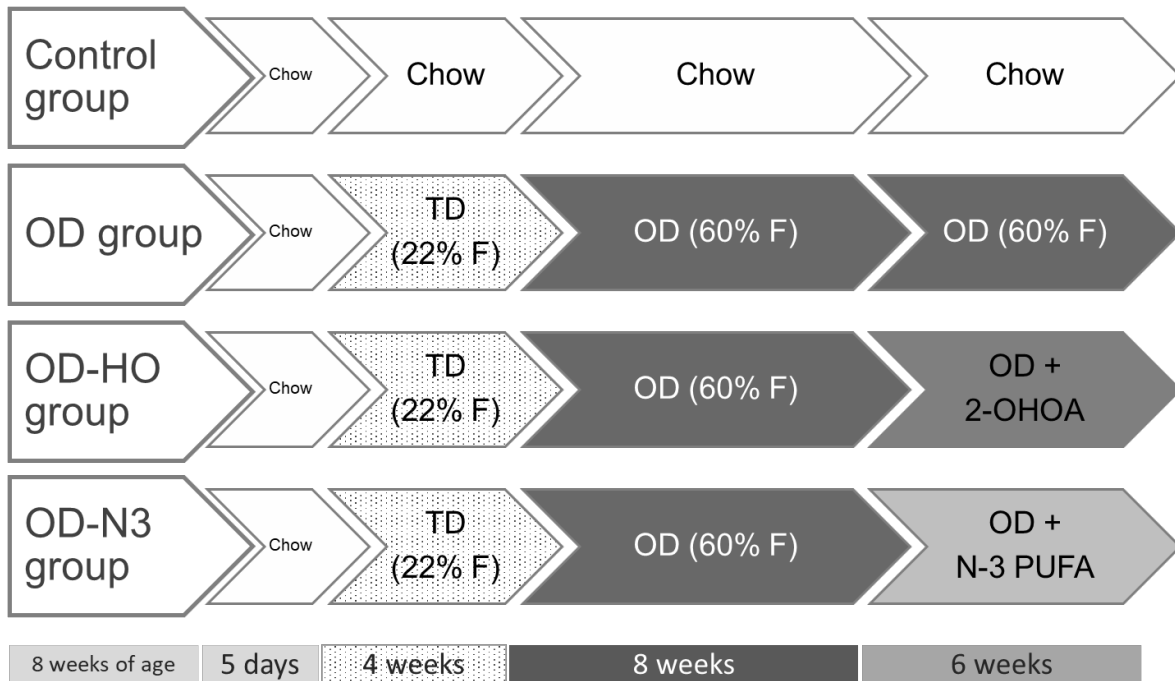


Figure 2

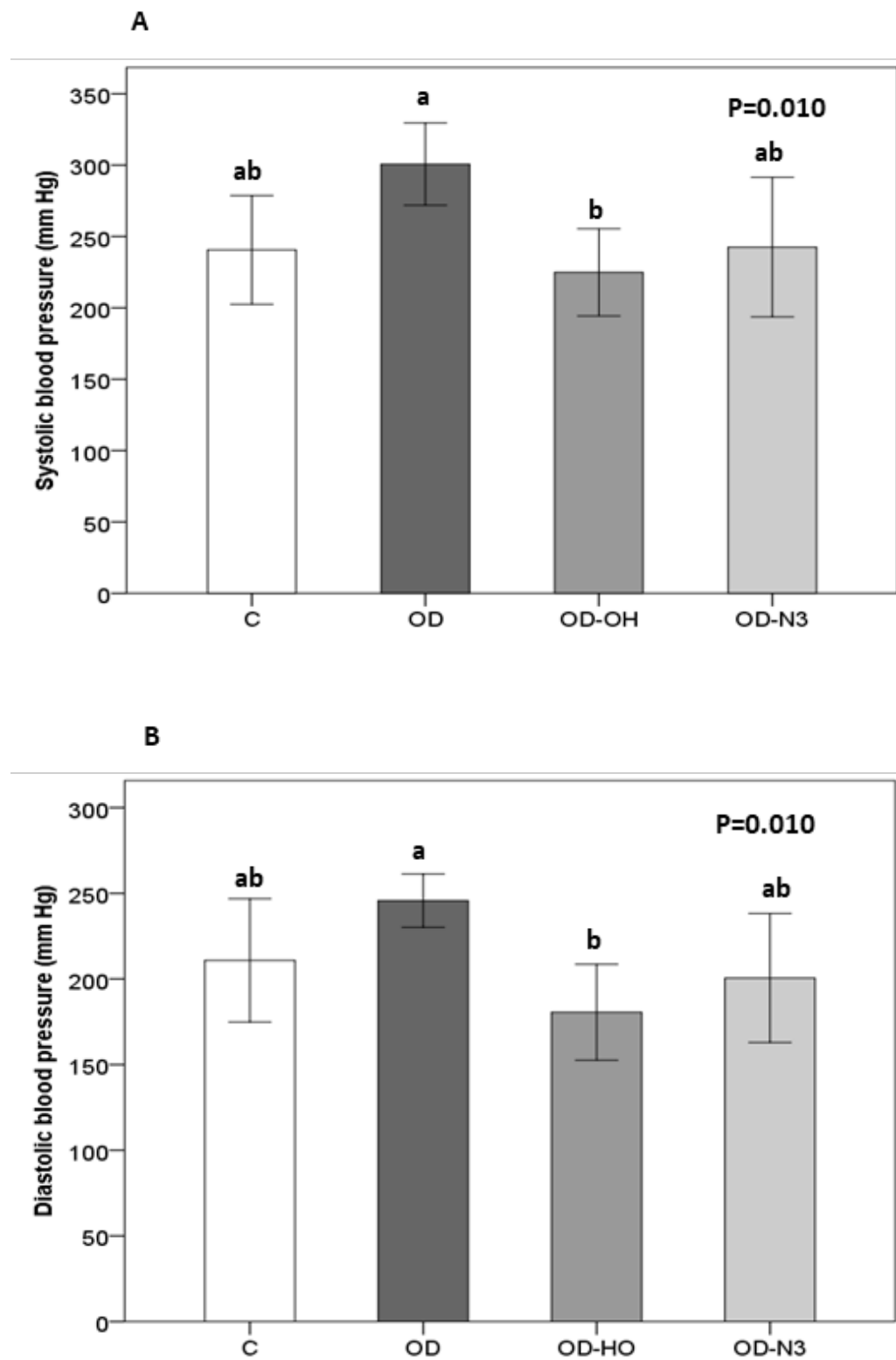


Figure 3

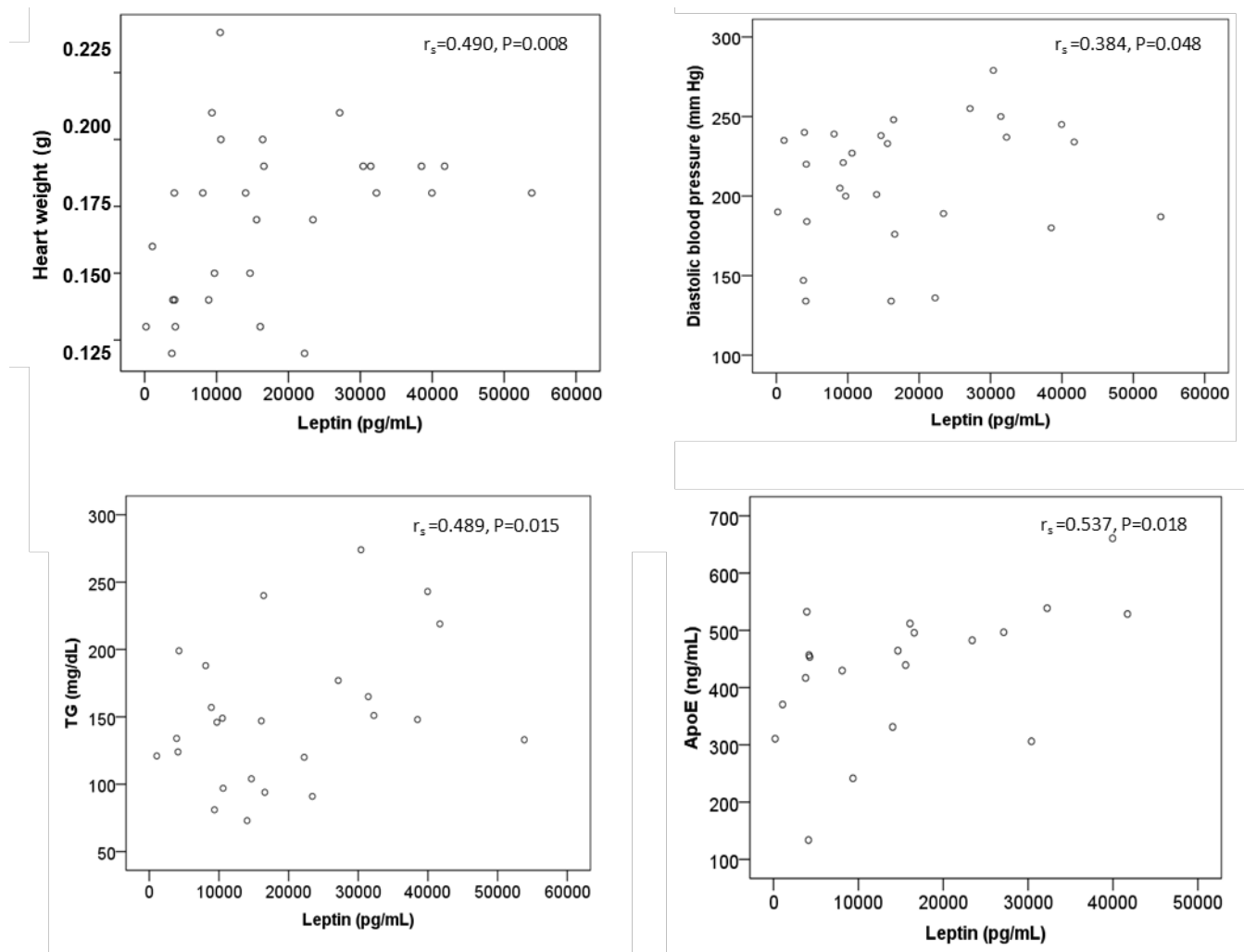




Table 1. Cardiovascular risk biomarkers in all groups at the end of the supplementations.

	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) <sup>b</sup>	7	50.8 (11.3) <sup>a</sup>	7	28.4 (3.5) <sup>c</sup>	7	48.2 (11.7) <sup>abc</sup>	<0.001
Periovarian fat (g)	8	0.62 (0.38) <sup>a</sup>	7	3.46 (2.59) <sup>ab</sup>	7	1.17 (0.69) <sup>ab</sup>	7	4.20 (2.35) <sup>b</sup>	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 <sup>a</sup> (0.14-0.18)	7	1.19 <sup>b</sup> (0.18-0.20)	7	0.14 <sup>a</sup> (0.12-0.18)	7	0.18 <sup>ab</sup> (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) <sup>ab</sup>	7	198.4 (62.6) <sup>a</sup>	6	125.5 (43.9) <sup>b</sup>	7	125.9 (34.9) <sup>b</sup>	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) <sup>a</sup>	7	28,963 (11,525) <sup>b</sup>	7	10,684 (7,207) <sup>ac</sup>	7	26,137 (15,508) <sup>bc</sup>	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) <sup>a</sup>	5.3 (1.8) <sup>b</sup>	11.7 (5.7) <sup>ab</sup>	8.3 (4.8) <sup>ab</sup>	0.002
Resistin	2.80 <sup>a</sup> (2.49-4.88)	0.89 <sup>b</sup> (0.57-1.95)	3.33 <sup>ab</sup> (0.80-4.37)	1.37 <sup>ab</sup> (1.04-3.00)	0.034	3.25 <sup>a</sup> (1.96-5.80)	0.59 <sup>c</sup> (0.42-1.26)	1.36 <sup>ab</sup> (1.16-3.51)	0.99 <sup>bc</sup> (0.58-1.83)	0.005
PAI1	16.3 (13.1)	16.6 (12.1)	18.9 (13.9)	5.5 (6.2)	NS	11.7 (3.2-16.7)	4.9 (4.5-6.9)	25.12 (7.1-37.5)	4.7 (1.9-8.5)	NS
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 (0.006-0.015)	0.033 (0.012-0.057)	0.021 (0.010-0.047)	0.009 (0.005-0.020)	NS	0.012 (0.005-0.020)	0.008 (0.003-0.034)	0.025 (0.012-0.060)	0.008 (0.004-0.017)	NS
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 $\alpha$ -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.