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

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

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**New findings**

What is the central question of this study?

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

What is the main finding and its importance?

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

## Abstract

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

## Introduction

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

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

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

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

## Methods

### Ethical Approval

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 Spanish Royal Decree 1201/2005 regarding the care and use of laboratory animals for experimental procedures. The authors acknowledge the ethical principles of *Experimental Physiology*, and confirm that the study was conducted in compliance with the animal ethics checklist as detailed by Grundy (2015).

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

### Animal origin and housing conditions

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

Experimental groups and diets

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

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

Collection of spleen and leukocyte suspensions

Once animals were euthanized, their spleens were collected aseptically and freed from fat. One fragment of each spleen was stored at –80 ºC for the study of oxidative stress parameters. Another fragment of spleen was minced with scissors and gently pressed through a mesh screen (Sigma, St Louis, USA) to obtain cell suspensions. The cell suspensions were centrifuged in a gradient of Ficoll-Hypaque (Sigma) with a density of 1.070 g/ml; cells from the interface were collected and suspended in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated foetal calf serum (Gibco, Canada) and 1% gentamicin (100 µg/ml, Gibco). After a wash step, leukocytes were counted in a Neubauer chamber and their number adjusted to $10^6$ cell/ml. Cell viability was routinely measured before each experiment by the trypan-blue exclusion test, and was higher than 95% in all experiments. All incubations were performed at 37 ºC in a humidified atmosphere of 5% $CO_2$.

Analysis of oxidative stress

GSH and GSSG levels

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

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

Xanthine oxidase (XO) activity

XO activity was assayed by fluorescence in homogenates of spleen, using a commercial kit (Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). In the assay, XO catalyses the oxidation of purine bases (xanthine) to uric acid and superoxide anions. The superoxide anion spontaneously degrades in the reaction mixture to $H_2O_2$, which in the presence of horseradish peroxidase (HRP) reacts with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Tissue samples were homogenized in phosphate buffer (50 mM, pH=7.4) containing 1 mM EDTA and normalized to total protein. The homogenate was centrifuged (5,000 *g*) and the supernatant (50 µl) was collected and incubated with 50 µl working solution of Amplex Red reagent (100 µM) containing HRP (0.4 U/ml) and xanthine (200 µM). After 30 min of incubation at 37 ºC, measurement of fluorescence was performed in a microplate reader (Fluostar Optima, BMG Labtech, Biomedal, Spain), using excitation at 530 nm and emission detection at 595 nm. XO supplied in the kit was used as the standard. The results were expressed as international milliunits of enzymatic activity per milligram of protein (mU XO/mg protein). Protein content of the samples had been previously assessed by the bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Madrid, Spain). All assays were performed in duplicates.

Lipid peroxidation

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

Leukocyte functions

Chemotaxis assay

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

NK activity assay

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

where E is the mean absorbance in the presence of effector cells; ES is the mean absorbance of effector cells incubated alone (effector spontaneous release); TS is the mean absorbance in target cells incubated with medium alone (target spontaneous release), and M is the mean of maximum absorbance after incubating target cells with lysis solution (target maximum release).

Lymphoproliferation assay

Following the method previously described (De la Fuente *et al*., 2004), aliquots (200 µl) of leukocytes ($10^6$ cells/ml complete medium) were seeded in 96-well flat-bottomed microtiter plates (Numc, Roskilde, Denmark). Then, 20 µl of concanavaline A (ConA, 1µg/ml, Sigma, St Louis, MO) or 20 µl of lipopolysaccharide (LPS, *Escherichia coli* 055:B5, 1µg/ml, Sigma) were added per well. In order to assess spontaneous proliferation, 20 µl of complete medium were added to some wells instead of the mitogens. After 48 h of incubation at 37 °C in an atmosphere of 5% $CO_2$, 0.5 µCi 3H-thymidine

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

Statistical analysis

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

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

## Results

Effect of the treatments on body weight gain

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

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

Effect of the treatments on the oxidative stress parameters in spleen

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

Effect of treatments on spleen leukocytes functions

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

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

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

## Discussion

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

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

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

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

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

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

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

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

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

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

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

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## Additional information

### Competing interests

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

### Author contributions

M.d.l.F., L.E.D and A.M. were responsible of the conception and design of the research; A.M. was the research coordinator; A.G., L.E.D., C.H. and O.H., performed the experiments; A.G., N.R., C.H., 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 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 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 final version of the manuscript.

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# FIGURES

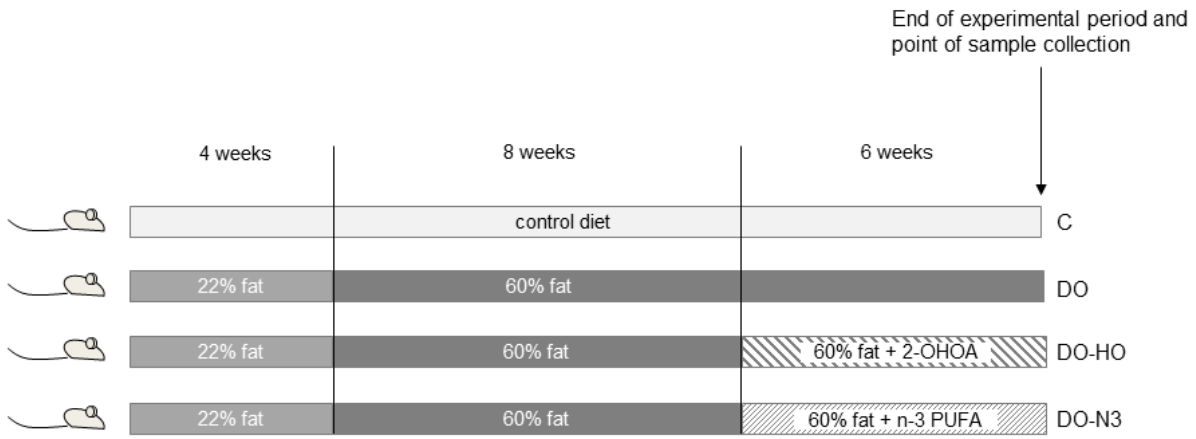



**Figure 1.** Experimental design and timing. 2-OHOA: 2-hydroxyoleic acid; PUFA: polyunsaturated fatty acids; C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA.

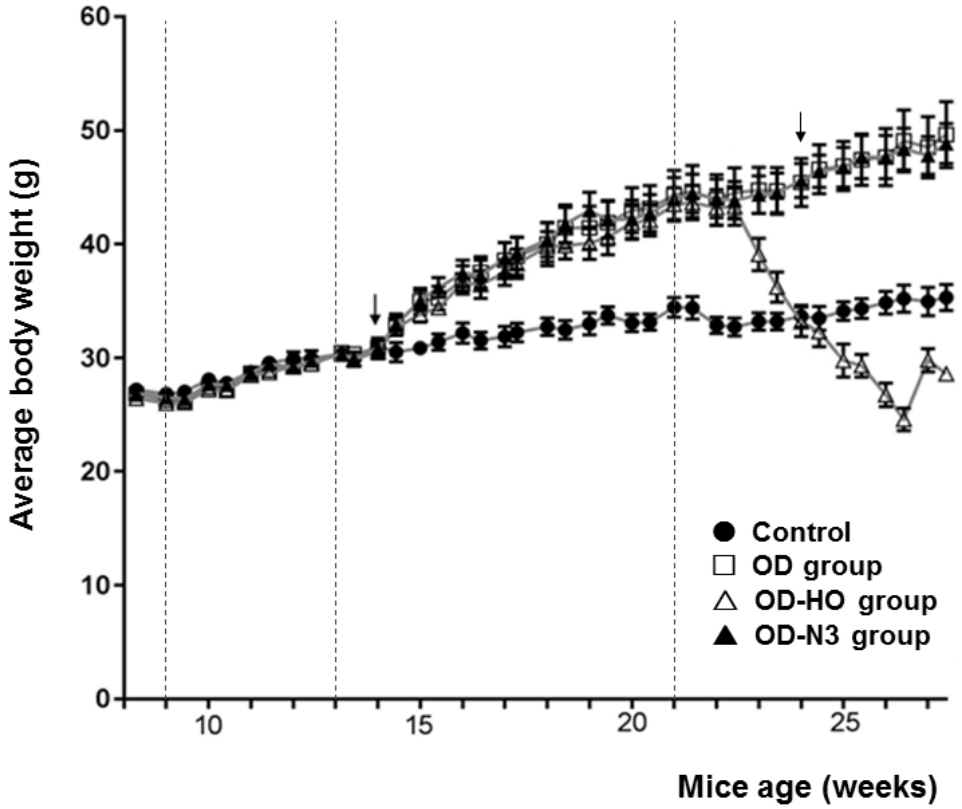
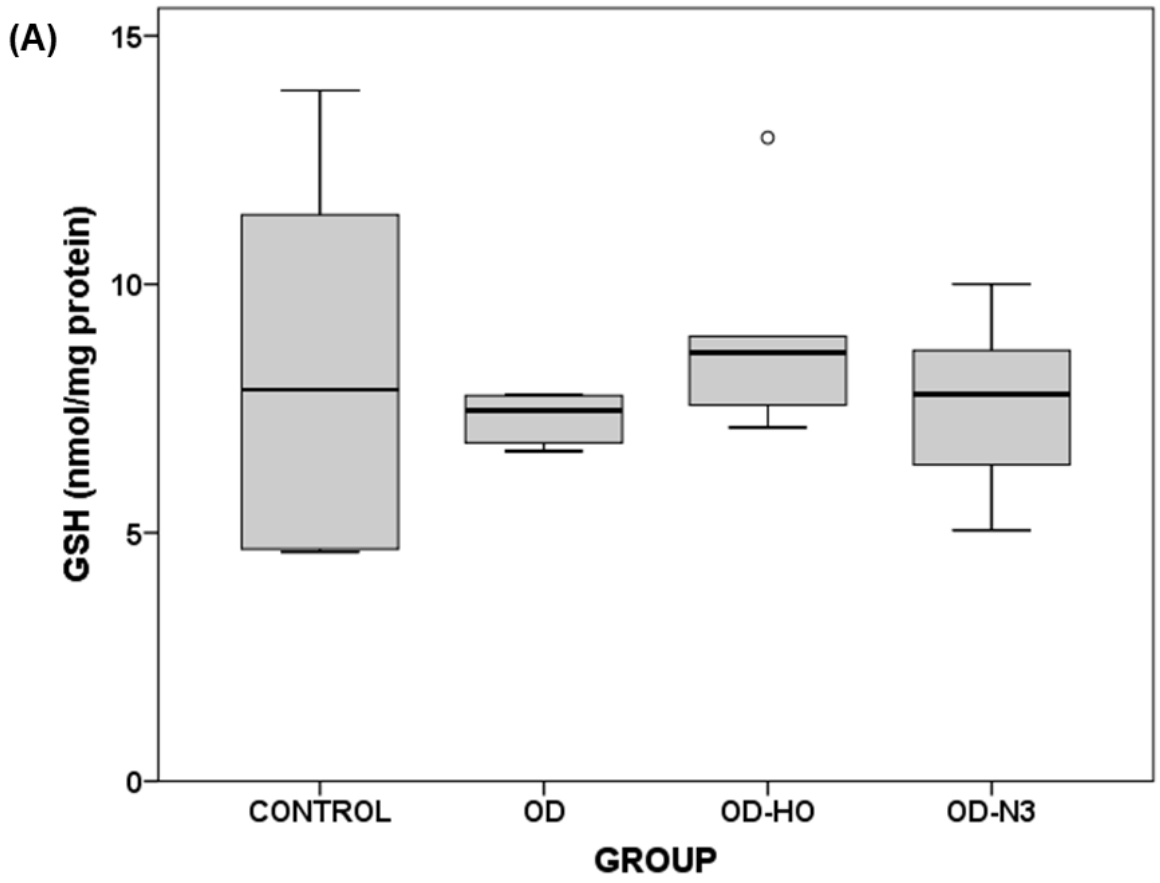



**Figure 2.** Evolution of the body weight of mice in the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=8 animals/group). The dotted lines indicate, left to right, the beginning of the administration of the moderate-fat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

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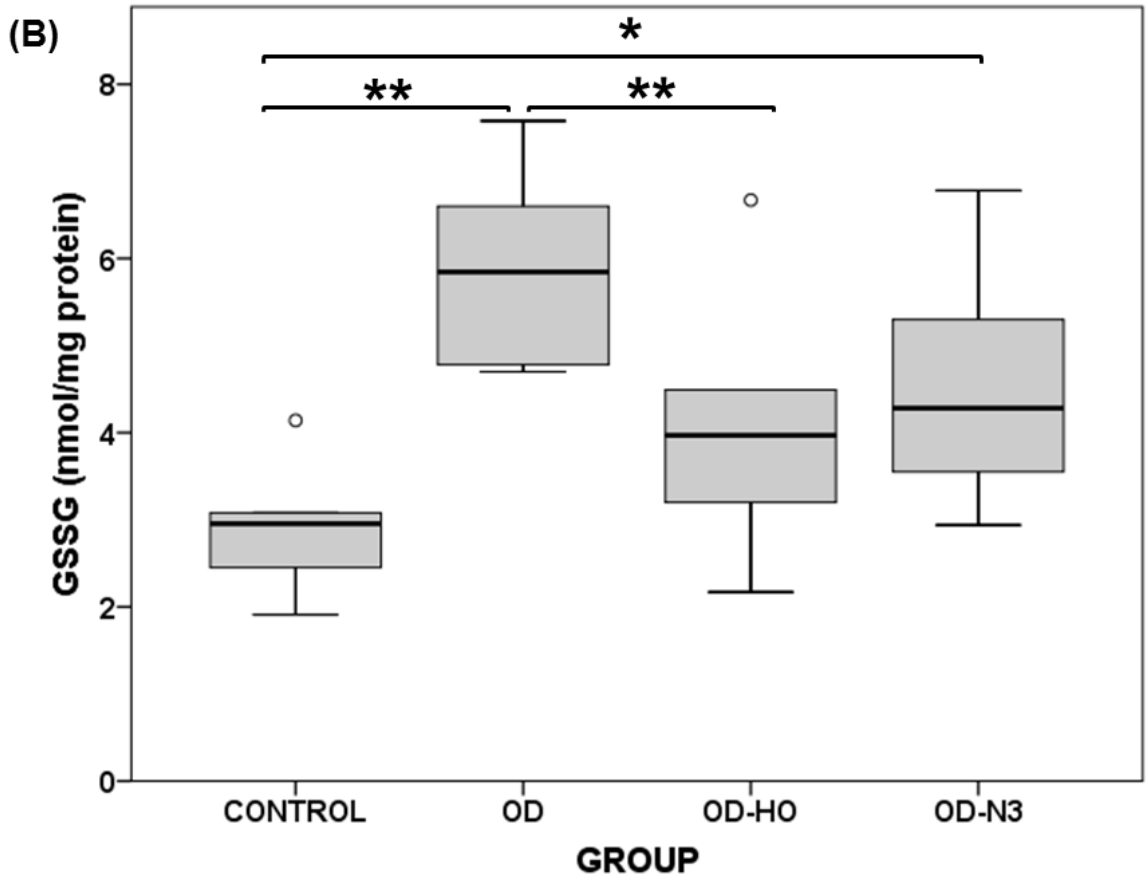


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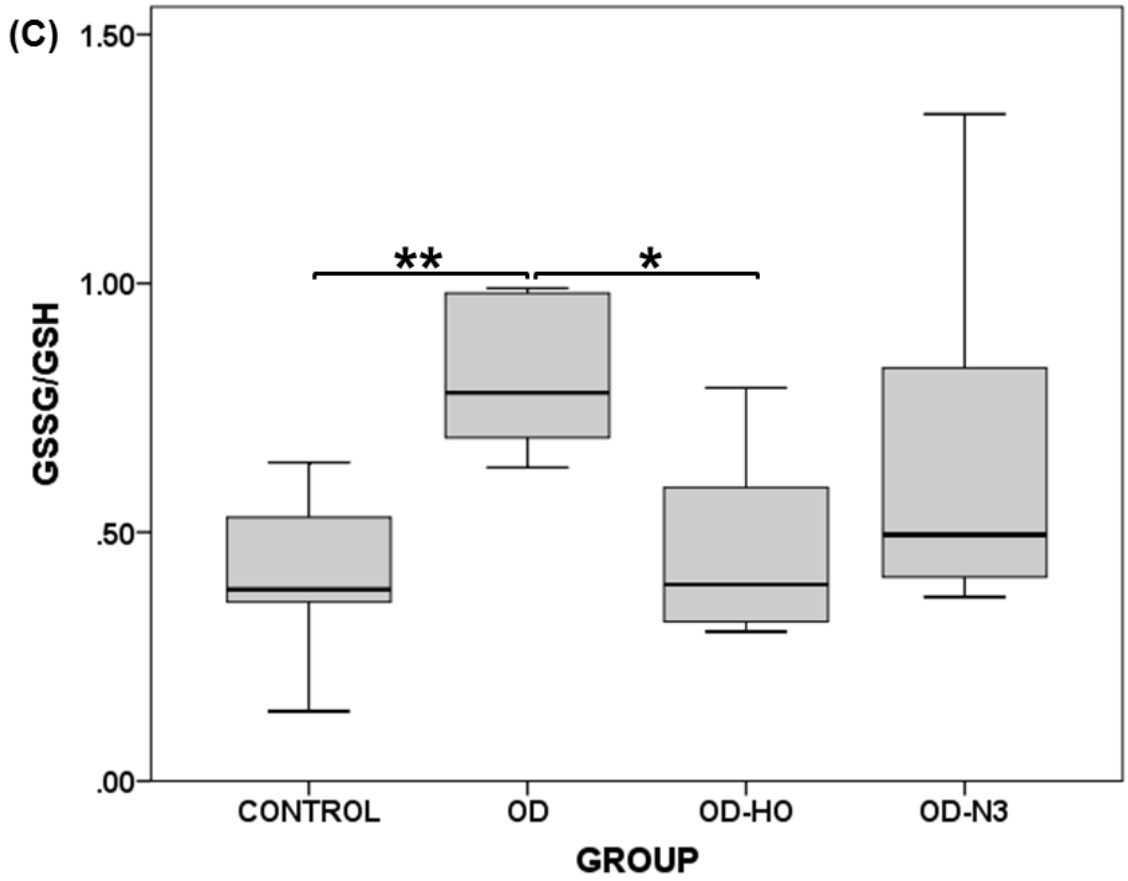



**Figure 3.** Levels of reduced (GSH) (A) and oxidized glutathione (GSSG) (B) and the GSSG/GSH ratios in the spleen homogenates of mice from the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=7 animals/group). All assays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

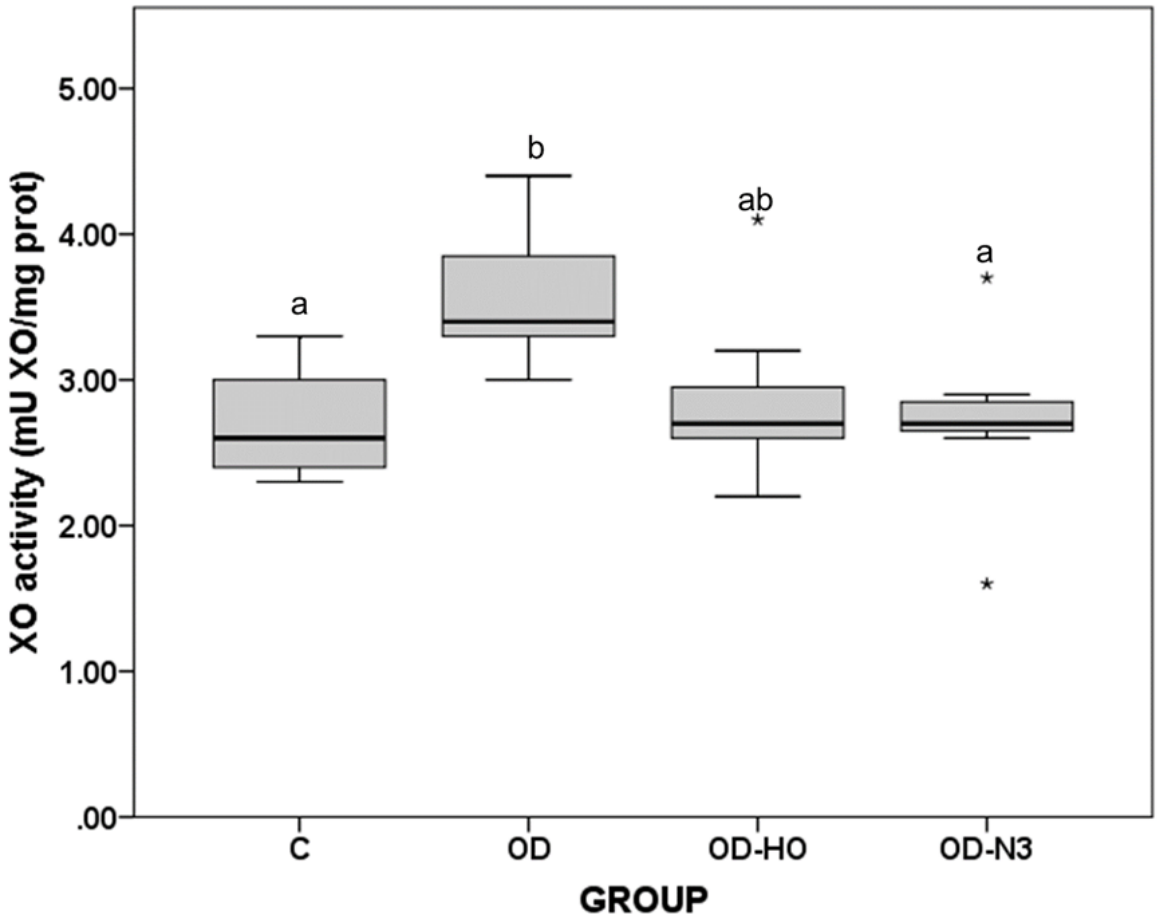



**Figure 4.** Xanthine Oxidase (XO) activity in the spleen homogenates of mice from the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=7 animals/group). All assays were performed in duplicates. Boxplots represent means,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

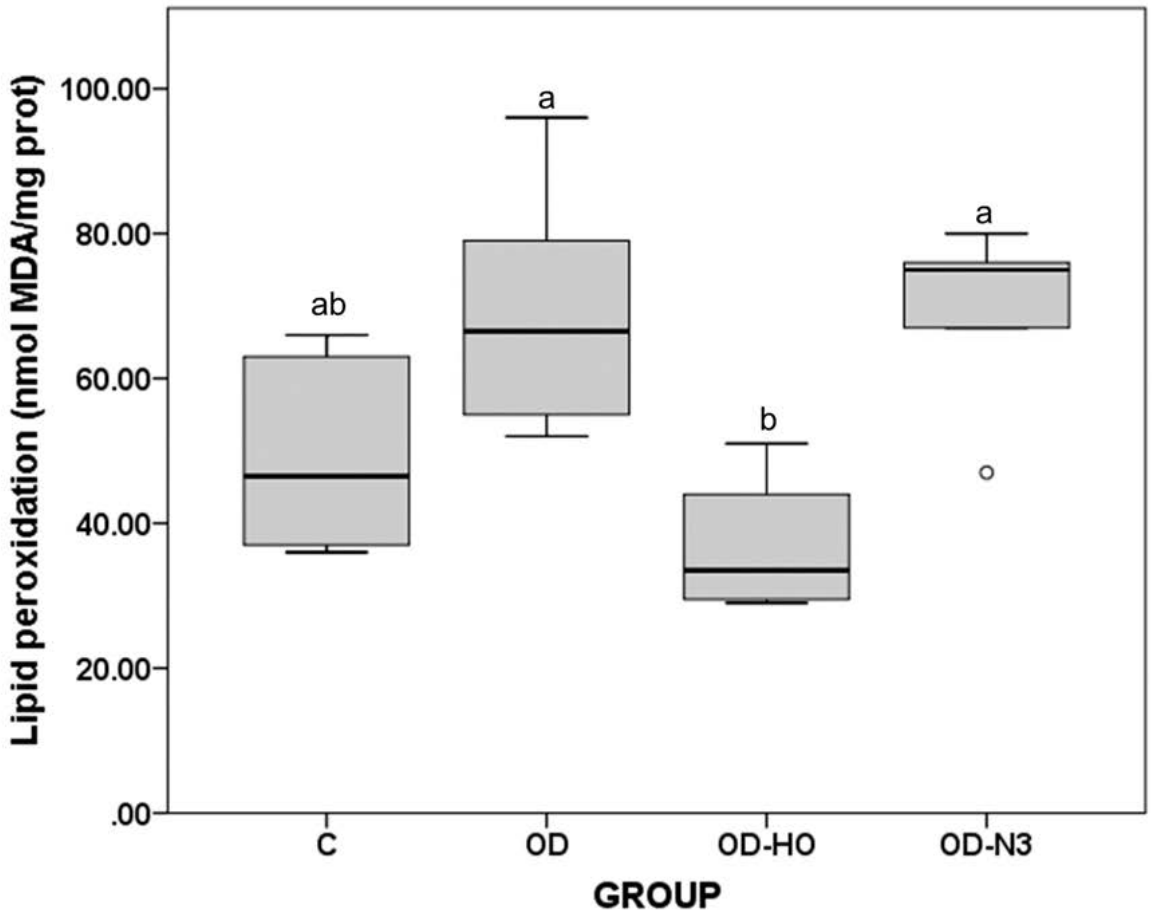



**Figure 5.** Lipid peroxidation, measured as levels of malondialdehyde (MDA), in the spleen homogenates of mice from the four experimental groups (C: control [n=6]; OD: obesogenic diet [n=6]; OD-HO: obesogenic diet + 2-OHOA [n=4]; OD-N3: obesogenic diet + n-3 PUFA [n=5]). All assays were performed in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

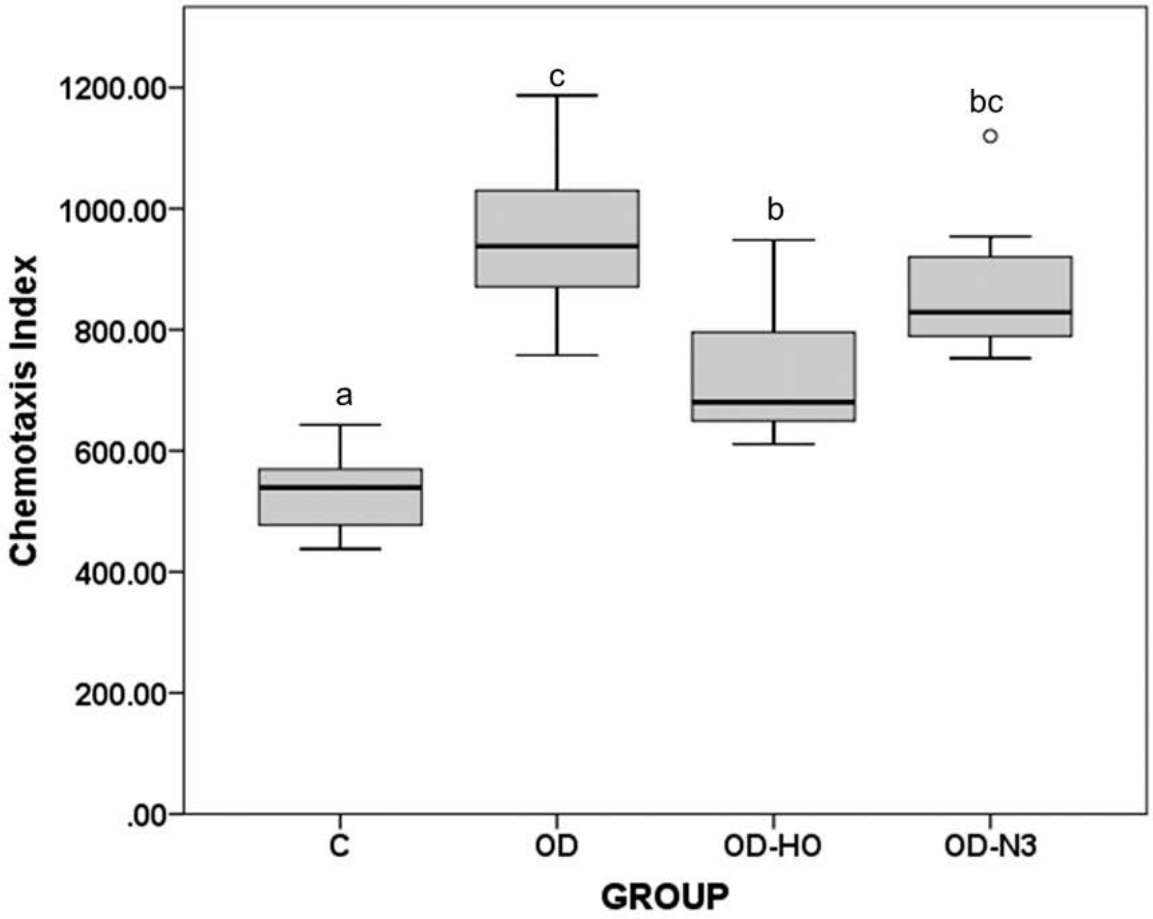



**Figure 6.** Chemotaxis index of spleen leukocytes of mice from the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=8 animals/group). All assays were performed in duplicates. Boxplots represent means, 95% CI, minimum and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

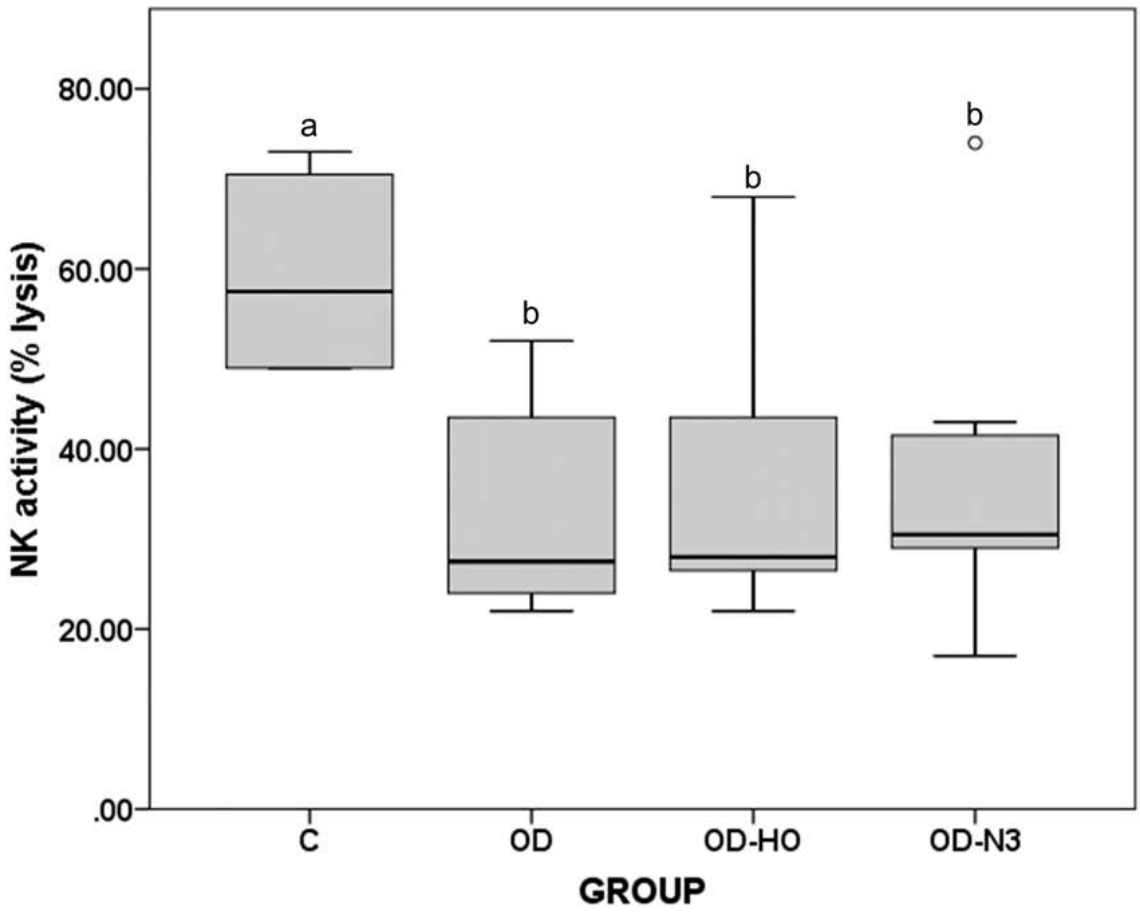
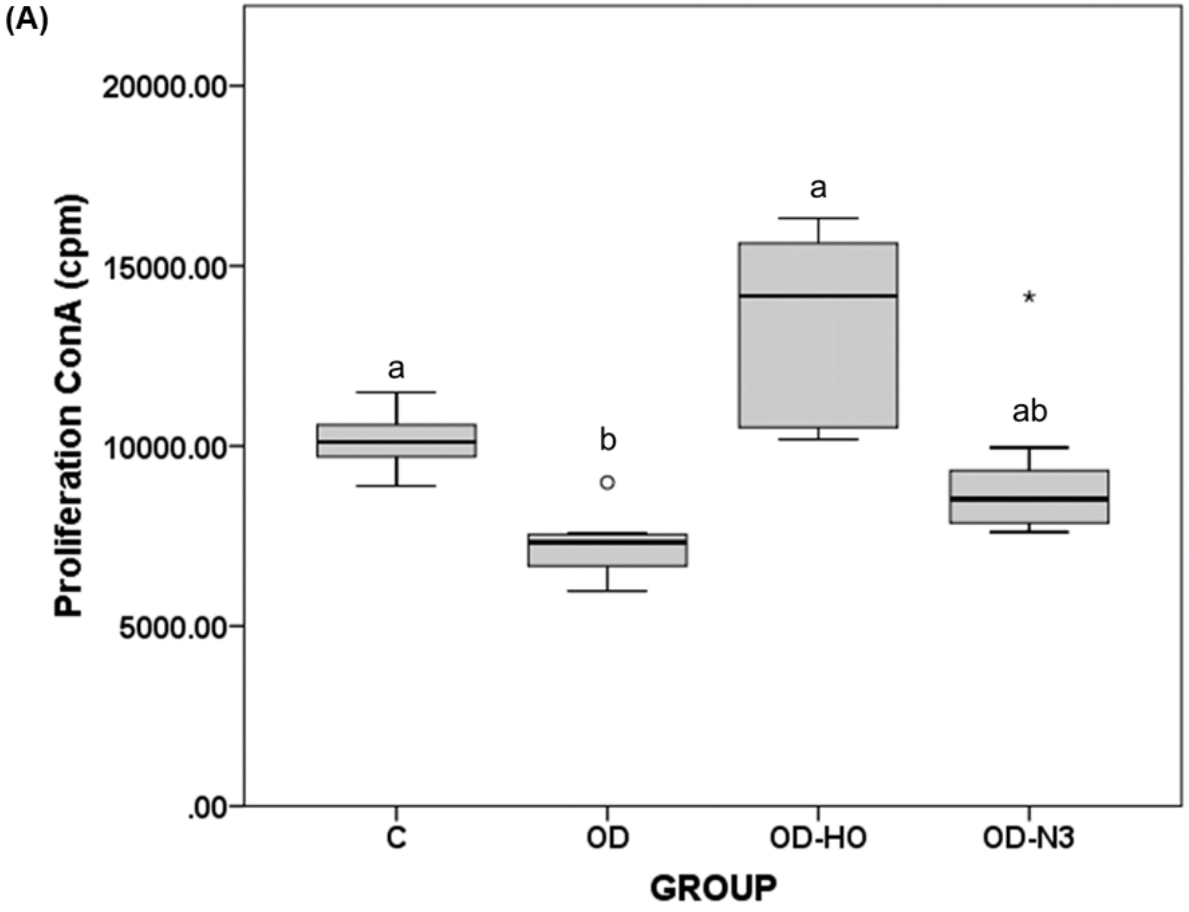



**Figure 7.** Natural killer (NK) activity of spleen leukocytes of mice from the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=8 animals/group). All assays were performed in triplicates. Boxplots represent means, 95% CI,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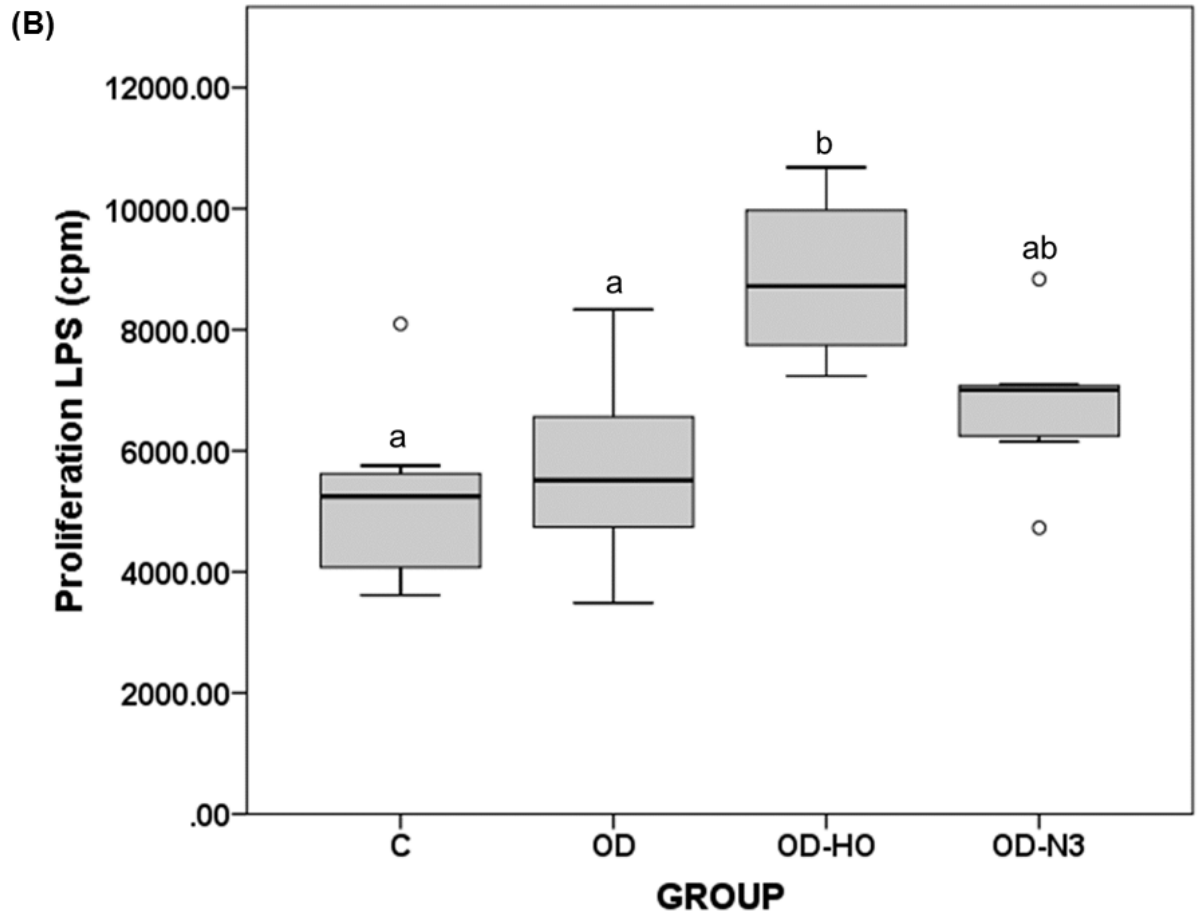


**Figure 8.** Lymphoproliferative response to concanavalin A (ConA) (A) and to lipopolysaccharide (LPS) (B) of spleen leukocytes of mice from the four experimental groups (C: control; OD: obesogenic diet; OD-HO: obesogenic diet + 2-OHOA; OD-N3: obesogenic diet + n-3 PUFA; n=7 animals/group). All assays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