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**Gheorghe, A, Pérez de Heredia, F, Hunsche, C, Redondo, N, Díaz, LE, Hernández, O, Marcos, A and De la Fuente, M**

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### Article

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1 **Oxidative stress and immunosenescence in spleen of obese mice can be reversed by 2-**  
2 **hydroxyoleic acid**

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1 **New findings**

2 What is the central question of this study?

3 Evidence is growing for the link between obesity, immune dysfunction and oxidative stress, but it is  
4 still not known how the properties and functions of spleen and spleen leukocytes are affected.

5 What is the main finding and its importance?

6 Obesity led to premature immunosenescence, manifested as oxidative stress and changes in leukocyte  
7 functions in mouse spleen. The oleic acid derivative 2-hydroxyoleate, and to a lesser extent a  
8 combination of EPA+DHA, could reverse most of the observed alterations, suggesting a potential  
9 therapeutic tool for obesity-related immune dysfunction and redox imbalance.

10

11

## 1 **Abstract**

2 We aimed to investigate the effects of obesity on oxidative stress and leukocyte function in spleen of  
3 mice, and to assess whether supplementation with 2-hydroxyoleic acid (2-OHOA) or n-3  
4 polyunsaturated fatty acids (PUFA) could reverse those effects. Female ICR/CD1 mice (8 weeks old,  
5 n=24) received an obesogenic diet (22% fat for 4 weeks and 60% fat for 14 weeks). After 6 weeks,  
6 mice were split in three groups (n=8/group): no supplementation, 2-OHOA supplementation  
7 (1500 mg/kg) and n-3 PUFA supplementation (EPA+DHA, 3000 mg/kg diet). Eight mice were fed  
8 standard diet for the whole duration of the study (control group). At the end of the experiment, the  
9 following variables were assessed in spleens: levels of reduced (GSH) and oxidized (GSSG)  
10 glutathione, GSH/GSSG, xanthine oxidase (XO) activity, lipid peroxidation, lymphocyte chemotaxis,  
11 natural killer (NK) activity and mitogen (ConA and LPS)-induced lymphocyte proliferation. Obese  
12 animals presented higher GSSG levels (P=0.003), GSSG/GSH ratio (P=0.013), lipid peroxidation  
13 (P=0.004), XO activity (P=0.015) and lymphocyte chemotaxis (P<0.001), and lower NK activity  
14 (P=0.003) and proliferation in response to ConA (P<0.001) than controls. 2-OHOA reversed totally  
15 or partially most of the changes (body weight, fat content, GSSG levels, GSH/GSSG, lipid  
16 peroxidation, chemotaxis and proliferation, all P<0.05), while n-3 PUFA reversed the increase in XO  
17 activity (P=0.032). In conclusion, 2-OHOA, and to a lesser extent n-3 PUFA, could ameliorate the  
18 oxidative stress and alteration of leukocyte function in spleen of obese mice. Our findings support a  
19 link between obesity and immunosenescence and suggest a potential therapeutic tool for obesity-  
20 related immune dysfunction.

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23

## 1 **Introduction**

2 Nutritional status is a key factor for a correct function of the immune system and the maintenance of  
3 health (De la Fuente & Miquel, 2009). States of malnutrition have been linked to higher vulnerability  
4 to infections and immune dysfunction. Malnutrition, however, is not restricted to nutrient deficiencies  
5 anymore, but it broadly refers to inadequate nutrition and nutritional imbalance, including excessive  
6 energy intake and obesity. Indeed, obesity has been associated with impaired immune function, which  
7 is reflected in an enhanced, non-resolved inflammatory response and compromised immune  
8 surveillance (Karlsson *et al.*, 2010; De la Fuente & De Castro, 2012; Perez de Heredia *et al.*, 2012;  
9 Hunsche *et al.*, 2016). Obesity is also associated with oxidative stress when maintained for a long  
10 time, and it can be mediated by either a decrease in antioxidant defences and/or increased formation  
11 of oxidants. Oxidative stress in turn can damage cellular structures and trigger an inflammatory  
12 response, closing a detrimental feedback loop (Perez de Heredia *et al.*, 2012; Matsuda & Shimomura,  
13 2013; Savini *et al.*, 2013; Vida *et al.*, 2014). Research on obesity and immunity has focused mainly  
14 on circulating leukocytes, but immune organs themselves can be compromised. The largest secondary  
15 immune organ in mammals is the spleen; it hosts macrophages, dendritic cells, plasma cells and a  
16 fourth of the body's lymphocytes, and is involved in several functions, such as activation of T and B  
17 cells in response to blood-born antigens, antibody production, or clearance of circulating apoptotic  
18 cells (thus contributing to peripheral immune tolerance) (Cesta, 2006). Therefore, it is important to  
19 study the impact of obesity in the spleen and in spleen leukocytes.

20 The last decades have witnessed a dietary transition toward a westernized dietary pattern,  
21 coincident with the rise of overweight and obesity in both developed and developing countries  
22 (Cuevas *et al.*, 2009; Bezerra *et al.*, 2014). In contrast, the Mediterranean diet has been linked to  
23 lower rates of obesity (Schroder *et al.*, 2004), inflammation and oxidation (Savini *et al.*, 2013). The  
24 Mediterranean diet has been reported to modulate the immune response and to exert anti-  
25 inflammatory and antioxidant properties (Minich & Bland, 2008). This may be in great part due to its  
26 high content in monounsaturated (n-9 MUFA) and polyunsaturated (n-3 PUFA) fatty acids, which

1 have shown immunomodulatory actions (de Pablo *et al.*, 1998; Padovese & Curi, 2009; Paschoal *et*  
2 *al.*, 2013). N-3 PUFA have been more extensively studied in this respect (Calder & Grimble, 2002),  
3 while less attention has been paid to the effects of MUFA on the immune system, although they may  
4 contribute to reducing oxidative stress (Fitó *et al.*, 2007). Evidence regarding whether dietary MUFA  
5 and n-3 PUFA affect the redox state and the function of leukocytes in spleen in obesity is nevertheless  
6 still scarce.

7 The aims of the current study were to investigate the effects of obesity on markers of oxidative  
8 stress and leukocyte functions in spleen of mice, and to evaluate the impact of subsequent  
9 supplementation with n-9 MUFA and n-3 PUFA on these parameters.

10

## 11 **Methods**

### 12 Ethical Approval

13 All experimental procedures were approved by the Committee for Animal Experimentation of the  
14 University Complutense of Madrid (ref. CEA-UCM 06/2012), and conducted in accordance with the  
15 guidelines and protocols of the Spanish Royal Decree 1201/2005 regarding the care and use of  
16 laboratory animals for experimental procedures. The authors acknowledge the ethical principles of  
17 *Experimental Physiology*, and confirm that the study was conducted in compliance with the animal  
18 ethics checklist as detailed by Grundy (2015).

19 Measures were taken to ensure the well-being of animals and to minimize pain and suffering  
20 to the best of our possibilities (see methodological description below). Organ and tissue samples were  
21 obtained *post-mortem*. Animals were euthanized at the end of the study, by decapitation in the  
22 morning (8:00 a.m.), and no anaesthetic was used to this effect to save unnecessary suffering to the  
23 animals. This procedure is in agreement with the dispositions of the European Directive 2010/63/EU.

24

### 25 Animal origin and housing conditions

1 Thirty-two female ICR/CD1 mice, 8 weeks of age, were purchased from Harlan Interfauna Iberica  
2 (Barcelona, Spain). The animals were housed in polyurethane cages (4 animals per cage) and  
3 maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at  
4 8:00 pm, relative humidity of 50-60%, temperature of 22±2 °C and adequate ventilation). During the  
5 first 5 days of acclimatization to the new environment, all mice were fed a standard maintenance diet  
6 (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014, Harlan Interfauna Iberica).

7

### 8 Experimental groups and diets

9 Animals were split into two groups: 8 mice kept receiving the maintenance diet for the entire duration  
10 of the study (18 weeks), constituting the control group, while the rest received a moderately fat-rich  
11 diet for 4 weeks (3.3 kcal/g, 22% calories from fat, 23% protein, 55% carbohydrates, ref. Teklad  
12 Global 2019, Harlan Interfauna Iberica), followed by an obesogenic diet (60% fat, 18.4% protein,  
13 21.3% carbohydrates, ref. TD. 06414, Harlan Interfauna Iberica) for a further 8 weeks (figure 1).

14 At this point, the high-fat diet-fed mice were split into three groups: 8 mice kept receiving the  
15 obesogenic diet for the rest of the experiment (OD group), 8 mice were given the diet supplemented  
16 with 2-OHOA (1500 mg/kg diet) (OD-HO group), and 8 mice were given the diet supplemented with  
17 a combination of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids (1500  
18 + 1500 mg/kg diet), for 6 additional weeks. The 2-OHOA is a synthetic derivative of oleic acid with  
19 a hydroxyl group in the  $\alpha$ -position, and it is also known as 2-hydroxy-D9-cis-octadecenoic acid. The  
20 n-3 PUFA were extracted from fish (anchovy). All fatty acid supplements were provided by BTSA-  
21 Biotecnologías Aplicadas, S.L. Upon reception of the supplements (2-OHOA in powdered form and  
22 n-3 PUFA in oil form), these were mixed at our facilities with the diet, which was of a malleable  
23 consistency, and then pelleted before being presented to the mice. Animals had free access to water  
24 and food during the entire study, and food intake was monitored on a weekly basis.

25

### 26 Collection of spleen and leukocyte suspensions

1  
2 Once animals were euthanized, their spleens were collected aseptically and freed from fat. One  
3 fragment of each spleen was stored at  $-80^{\circ}\text{C}$  for the study of oxidative stress parameters. Another  
4 fragment of spleen was minced with scissors and gently pressed through a mesh screen (Sigma, St  
5 Louis, USA) to obtain cell suspensions. The cell suspensions were centrifuged in a gradient of Ficoll-  
6 Hypaque (Sigma) with a density of  $1.070\text{ g/ml}$ ; cells from the interface were collected and suspended  
7 in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with L-glutamine (PAA,  
8 Pasching, Austria) and supplemented with 10% heat-inactivated foetal calf serum (Gibco, Canada)  
9 and 1% gentamicin ( $100\text{ }\mu\text{g/ml}$ , Gibco). After a wash step, leukocytes were counted in a Neubauer  
10 chamber and their number adjusted to  $10^6\text{ cell/ml}$ . Cell viability was routinely measured before each  
11 experiment by the trypan-blue exclusion test, and was higher than 95% in all experiments. All  
12 incubations were performed at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ .

13

14 Analysis of oxidative stress

15 GSH and GSSG levels

16 Reduced glutathione (GSH) is one of the most important anti-oxidant defence mechanisms in the  
17 organism, and as such a relevant marker of its antioxidant capacity. Both reduced and oxidized  
18 (GSSG) glutathione were determined in spleen using a fluorometric method (Hissin & Hilf, 1976).  
19 This is based on the reaction of a fluorescence probe, o-phthaldialdehyde (OPT), with GSH at  $\text{pH}=8$   
20 and with GSSG at  $\text{pH}=12$ , which generates a fluorescence derivative. The spleen samples were  
21 homogenized ( $50\text{ mg/ml}$ ) in sodium phosphate-EDTA buffer ( $0.1\text{ M}$ ,  $\text{pH}=8$ ) and proteins were  
22 precipitated by adding  $5\text{ }\mu\text{l}$  of 60% perchloric acid. Homogenized spleen samples were centrifuged  
23 ( $9,500\text{ g}$ ,  $10\text{ min}$ ,  $4^{\circ}\text{C}$ ) and supernatants were maintained in ice for measurement of GSH and GSSG  
24 levels. For GSH levels determination,  $10\text{ }\mu\text{l}$  of the supernatant,  $190\text{ }\mu\text{l}$  of sodium phosphate-EDTA  
25 buffer and  $20\text{ }\mu\text{l}$  of OPT solution ( $1\text{ mg/ml}$  in methanol) were added to a 96-well black microplate  
26 and incubated at room temperature for 15 minutes. Fluorescence was determined in a microplate



1 reader using excitation at 350 nm and emission detection at 420 nm. For the determination of GSSG  
2 levels, 10  $\mu$ l of the supernatant and 4  $\mu$ l of N-ethylmaleimide (NEM, 0.04 M) were added to a 96-  
3 well black microplate and incubated at room temperature for 30 minutes. Then, 186  $\mu$ l of sodium  
4 hydroxide (NaOH, 0.1 N) with 20  $\mu$ l of OPT solution were added to each well. After incubation (room  
5 temperature, 15 min), fluorescence was measured as previously described for GSH determination.  
6 The results were analysed with GSH and GSSG standard curves at different concentrations and  
7 expressed as nmol/mg protein. Protein concentration of the samples was measured following the  
8 bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Madrid, Spain). The GSSG/GSH ratio  
9 was then calculated for each sample. All assays were performed in duplicates.

10

#### 11 Xanthine oxidase (XO) activity

12 XO activity was assayed by fluorescence in homogenates of spleen, using a commercial kit (Amplex  
13 Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). In the assay, XO  
14 catalyses the oxidation of purine bases (xanthine) to uric acid and superoxide anions. The superoxide  
15 anion spontaneously degrades in the reaction mixture to H<sub>2</sub>O<sub>2</sub>, which in the presence of horseradish  
16 peroxidase (HRP) reacts with Amplex Red reagent to generate the red-fluorescent oxidation product,  
17 resorufin. Tissue samples were homogenized in phosphate buffer (50 mM, pH=7.4) containing 1 mM  
18 EDTA and normalized to total protein. The homogenate was centrifuged (5,000 g) and the  
19 supernatant (50  $\mu$ l) was collected and incubated with 50  $\mu$ l working solution of Amplex Red reagent  
20 (100  $\mu$ M) containing HRP (0.4 U/ml) and xanthine (200  $\mu$ M). After 30 min of incubation at 37 °C,  
21 measurement of fluorescence was performed in a microplate reader (Fluostar Optima, BMG Labtech,  
22 Biomedal, Spain), using excitation at 530 nm and emission detection at 595 nm. XO supplied in the  
23 kit was used as the standard. The results were expressed as international milliunits of enzymatic  
24 activity per milligram of protein (mU XO/mg protein). Protein content of the samples had been  
25 previously assessed by the bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Madrid,  
26 Spain). All assays were performed in duplicates.

1

## 2 Lipid peroxidation

3 Lipid peroxidation levels were determined by measuring the formation of malondialdehyde (MDA)  
4 using a colorimetric assay kit (BioVision, Inc., Mountain View, CA, USA). The spleen samples were  
5 homogenized (10 mg) in 300  $\mu$ l of MDA lysis buffer with 3  $\mu$ l butylhydroxytoluene (BHT) (X100)  
6 and then centrifuged (13,000 g, 10 min) to remove insoluble material. An aliquot (200  $\mu$ l) of each  
7 supernatant was added to 600  $\mu$ l of thiobarbituric acid (TBA) and incubated at 95 °C for 60 minutes.  
8 The samples were then maintained in an ice bath for 10 minutes and 200  $\mu$ l from each 800  $\mu$ l reaction  
9 mixture were placed into a 96-well microplate for spectrophotometric measurement at 532 nm. The  
10 results were analyzed with MDA standard curve at different concentrations and expressed as nmol/mg  
11 protein. Protein concentration of the samples was measured following bicinchoninic acid protein  
12 assay kit protocol (Sigma-Aldrich, Madrid, Spain). All assays were performed in duplicates.

13

## 14 Leukocyte functions

## 15 Chemotaxis assay

16 The assay was carried out following the method previously described by De la Fuente and colleagues  
17 (2004). Chambers with two compartments separated by a filter of 3  $\mu$ m pore diameter (Millipore,  
18 Ireland) were used. Aliquots of 300  $\mu$ l of leukocyte suspensions were placed in the upper  
19 compartment, and 400  $\mu$ l of the chemoattractant fMet-Leu-Phe (fMLF, Sigma), at a concentration of  
20  $10^{-8}$  M, were placed in the lower compartment. After 3 h incubation, the filters were fixed and stained,  
21 and the number of lymphocytes on the lower face of the filters was counted in one third of them, with  
22 an optical microscope, and recorded as the chemotaxis index (CI). All the samples were assayed in  
23 duplicate.

24

## 25 NK activity assay

1 An enzymatic colorimetric assay was used for measurements of cytolysis of target cells (Cytotox 96  
2 TM Promega, Boeriner Ingelheim, Germany), based on the determination of the activity of the  
3 enzyme lactate dehydrogenase (LDH), and using tetrazolium salts, as previously published (De la  
4 Fuente *et al.*, 2004). Briefly, target cells (YAC-1 cells from a murine lymphoma) were seeded in 96-  
5 well U-bottom culture plates (Nunclon, Denmark) in RPMI 1640 medium without phenol red, at a  
6 concentration of  $10^4$  cell/well. Effector cells (leukocytes from spleen) were added at a concentration  
7 of  $10^5$  cell/well, thus obtaining an effector/target rate of 10/1. The plates were centrifuged at 250 g  
8 for 4 minutes to facilitate cell-to-cell contact and then incubated for 4 h. After incubation, plates were  
9 centrifuged again at 250 g for 4 minutes and LDH activity was measured in the supernatants  
10 (50  $\mu$ l/well) by addition of the enzyme substrate with absorbance recording at 490 nm. The results  
11 were expressed as percentage of lysis. Each sample was assayed in triplicate. Three kinds of control  
12 measurements were performed: a target spontaneous release, a target maximum release and an  
13 effector spontaneous release. To determine the percentage of lysis of target cells, the following  
14 equation was used: % lysis =  $([E-ES-TS]/ [M-ES-TS]) \times 100$   
15 where E is the mean absorbance in the presence of effector cells; ES is the mean absorbance of  
16 effector cells incubated alone (effector spontaneous release); TS is the mean absorbance in target cells  
17 incubated with medium alone (target spontaneous release), and M is the mean of maximum  
18 absorbance after incubating target cells with lysis solution (target maximum release).

19

## 20 Lymphoproliferation assay

21 Following the method previously described (De la Fuente *et al.*, 2004), aliquots (200  $\mu$ l) of leukocytes  
22 ( $10^6$  cells/ml complete medium) were seeded in 96-well flat-bottomed microtiter plates (Numc,  
23 Roskilde, Denmark). Then, 20  $\mu$ l of concanavaline A (ConA, 1 $\mu$ g/ml, Sigma, St Louis, MO) or 20  $\mu$ l  
24 of lipopolysaccharide (LPS, *Escherichia coli* 055:B5, 1 $\mu$ g/ml, Sigma) were added per well. In order  
25 to assess spontaneous proliferation, 20  $\mu$ l of complete medium were added to some wells instead of  
26 the mitogens. After 48 h of incubation at 37 °C in an atmosphere of 5% CO<sub>2</sub>, 0.5  $\mu$ Ci 3H-thymidine

1 (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semiautomatic  
2 microharvester 24 h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala,  
3 Sweden). The results were expressed as <sup>3</sup>H-thymidine uptake (cpm). All assays were performed in  
4 triplicates.

5

## 6 Statistical analysis

7 We tested the following hypotheses: 1) spleen from obese animals would be subjected to higher  
8 oxidative stress than those from controls; 2) leukocytes from spleens of obese animals would present  
9 altered chemotaxis, NK activity and proliferation in response to mitogens in relation to controls; 3)  
10 supplementation with 2-OHOA and n-3 PUFA would reverse the observed changes.

11 Sample size was 8 per experimental group (unless otherwise specified), with assays being  
12 conducted in triplicates (NK activity and lymphoproliferation) or duplicates (the rest of assays). In  
13 those cases, the average value of the replicas was used. The results are expressed as mean  $\pm$  standard  
14 deviation (SD), or median and interquartile range (IQR), depending upon normality of the data, which  
15 was checked by the Shapiro-Wilk test. For normally distributed variables (XO activity, MDA levels,  
16 CI, NK activity and proliferation), one-way ANOVA with Bonferroni *post-hoc* test were conducted  
17 to compare the four experimental groups. For non-normally distributed variables (body weight, GSH  
18 and GSSG levels and GSSG/GSH), the Kruskal-Wallis test was used to compare the four  
19 experimental groups, and Mann-Whitney was used to run pairwise comparisons when the Kruskal-  
20 Wallis test was significant. Significance level was always set at  $P < 0.05$ . All statistical tests were  
21 performed using IBM SPSS v23.

22

## 23 Results

### 24 Effect of the treatments on body weight gain

25 The animals fed the obesogenic diet started gaining significantly more weight than the controls after  
26 five weeks of high-fat feeding ( $P = 0.036$ ). Supplementation with 2-OHOA resulted in progressive

1 reduction of body weight, and at the end of the experiment the average weight of the OD-HO group  
2 was significantly lower than that of controls ( $P=0.009$ ). The supplementation with n-3 PUFA did not  
3 have a significant effect on body weight when compared to the OD group (figure 2).

4

#### 5 Effect of the treatments on the oxidative stress parameters in spleen

6 The levels of reduced glutathione (GSH) were similar among experimental groups ( $P=0.518$ ) (figure  
7 3A). On the contrary, the levels of oxidized glutathione (GSSG) and the GSSG/GSH ratios were  
8 significantly different between groups ( $P=0.003$  and  $P=0.013$ , respectively). The OD group presented  
9 the highest values; treatment with 2-OHOA seemed to prevent or reverse the increase in both GSSG  
10 levels and GSSG/GSH, while n-3 PUFA seemed to ameliorate only the rise in GSSG/GSH (figures  
11 3B and 3C). Similarly, xanthine oxidase activity (figure 4) was different among experimental groups  
12 ( $P=0.015$ ). The highest value corresponded to the OD group ( $P=0.032$  vs control), and the increase  
13 was abolished by supplementation with n-3 PUFA ( $P=1.000$  and  $P=0.032$  vs control and OD groups,  
14 respectively), and partially by 2-OHOA ( $P=1.000$  and  $P=0.114$  vs control and OD groups,  
15 respectively). Lipid peroxidation (measured as malondialdehyde [MDA] levels) was also different  
16 among treatments ( $P=0.004$ ), the differences being found between the OD and OD-HO groups  
17 ( $P=0.012$ ), and between the OD-HO and OD-N3 groups ( $P=0.017$ ) (figure 5).

18

#### 19 Effect of treatments on spleen leukocytes functions

20 Significant differences were found among the experimental groups for the chemotaxis index (CI) of  
21 spleen lymphocytes ( $P<0.001$ ), which was lowest in controls, and highest in the OD group.  
22 Supplementation with 2-OHOA partially prevented or reversed the increase in the CI ( $P=0.002$  vs the  
23 OD group), while no significant effect could be attributed to n-3 PUFA supplementation ( $P=0.863$  vs  
24 the OD group) (figure 6). In contrast, the natural killer (NK) activity of spleen leukocytes was higher  
25 in the control group than in all groups fed the obesogenic diet ( $P=0.003$ ), with no statistical

1 differences observed between the OD group and the groups that received the fatty acid supplements  
2 (P=1.000 for both *post-hoc* contrasts) (figure 7).

3 With regards to the proliferative capacity of lymphocytes, no significant differences were  
4 observed between groups in basal conditions (control group:  $1395 \pm 550$ ; OD:  $1283 \pm 463$ ; OD-HO:  
5  $1363 \pm 346$ ; and OD-N3:  $1193 \pm 361$  cpm). In response to stimulation by ConA, the OD group  
6 showed the lowest values of proliferation (P<0.001), a decrease that was totally reversed by  
7 supplementation with 2-OHOA and partially by supplementation with n-3 PUFA (figure 8A). In  
8 response to LPS stimulation, the obesogenic diet *per se* did not result in a significant alteration of the  
9 lymphoproliferative response, but the supplementation with 2-OHOA was accompanied by higher  
10 levels of proliferation, in comparison with both the control group (P<0.001) and the OD group  
11 (P=0.002) (figure 8B).

12

### 13 **Discussion**

14 The results of the current study support that the induction of dietary obesity during the juvenile period  
15 leads to the development of obesity, oxidative stress and impaired leukocyte function in spleen in  
16 adulthood. The supplementation with 2-OHOA, and to a lesser extent with n-3 PUFA (EPA+DHA),  
17 was able to partially or completely ameliorate the alteration of most leukocyte functions, and to  
18 improve the oxidative stress status in the obese mice.

19 We found that 2-OHOA supplementation, but not n-3 PUFA, led to a progressive decrease in  
20 body weight. This was not accompanied by a decrease in food intake (data not shown). Our results  
21 agree with Vögler and colleagues (2008), who previously reported that 2-OHOA-treated mice  
22 experienced a decrease in body weight through reduction of adipose fat mass. With regards to n-3  
23 PUFA, despite a considerable body of evidence (Thorsdottir *et al.*, 2007; Buckley & Howe, 2010), a  
24 recent meta-analysis indicated that PUFA supplementation does not promote anti-obesity effects in  
25 overweight/obese individuals, in agreement with our results (Du *et al.*, 2015).

1           The obese animals in our study presented increased oxidative stress and altered function of  
2 leukocytes in spleen. Obesity is a risk factor for the progressive deterioration of cellular immune  
3 functions (Tarantino *et al.*, 2013), and has been associated with premature immunosenescence, a  
4 situation of both oxidative and inflammatory stress (De la Fuente & De Castro, 2012; De la Fuente,  
5 2014). The pathophysiological mechanisms by which cellular immune functions are affected by  
6 obesity are still under investigation but the spleen may play an important role (Tarantino *et al.*, 2013).  
7 The increased oxidative stress in our mice was manifested by higher GSSG, GSSG/GSH ratio, lipid  
8 peroxidation and xanthine oxidase activity levels. This suggests that obesity was accompanied by  
9 elevated formation of oxidants rather than a decrease in the levels of antioxidants, at least in the  
10 spleen. This is the first time to our knowledge that GSH, GSSG, and the GSSG/GSH ratio have been  
11 investigated in the spleen of dietary obese mice, but our results agree with previous studies that looked  
12 at other organs and/or species (Capel & Dorrell, 1984; Kolesnikova *et al.*, 2013; Hunsche *et al.*, 2016).  
13 An increased GSSG/GSH ratio has been associated with a number of diseases, including type-2  
14 diabetes (Lee *et al.*, 2008), and it can therefore be a useful health marker in the assessment of obesity-  
15 related comorbidities. In a similar manner, the activity of XO in thymus has been reported to be higher  
16 in obese rats than in normal weight controls (De la Fuente & De Castro, 2012), and to be elevated in  
17 obese children when compared to non-obese children (Chiney *et al.*, 2011). In our study, the  
18 supplementation with unsaturated fatty acids proved to be effective at improving the oxidative state  
19 of obese mice; 2-OHOA supplementation reduced both GSSG levels and GSSG/GSH ratio, as well  
20 as MDA levels (the marker of lipid peroxidation), when compared to the OD group, while XO activity  
21 was decreased by n-3 PUFA supplementation (in relation to the OD group). Our results are in  
22 agreement with previous evidence showing that olive oil consumption can favour tissue antioxidant  
23 defence mediated by the glutathione system (De La Cruz *et al.*, 2000), reduce lipid peroxidation levels  
24 (El-Kholy *et al.*, 2014), and improve plasma antioxidant capacity (Pitsavos *et al.*, 2005).

25           In our study, obese animals presented as well higher chemotaxis, lower cytotoxic activity and  
26 lower mitogen-induced proliferation in spleen leukocytes. The chemotaxis capacity enables the

1 migration of circulating immune cells into tissues and their accumulation in infection or injury sites,  
2 in order to produce an adequate inflammatory and defensive response (Doherty *et al.*, 1987).  
3 Understanding the significance of increased chemotaxis in our obese mice would require further  
4 research, as evidence available is not unanimous in this respect. On the one hand, decreased  
5 chemotaxis has been reported in neutrophils from genetically obese mice (Kordonowy *et al.*, 2012),  
6 and in peritoneal immune cells from aged mice, which are in a state of oxidative stress (De la Fuente  
7 & Miquel, 2009). On the other hand, a rise in the chemotactic indices of spleen lymphocytes was  
8 observed in a mouse model of Alzheimer's disease, another condition with a high oxidative situation  
9 (Giménez-Llort *et al.*, 2008), while another study showed enhanced immune cell chemotaxis in mice  
10 fed a high-fat diet (Qiao *et al.*, 2009). High-fat consumption has been reported to induce cellular  
11 adherence activation (Esser *et al.*, 2013), which in turn is linked to oxidative stress (De la Fuente &  
12 Miquel, 2009). The increased chemotaxis observed in our study, therefore, is likely to be related to  
13 the oxidative stress state of the obese mice. It is important, however, to highlight that changes in  
14 chemotactic function can be dependent on the type of immune cells and organs analysed.  
15 Supplementation with 2-OHOA, but not with n-3 PUFA, was able to partially prevent or reverse the  
16 rise in the chemotaxis index associated with obesity in our mice. In agreement with our findings,  
17 previous studies have reported no effect of n-3 PUFA on neutrophil chemotaxis (Schmidt *et al.*, 1996;  
18 Healy *et al.*, 2000; Hill *et al.*, 2007), although other authors did observe diminished neutrophil  
19 chemotaxis in response to these fatty acids (Lee *et al.*, 1985; Luostarinen *et al.*, 1992; Schmidt *et al.*,  
20 1992; Sperling *et al.*, 1993). In relation to 2-OHOA, our study is to our knowledge the first to report  
21 a significant amelioration of obesity-related changes in spleen leukocyte chemotaxis.

22 Natural killer cytotoxic activity was lower in all the groups fed the obesogenic diet. These  
23 results are consistent with previous studies indicating that high-fat-fed mice, obese rats and obese  
24 humans suffer from diminished NK cell cytotoxicity (Morrow *et al.*, 1985; Moriguchi *et al.*, 1998;  
25 Lamas *et al.*, 2004; O'Shea *et al.*, 2010; De la Fuente & De Castro, 2012). NK cells constitute the  
26 most important defensive line against malignant and virus-infected cells. Thus, a decrease in their



1 activity renders the animals more susceptible to infections and tumours. Interestingly, similar changes  
2 in NK activity have been observed in old and prematurely aging animals (De la Fuente *et al.*, 2004;  
3 De la Fuente & Miquel, 2009). In our study, neither 2-OHOA nor n-3 PUFA supplementation resulted  
4 in any improvement of NK activity, in agreement with previous work (Berger *et al.*, 1993; Yaqoob  
5 *et al.*, 2000). Therefore, we can speculate that changes in NK activity in our study were not directly  
6 linked to the oxidative stress status of the animals, but to other alterations associated with obesity  
7 and/or high fat intake.

8 Finally, obesity in our study was accompanied by decreased lymphoproliferative response  
9 after stimulation with the mitogen ConA, but not with LPS or under basal conditions. ConA is a T-  
10 cell mitogen, while LPS acts as a B-cell mitogen. These different mitogen actions could explain why  
11 the experimental treatments did not affect proliferation in all conditions, and suggest instead that  
12 obesity impacts specifically on certain types of immune cells (Perez de Heredia *et al.*, 2015). In  
13 addition, we did not observe statistically significant differences in the percentages of CD3<sup>+</sup> cells (T  
14 lymphocytes) and CD19<sup>+</sup> cells (B lymphocytes) among the experimental groups (data not shown),  
15 which would be in agreement with the lack of proliferative response to LPS and also with the similar  
16 levels of lymphoproliferation in the basal state. We can only speculate at this point, but our results  
17 could suggest that obesity and/or the high-fat diet did not affect the basal proliferation capacity of  
18 lymphocytes *per se*, but could impair the ability of the spleen to respond to an offense by increasing  
19 the population of T lymphocytes specifically. More research is needed to understand the pathways  
20 and mechanisms by which obesity can impact lymphocyte maturation in spleen. Supplementation  
21 with 2-OHOA led to restored lymphoproliferation levels in response to ConA, and also resulted in  
22 higher proliferative response to LPS stimulation in our study, while n-3 PUFA supplementation did  
23 not affect significantly either ConA- or LPS-stimulated proliferation. Other authors, by contrary, have  
24 reported lower olive oil-induced lymphoproliferative response to both ConA and LPS, when  
25 comparing to other types of fat (de Pablo *et al.*, 1998), and that n-3 PUFA could reduce *in vitro*  
26 lymphocyte proliferation (Peterson *et al.*, 1998). Further research is required as well in order to

1 confirm the effects of both 2-OHOA and n-3 PUFA in modulating the proliferation of spleen  
2 leukocytes.

3 Our study has certain limitations that should be addressed. Additional measures of anti-  
4 oxidant markers, like total anti-oxidant capacity of the spleen, could contribute to confirm our  
5 hypothesis that oxidative stress in obesity is due to increased generation of oxidants rather than  
6 decreased anti-oxidant defence mechanisms. The analysis of the activity of the enzymes glutathione  
7 peroxidase and glutathione reductase could contribute to explain the results obtained in relation to  
8 levels of GSH and GSSG. Similarly, the analysis of the activity of the superoxide dismutase could  
9 shed light on the results obtained in relation to xanthine oxidase. Unfortunately, the amount of tissue  
10 available limited the number of analyses that could be conducted in the spleens. In this line, it would  
11 have also been interesting to analyse antioxidant markers in plasma, in order to confirm the oxidative  
12 stress state associated with obesity, but again the amount of blood available from each mouse was  
13 very limited and it was necessary to conduct a set of humoral and metabolic determinations.

14 In conclusion, early induction of dietary obesity led to oxidative stress and impaired leukocyte  
15 function in mice, suggesting premature immunosenescence. Supplementation with 2-OHOA, and to  
16 a lesser extent with n-3 PUFA, was able to reduce body weight and to ameliorate the oxidative stress  
17 and alteration of several leukocyte functions in the spleen of obese mice.

18

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4

## 1 **Additional information**

## 2 **Competing interests**

3 The authors want to declare that the work was partially funded by BTSA-Applied Biotechnologies  
4 S.L., who was the manufacturer of the experimental fatty acid supplements. This funding was not  
5 provided directly to the researchers; it occurred in the context of a larger consortium coordinated by  
6 the CENIT (National Strategic Consortia for Technical Research) Program under the supervision of  
7 the Spanish Ministry of Science and Innovation.

8

## 9 **Author contributions**

10 M.d.l.F., L.E.D and A.M. were responsible of the conception and design of the research; A.M. was  
11 the research coordinator; A.G., L.E.D., C.H. and O.H., performed the experiments; A.G., N.R., C.H.,  
12 O.H. and F.P.d.H. participated in the data analysis; F.P.d.H., N.R. and A.G. interpreted the results of  
13 experiments; A.G. and F.P.d.H. prepared the figures; F.P.d.H., A.G., L.E.D. and M.d.l.F. drafted the  
14 manuscript; F.P.d.H., A.G., L.E.D., M.d.l.F., C.H., N.R. and A.M. revised, edited and approved the  
15 final version of the manuscript.

16

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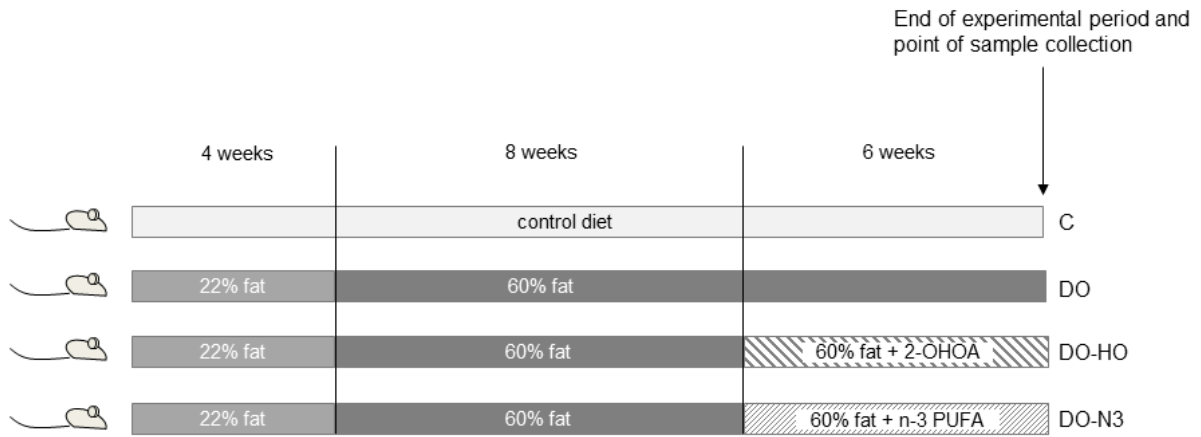
18 Work was supported by CENIT (National Strategic Consortia for Technical Research) Program and  
19 BTSA-Applied Biotechnologies S.L., the Spanish Ministry of Economy and Competitiveness  
20 (MICINN; BFU2011-3036), the RETICEF (RD12/0043/0018) and FIS (PI15/01787) from the Carlos  
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23

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3 National Research Council) for their support and assistance in the statistical analysis of data.

4

1 **FIGURES**

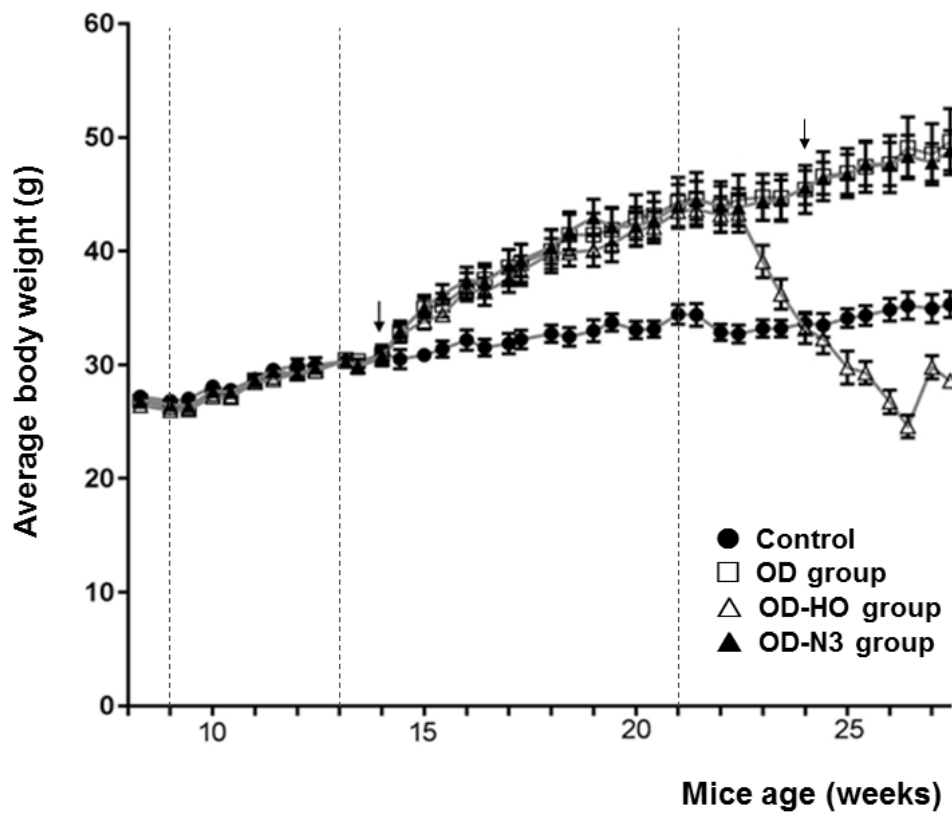
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3 **Figure 1.** Experimental design and timing. 2-OHOA: 2-hydroxyoleic acid; PUFA: polyunsaturated  
 4 fatty acids; C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3:  
 5 obesogenic diet + n-3 PUFA.

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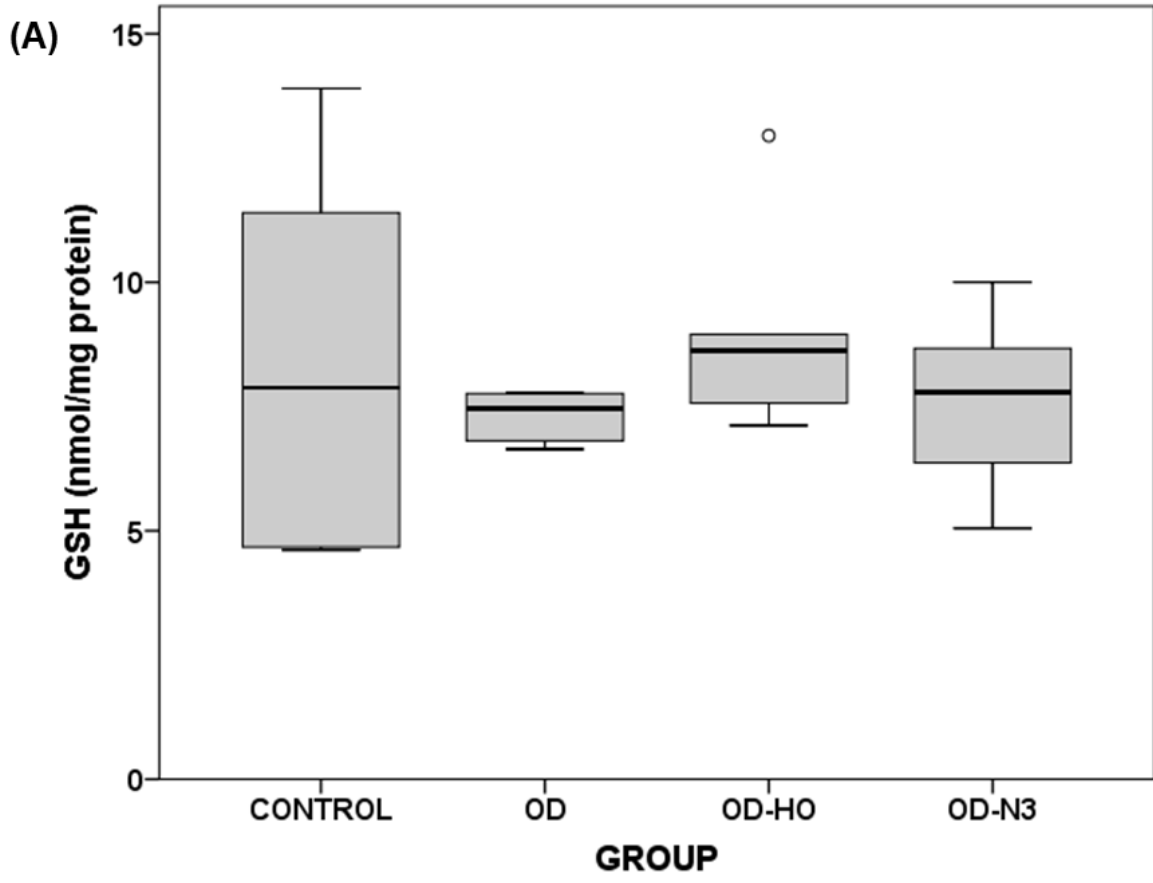


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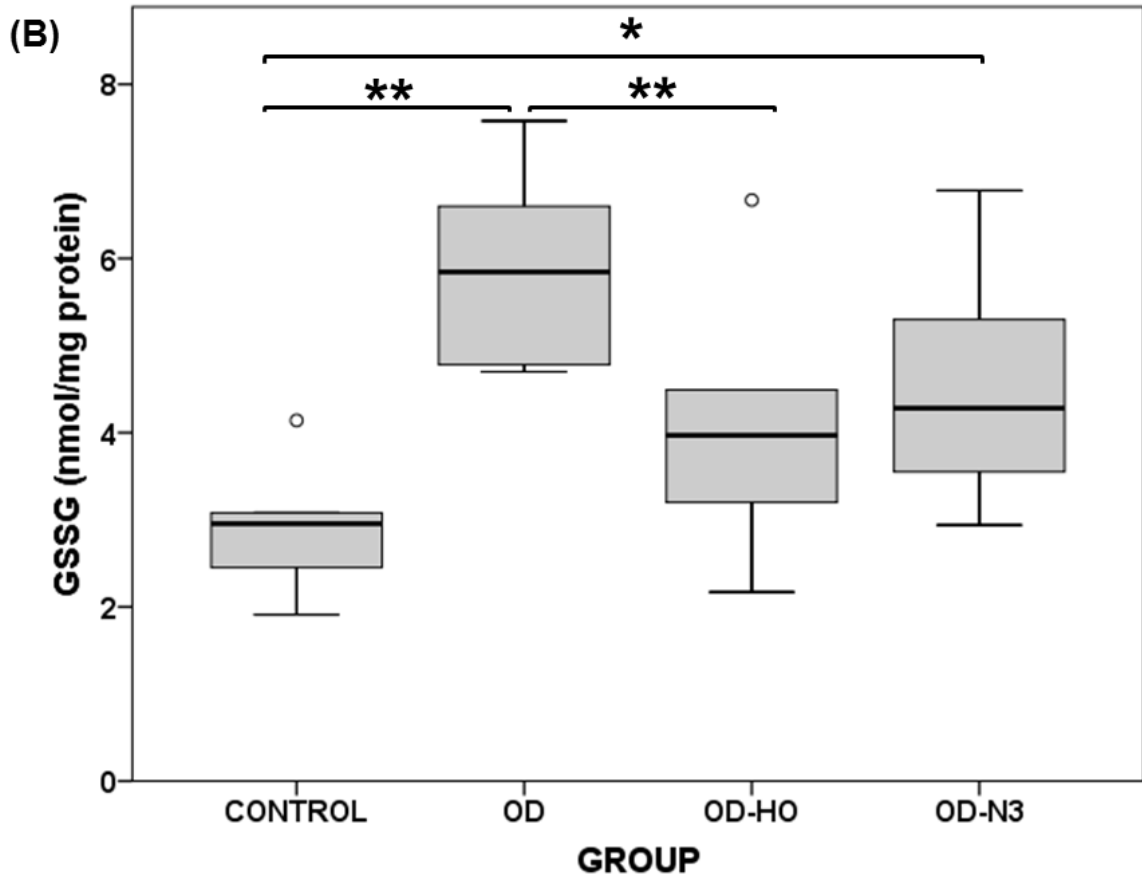
2 **Figure 2.** Evolution of the body weight of mice in the four experimental groups (C: control; OD:  
3 obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=8  
4 animals/group). The dotted lines indicate, left to right, the beginning of the administration of the  
5 moderate-fat diet, the high-fat diet and the supplements. Arrows indicate the weeks when the average  
6 body weights started to differ significantly between controls and obesogenic diet-fed animals  
7 ( $P=0.036$ ), and between the OD-HO group and the other two obesogenic-diet fed groups ( $P=0.011$ ),  
8 as analysed by Kruskal-Wallis.

9

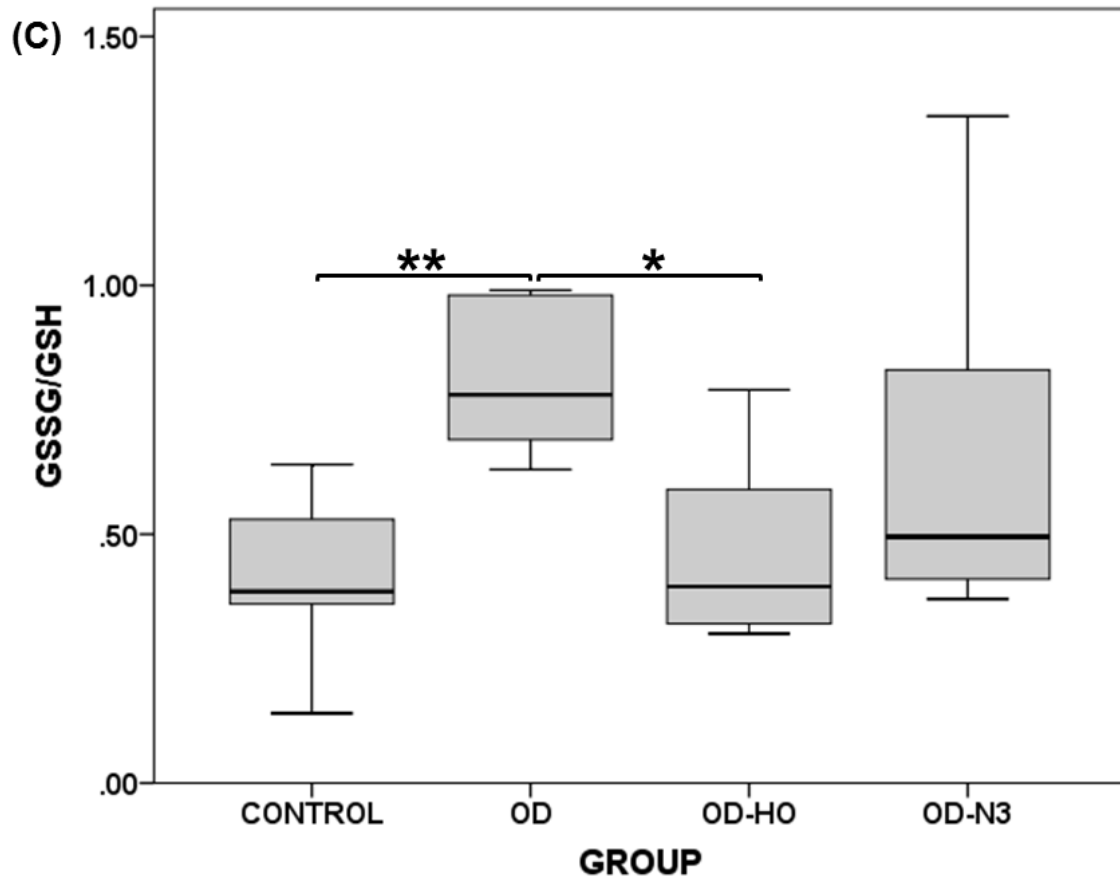
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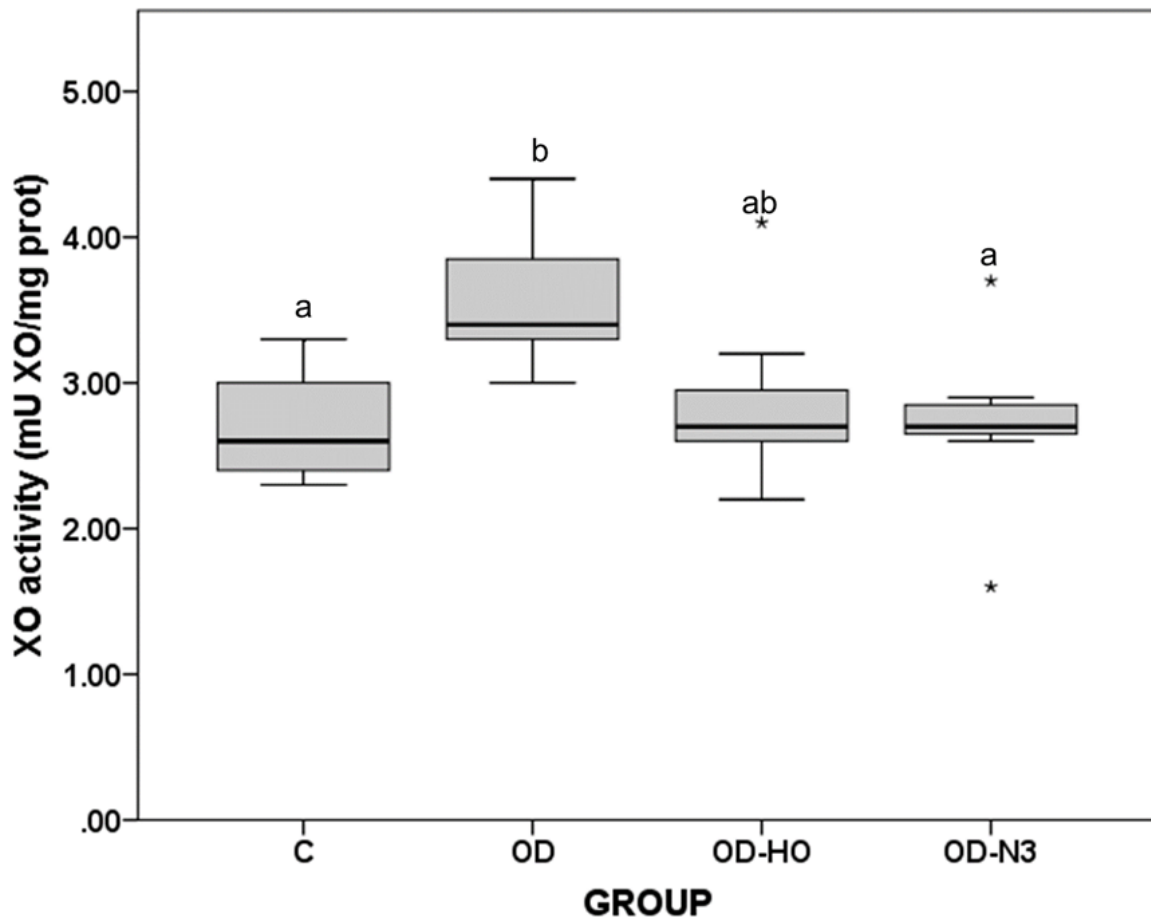
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2 **Figure 3.** Levels of reduced (GSH) (A) and oxidized glutathione (GSSG) (B) and the GSSG/GSH  
 3 ratios in the spleen homogenates of mice from the four experimental groups (C: control; OD:  
 4 obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=7  
 5 animals/group). All assays were performed in duplicates. Differences between treatments analysed  
 6 by Mann-Whitney, \*P<0.05, \*\*P<0.01.

7

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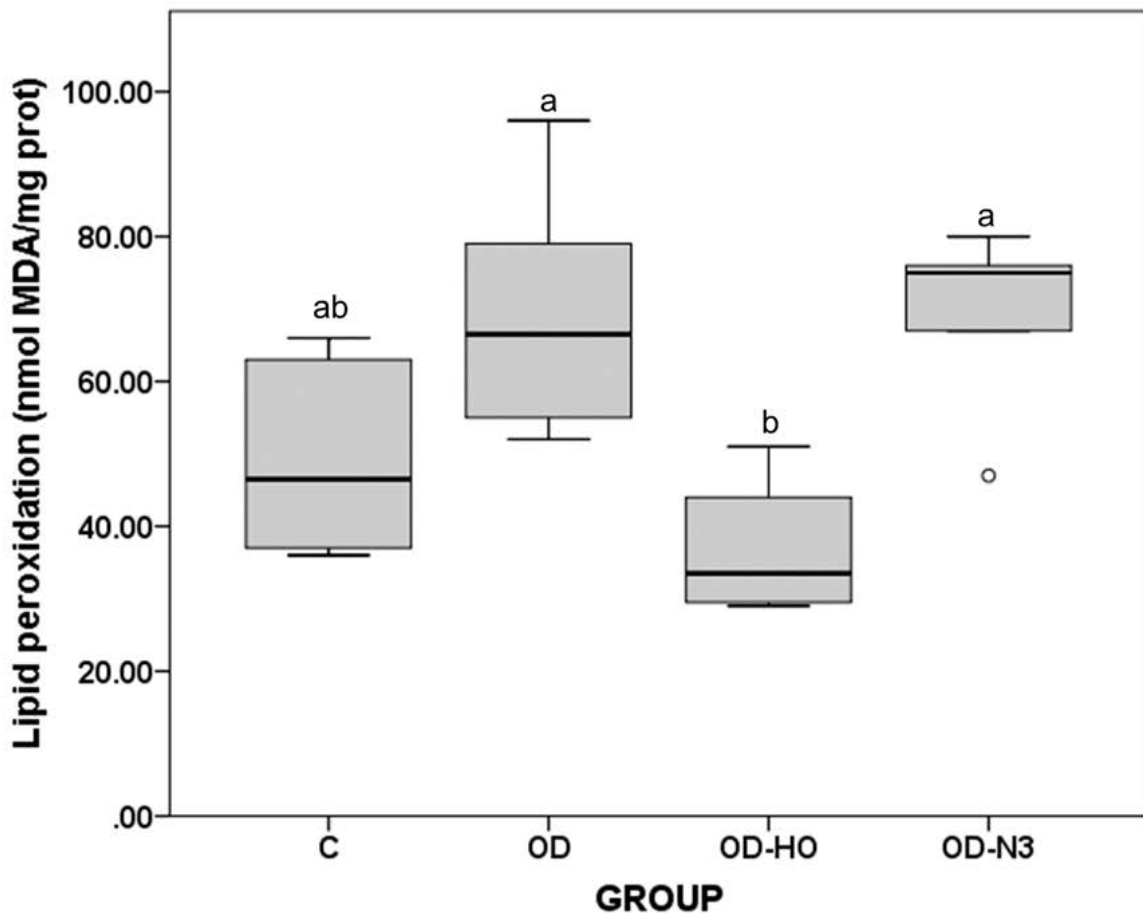




1

2 **Figure 4.** Xanthine Oxidase (XO) activity in the spleen homogenates of mice from the four  
 3 experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-  
 4 N3: obesogenic diet + n-3 PUFA; n=7 animals/group). All assays were performed in duplicates.  
 5 Boxplots represent means, 95% CI, minimum and maximum values. Different superscript letters  
 6 indicate significant differences as analysed by one-way ANOVA with *post-hoc* correction by  
 7 Bonferroni,  $P < 0.05$ .

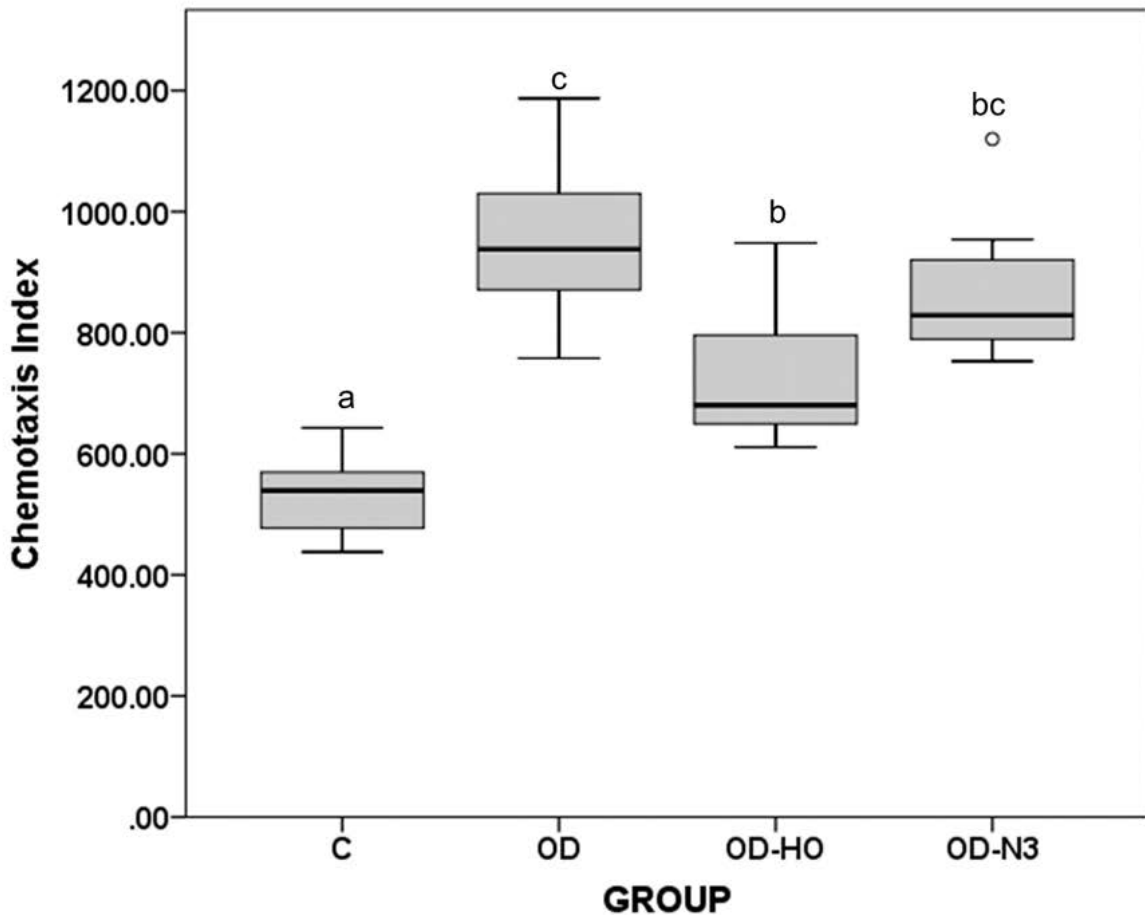
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2 **Figure 5.** Lipid peroxidation, measured as levels of malondialdehyde (MDA), in the spleen  
 3 homogenates of mice from the four experimental groups (C: control [n=6]; OD: obesogenic diet  
 4 [n=6]; OD-HO: obesogenic diet + 2-OHOA [n=4]; OD-N3: obesogenic diet + n-3 PUFA [n=5]). All  
 5 assays were performed in duplicates. Boxplots represent means, 95% CI, minimum and maximum  
 6 values. Different superscript letters indicate significant differences as analysed by one-way ANOVA  
 7 with *post-hoc* correction by Bonferroni,  $P < 0.05$ .

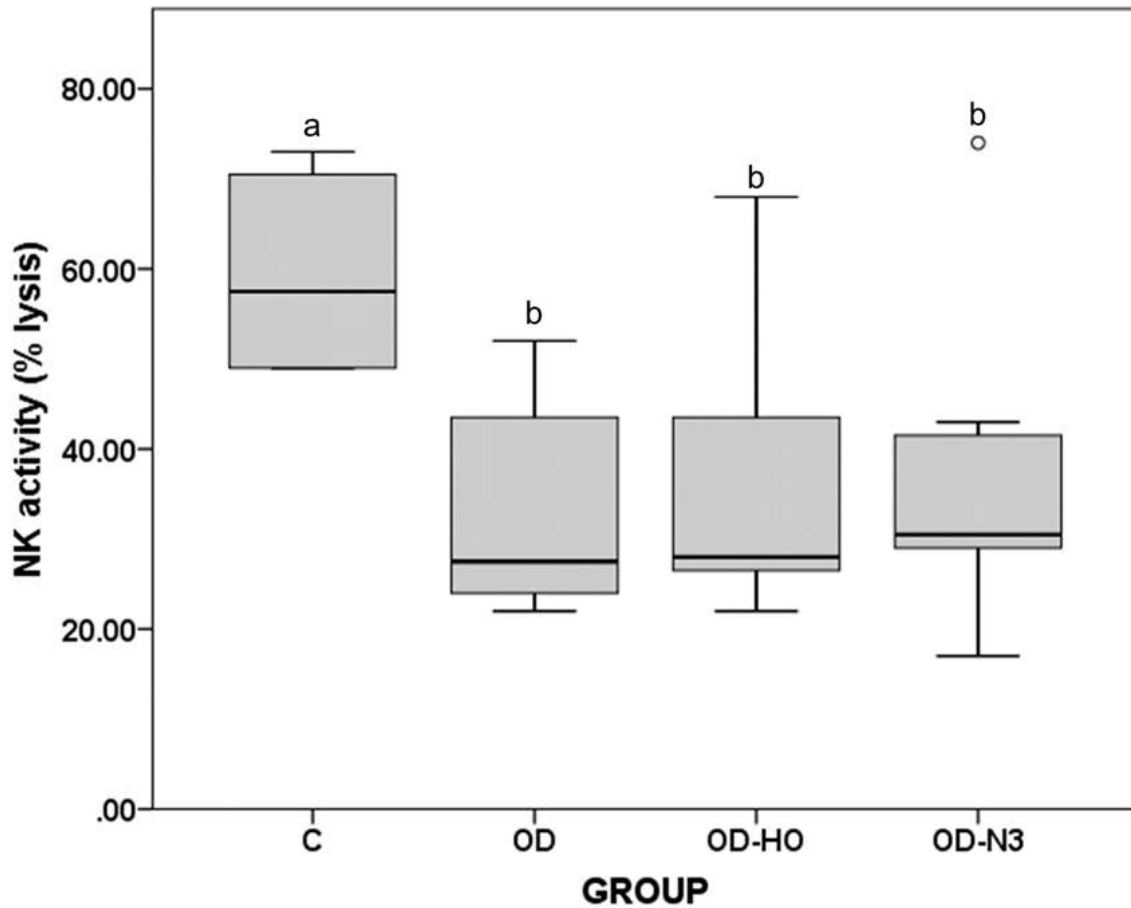
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2 **Figure 6.** Chemotaxis index of spleen leukocytes of mice from the four experimental groups (C:  
 3 control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3  
 4 PUFA; n=8 animals/group). All assays were performed in duplicates. Boxplots represent means, 95%  
 5 CI, minimum and maximum values. Different superscript letters indicate significant differences as  
 6 analysed by one-way ANOVA with *post-hoc* correction by Bonferroni,  $P < 0.05$ .

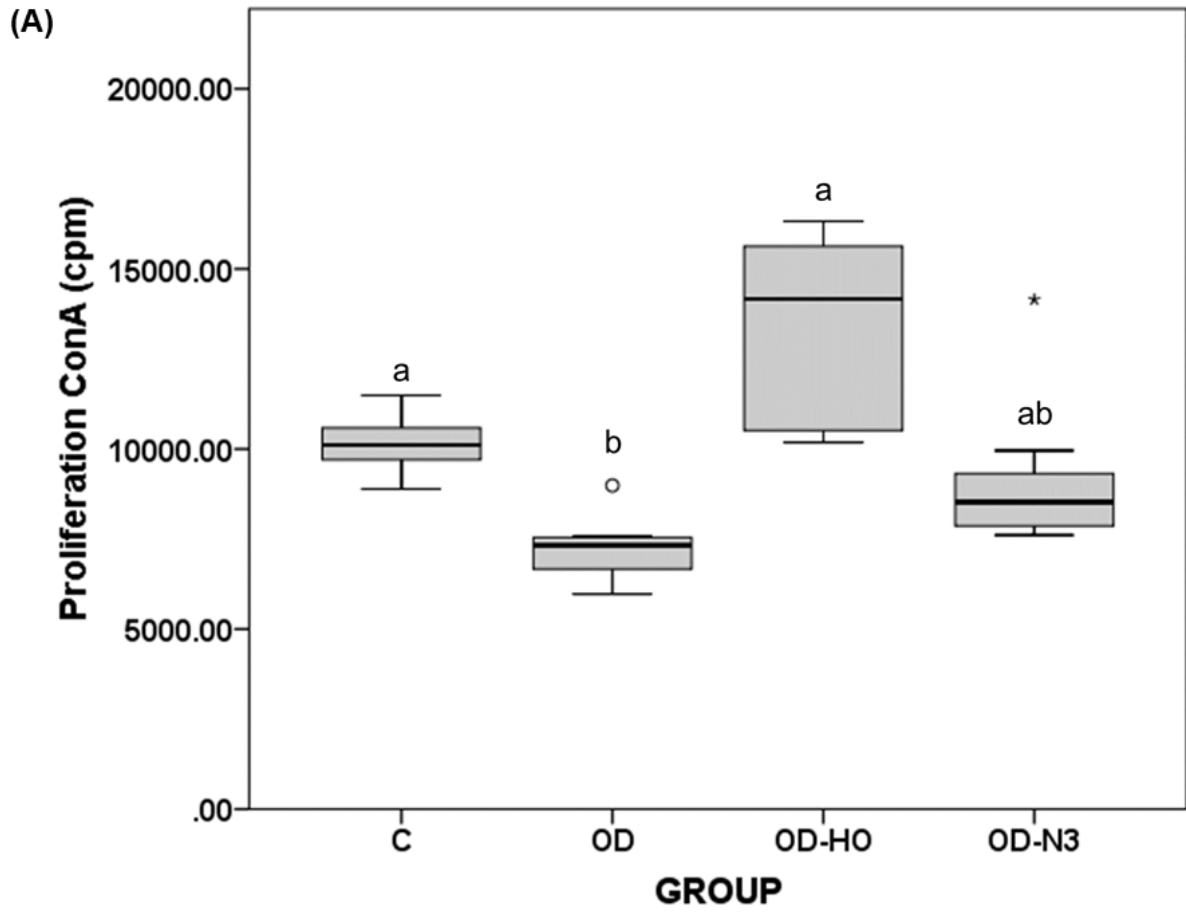
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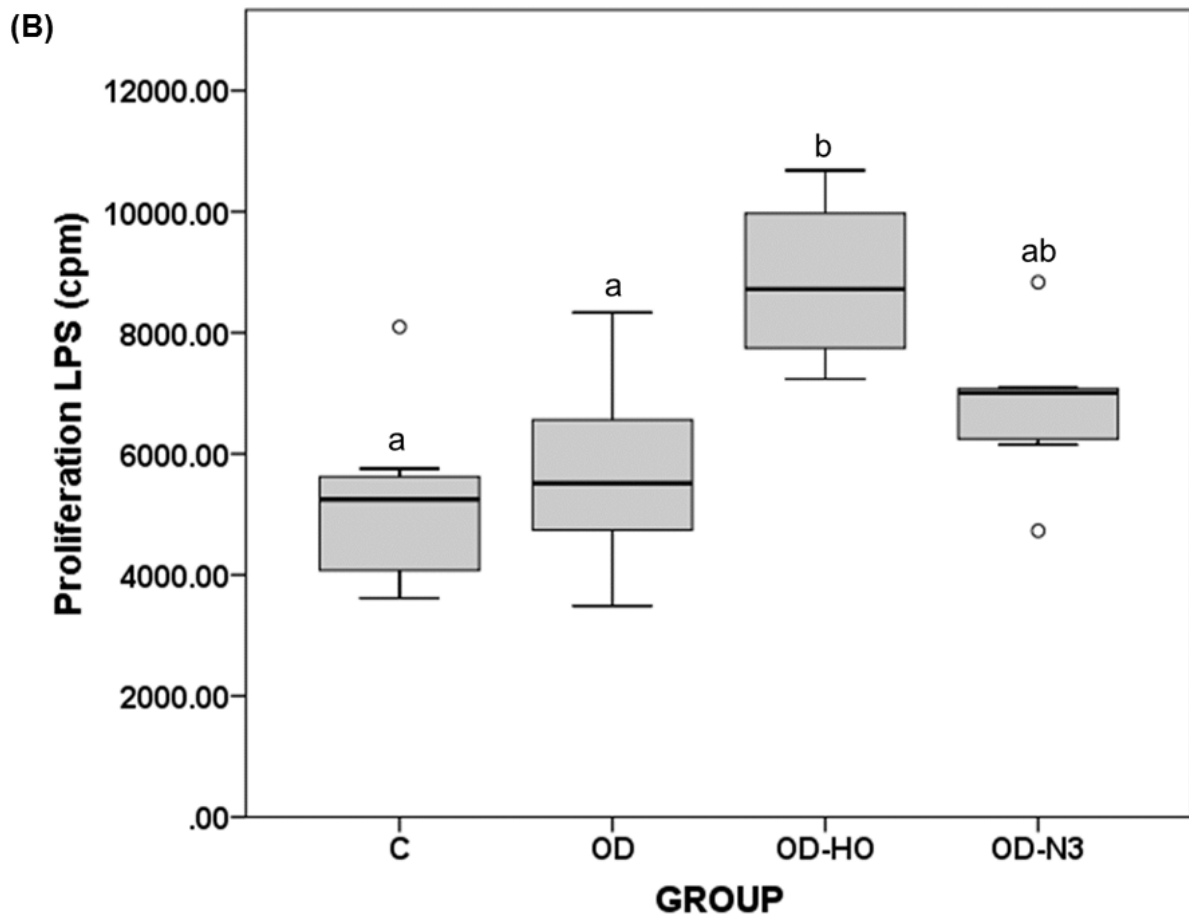
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2 **Figure 7.** Natural killer (NK) activity of spleen leukocytes of mice from the four experimental groups  
3 (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet +  
4 n-3 PUFA; n=8 animals/group). All assays were performed in triplicates. Boxplots represent means,  
5 95% CI, minimum and maximum values. Different superscript letters indicate significant differences  
6 as analysed by one-way ANOVA with *post-hoc* correction by Bonferroni,  $P < 0.05$ .

7



1



1

2 **Figure 8.** Lymphoproliferative response to concanavalin A (ConA) (A) and to lipopolysaccharide  
 3 (LPS) (B) of spleen leukocytes of mice from the four experimental groups (C: control; OD:  
 4 obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=7  
 5 animals/group). All assays were performed in triplicates. Boxplots represent means, 95% CI,  
 6 minimum and maximum values. Different superscript letters indicate significant differences as  
 7 analysed by one-way ANOVA with *post-hoc* correction by Tamhane (ConA) or Bonferroni (LPS),  
 8  $P < 0.05$ .