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Effects of Diets High in Corn Oil or in Extra Virgin Olive Oil on Oxidative Stress in an Experimental Model of Breast Cancer

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Abstract:

Experimental evidence highlights the importance of dietetic factors on breast cancer. In this work we aimed to analyze the effects two oils, corn oil (rich in n-6 polyunsaturated fatty acids -PUFA-) and extra virgin olive oil (EVOO), on oxidative stress in an animal model of breast carcinogenesis. Female rats were fed a low-fat control, a high-corn oil, or a high-EVOO diet from weaning or after induction with 7,12-dimethylbenz[a]anthracene at 53 days. Animals were euthanized at 36, 51, 100 and 246 days of age. We analyzed antioxidant enzymes (mRNA and activity of superoxide dismutase, glutathione peroxidase and catalase), non-enzymatic capacity (oxidized and reduced glutathione) and DNA damage (8-oxo-dG) in tumors and mammary gland at different ages. We also analyzed lipid peroxidation (isoprostanes in serum and lipofuscin in liver). Results indicated a decrease in the enzymatic antioxidant capacity and increased oxidative stress in mammary gland of healthy young animals after a short period of high-fat diets intake, followed by an adaptation to chronic dietary intervention. After induction both diets, especially the one high in n-6 PUFA, increased the oxidized glutathione. In tumors no clear effects of the high-fat diets were observed, although in the long-term lipofuscin and 8-oxo-dG suggested greater oxidative damage by effect of the n-6 PUFA-rich diet. Considering the differential effects of these diets on mammary carcinogenesis that we have previously reported, this study suggests that these high-fat diets could have an effect on oxidative stress that would lead to different signaling pathways.

Keywords: dietary lipids, high fat diet, breast cancer, oxidative stress, extra virgin olive oil.

Declarations:

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Introduction

Breast cancer is the most common cancer in women worldwide [1]. Besides genetic/epigenetic and endocrine factors, a wealth of knowledge strongly support an effect of environmental factors in the etiology of this disease, with dietary fat being one of the most important [2,3]. Actually, there is evidence that increasing adherence to the Mediterranean pattern is associated to a reduction of overall and cancer mortality [4, 5]. This dietary pattern is characterized by the high consumption of extra virgin olive oil (EVOO), with attributed health benefits due to its high content of monounsaturated fatty acids (MUFA) and phenolic compounds [3, 6]. Moreover, experimental studies have demonstrated the influence of the type and quantity of dietary lipids on mammary carcinogenesis, being the diets rich in n-6 polyunsaturated fatty acids (PUFA) and animal saturated fat clear promoters, whereas n-3 PUFA have an inhibitory influence [3]. Several molecular mechanisms have been proposed to understand the influence of diet on malignant diseases, including an influence on oxidative stress, cell signaling transduction pathways or gene transcription [7]. Elevated levels of reactive oxygen species (ROS) and alteration in redox balance cause oxidative stress and lipid peroxidation of PUFA, plying a major role in the initiation and progression of carcinogenesis, specifically the nutrition mediated carcinogenesis [8, 9]. There is a set of molecules which are responsible for transforming free radicals into stable and less damaging molecules including the scavenger enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and non-enzymatic antioxidants, such as glutathione (GSH) [10]. Some non-enzymatic antioxidants such as vitamins C and E, carotenoids and phenolic compounds are found in vegetal foods and have been identified as good protectors against free radicals-associated carcinogenesis [11]. Actually,

several of the EVOO minor compounds (squalene, or polyphenols such as ligstroside, oleuropein, hydroxytyrosol and tyrosol) have shown oxidative stress cell protection [12].

We have previously reported the differential effects that diets high in n-6 PUFA or in EVOO have on an in vivo experimental model of breast cancer in rat. The percentage of oil used in these high fat diets was 20% (weight by weight -w/w-), corresponding to 39.5% calories in the form of fat [13], a percentage similar to that found in the western dietary patterns and that is increasing in some populations [14]. Thus, the diet rich in n-6 PUFA has a strong stimulating effect on mammary tumors, while animals fed the diet rich in EVOO, despite having a high intake of fat, have similar carcinogenesis yield in comparison to the low fat controls [13]. Hence, elucidating the effects of dietary lipids could be of great interest in the prevention of cancer, also considering that dietary patterns in western countries are changing to higher fat intake. Thus, we aim to get insight into the molecular mechanisms of the influence of lipids on mammary carcinogenesis, investigating the effects of these high n-6 PUFA and high EVOO diets on the oxidative stress. To this end, we analyzed several parameters (enzymatic and non-enzymatic antioxidant status, lipid peroxidation and DNA damage) in tumors and in mammary gland at different ages. All these studies highlight the importance of dietary habits from childhood on health and the risk of disease.

Materials and Methods

Diets

Three semisynthetic diets were designed: a low-fat diet (LF, 3% corn oil weight by weight -w/w-), a high-corn oil diet (HCO, 20% corn oil) and a high-extra virgin olive oil diet (HEVOO, 3% corn oil+17% extra virgin olive oil). The content of carbohydrates in the form of

dextrose was 67.9% w/w (LF diet) or 45.9% (high-fat diets). The protein content, in the form of casein, was 18% w/w (LF) or 23% (high-fat diets). All three diets also contained 5% w/w cellulose, 5.9% w/w salt mixture and 0.24% w/w vitamin mixture. In order to maintain the normal lipidic metabolism, they were supplemented with choline (1800 mg/kg diet), methionine (0.51% w/w in LF and 0.66% in the high-fat diets), and folic acid (5 mg/kg diet). The definition, preparation and suitability of the experimental diets have been described previously [15-17]. Diets were prepared weekly and stored under nitrogen in the dark at 4°C.

Animals and experimental design

All animals received humane care under an institutionally approved experimental animal protocol, following the legislation applicable in Spain. Female Sprague–Dawley Crl:SD rats [Charles River Lab; stain Crl: OFA(SD), n = 167] were purchased from Charles River Lab (L'Arbresle Cedex, France) on post-natal day 23 and fed with the experimental diets and water ad libitum. Animals were randomly distributed in five groups according to the type and timing of dietary intervention (Figure 1): A control group fed the low-fat diet from weaning (LF group), two groups fed the high-corn oil diet from weaning (HCO group) or after carcinogen induction (LF-HCO group), and two groups fed the a high-extra virgin olive oil diet from weaning (HEVOO group) or after carcinogen induction (LF-HEVOO group). Mammary tumors were induced at 53 days of age by oral gavage with a single dose of 5 mg of 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil. Animals were euthanized by decapitation at 24 days (post-weaning, one group, n=6), 36 and 51 days of age (non-induced animals, three different experimental conditions, n=6 each), 100 days (five groups, n=5 each) and the end of the assay (236-256 days, median 246 days, five

groups, n=20 each). Blood was collected and plasma was obtained and flash frozen (stored at -80°C). Abdominal mammary glands, liver and mammary tumors were obtained, flash frozen and stored at -80°C. A portion of mammary gland was preserved in PBS at 4°C for the Comet assay. Moreover, a portion of mammary gland, liver and tumors were fixed in 4% buffered formalin for histological analyses. Tumor histopathology classification was performed as previously described [18], and only confirmed mammary adenocarcinomas were included in this study.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (QiaGen, Hilden, Germany) according to the manufacturer. RNA quality was analyzed by Agilent Bioanalyzer 2100 and by ethidium-bromide-stained agarose gel electrophoresis. Two micrograms of total RNA were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Twenty-five nanograms of cDNA was amplified by real-time PCR with TaqMan methodology in the iCycler iQ Real- Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Specific Gene TaqMan Assays for SOD1/CuZnSOD, SOD2/MnSOD, Gpx1/GSHPx, Gpx4/PHGpx, Cat and Hprt as the housekeeping control gene were obtained from Applied Biosystems. PCRs were performed at 10 min at 95°C followed by 15 s at 95°C and 60 s at 60°C for 40 cycles. Cycle thresholds (Ct) for each sample were obtained and $2^{-\Delta Ct}$ calculated.

Protein Extraction and Enzymatic activity

Two different protein extracts were obtained to perform the enzymatic activity assays. In the first protocol mammary gland or tumor tissues were homogenized in a Buffer of Enzymatic Activity (50 mM Tris-HCl pH 7.2, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl 2). Cell lysates were centrifuged at 1500 g to remove the nuclei, non-lysed cells and tissue debris. This protein extract, containing enzymes from cytosol and mitochondria, were used for total SOD activity determination. Protein extracts were stored at -80 °C. A second extraction protocol was performed from mammary gland and tumor tissues manually homogenized in Buffer of Enzymatic Activity supplemented with 1 mM DL-Dithiothreitol (DTT). Then, cell lysates were centrifuged at 10000 g to obtain a cytosolic extract that was stored at -80ªC. Theses extracts were used to determine the activity of GPx and catalase.

Enzymatic activities assays were performed in such protein extracts with the kits "Superoxide Dismutase Assay", “Glutathione Peroxidase Assay” and “Catalase Assay” (Cayman, Ann Arbor, MI, USA), following the manufacturer procedures.

Glutathione analysis

To determine the amount of GSH, 25 mg of gland tissue and mammary adenocarcinoma were homogenized in a 50 mM potassium phosphate and centrifuged at 10000g. Supernatant was recovered, 20 µl of 10 mM metacresol was first added followed by 300 µl of 1% fluoronitrobenzene (FDNB), incubated at RT for 4 hours into darkness, and quantified by High performance liquid chromatography (HPLC) analysis.

Mammary gland and tumor GSSG levels were determined from 25 mg of tissue homogenized in 6% perchloric acid buffer. Cell lysate was centrifuged at 10000 g. Supernatant was recovered and 50 µl of 10 mM metacresol was added, followed by 300 µl of 1% FDNB, and incubated at

RT for 4 hours. Samples were dried overnight at 50°C, eluted in 80% methanol and centrifuged at 10000 g. The supernatant was used to quantification GSSG levels by HPLC.

F_2-IsoP plasma levels

F_2-isoprostane levels were determined by immunoassay using the “8-isoprostane EIA” kit (Cayman) in plasma (frozen samples from blood obtained at different ages). Briefly, the sample, the tracer and the anti-8-isoprostane serum were incubated for 18 hours and the resulting complexes were linked to a monoclonal anti-rabbit IgG antibody ("Rabbit IgG mouse monoclonal antibody"). Then the Ellman's reagent containing the acetylcholinesterase substrate was added, and samples were read spectrophotometrically at 412 nm.

Lipofuscin histological analysis

This analysis was performed in 4μm-thickness sections of formaldehyde-fixed paraffin-embedded liver samples. To determine the presence of lipofuscin, Schmorl staining was used, which is specific to certain pigments with reducing characteristics such as melatonin and is typically found in von Kupffer cells. After staining of the samples following standard protocols, evaluation was made blindly based on the abundance of pigments stained inside the von Kupffer cells, on a 0-5 scale.

8-oxo-dG analysis

Genomic DNA from mammary glands and tumors were extracted with the "Speedtools Tissue DNA Extraction" kit (Biotools, Spain). Nuclear DNA was isolated from genomic DNA by precipitation with chloroform isoamyl alcohol (24: 1). Two hundred μg of DNA were first

digested with 100 units of DNase I for 1 hour at + 37 ° C, and then with 5 units of P1 nuclease for 1 hour at + 37 ° C. The enzymes were precipitated with 5 volumes of acetone and separated by centrifugation. To test 8-oxo-deoxyguanosine (8-oxo-dG) tissue levels, hydrolyzed DNA was dissolved in ultrapure water for HPLC and filtered through a 0.2 μm pore before applying the samples on a "Spherisorb ODS2 HPLC" column (Waters S.A. Chromatography) (4.6 x 250 mm); 5 μm particle size). The amount of 8-oxo-dG and deoxyguanosine (dG) was measured by electrochemistry and by UV absorbance at 254 nm, respectively.

Comet assay

A microscopic slide was layered with 1% agarose. Cell suspensions were obtained from blood and mammary gland samples. For mammary glands, tissue was first disintegrated and digested with 0.5% trypsin. The obtained cell suspensions were mixed with 1% low melting point agarose in different proportions according to the tissue. A few drops of the mixture were applied on the slide coated with the first layer of agarose. Cells were lysed with an alkaline solution, and slides were then run through gel electrophoresis system. Electrophoresis was performed at 25 V and 300 mA for 20 (blood) or 40 (mammary gland) minutes, following which the slides were neutralized, washed and stained with DAPI (4,6 Diamino-2-Phenylindole Dihydrachloride Hydrate). More than 90 cells per sample were analyzed using a Nikon E600 fluorescence microscope. The percentage of the signal obtained from the tail with respect to the total calculated using the Comet Assay IV software (Perceptive Instruments, Suffolk, UK).

Statistical analysis

Statistical analysis was performed with the SPSS software. We used non-parametric statistics based on distribution of each variable studied, determined by Kolmogorov-Smirnov's test, and variances equality among groups, determined by Levene's test. Analysis of quantitative data was performed with Friedman and Mann-Whitney's U test. Qualitative data were analyzed with Pearson's Chi-squared test. Differences were considered significant when $P<0.05$.

Results

Effects of diets and age on antioxidant capacity in mammary gland

The enzymatic antioxidant capacity of mammary gland was studied by analyzing the mRNA levels and activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Figure 2). Comparison of mRNA levels of two isoforms of SOD (SOD1 and SOD2), two isoforms of GPx (GPx1, GPx4) and CAT showed few and heterogeneous differences among experimental groups. Thus, for GPx1 we observed higher expression in HCO at 51 days and in LF-HEVOO at 100 days compared to control; for GPx4 the expression in the high-fat diet groups (especially in EVOO groups) was lower than control; and for catalase the HCO group had lower expression than control at 51 days. We observed a marked effect of age on enzyme expression, with lower mRNA levels of the enzymes around puberty (36 or 51 days) and an increase by the end of the assay (Figure 2A).

We also observed changes by dietary fat and by age in the enzymatic activity (Figure 2B). Total SOD activity increased in all groups from 24 to 36 days of age, to progressively decrease to 100-246 days. Up to 100 days, the high-fat diet groups had lower SOD activity than the control group (statistically significant at 36 days), while at the end of the study, SOD activity was higher in LF-HCO and LF-HEVOO. CAT activity decreased in all experimental groups from 24 to 100 days

of age, to increase at 246 days. All high-fat diet groups had lower CAT activity than the control group, especially at 36 and 51 days of age.

On the other hand, we studied the levels of glutathione in its oxidized (GSSG) and reduced (GSH) forms (Figure 3). Both GSSG and GSH in non-induced mammary gland (36 and 51 days) were higher in HEVOO (and in HOO at 36 days), but differences did not reach statistical significance. The oxidized *vs* reduced (GSSG/GSH) ratio was significantly increased in HCO group at 36 days. In the induced mammary gland results were heterogeneous, and the GSSG/GSH ratio was significantly increased in HCO vs HEVOO, and in LF-HEVOO vs HEVOO and control.

Effects of diets on antioxidant capacity in tumors

Results of the effects of the experimental high-fat diets on mammary adenocarcinomas at the end of the assay are shown in Figure 4. No significant differences in mRNA expression among groups in SOD and CAT enzymes were observed. GPx1 analysis showed a decrease in mRNA levels in all high-fat diet groups in comparison to control, significantly in the EVOO groups. GPx4 was also similar among groups, with the only difference between HCO and LF-HEVOO (Figure 4A). The analysis of enzyme activities did not show significant differences compared to the control (Figure 4B). The comparison among high-fat diet groups showed higher SOD activity in LF-HEVOO than in LF-HCO, whereas HEVOO had lower GPx activity than LF-HCO and lower CAT activity than HCO.

The study of non-enzymatic antioxidant capacity showed higher levels of oxidized glutathione (GSSG) in all the high-fat diet groups, whereas the reduced one (GSH) was increased only in HEVOO group. No significant differences were observed in the ratio among groups (Figure 4C).

Effect of diets on lipid peroxidation

Lipid peroxidation was evaluated by determining isoprostanes in blood and lipofuscin in liver. Analysis of free isoprostanes in serum showed highly fluctuating levels, with no significant differences at 36 and 51 days, and higher levels in high-fat diet groups at 100 days but lower levels by the end of the assay (Figure 5A).

Few samples showed detectable levels of lipofuscin. The number of positive samples was: 1/18 (5.6%) in LF group; 9/19 (47.4%) in HCO group; 4/19 (21.1%) in LF-HCO group; 2/17 (11.8%) in HEVOO group; 2/19 (10.53%) in LF-HEVOO group. Qualitative statistical analysis indicated significant greater number of samples displaying positive levels of lipofuscin in HCO in comparison to control and EVOO groups (HEVOO and LF-HEVOO). Although the staining intensity was also higher in the HCO group (Figure 5B), the values could not be analyzed quantitatively due to the low number of positive samples,

Effect of diets on DNA damage

Genomic damage was assessed by the quantification of 8-oxo-dG in mammary gland and tumor, and single- and double-stand DNA breaks by comet assay in mammary gland and blood. The quantification of 8-oxo-dG in mammary gland showed lower levels in high fat diet groups at 36 days but higher levels in the induced gland (100 days and the end of the assay) (Figure 5C). In tumors, 8-oxo-dG levels were higher in the LF-HCO group (Figure 5D). Finally, comet assay indicated higher DNA damage in mammary gland than in white blood cells, and no marked effects by dietary lipids in such gland (the only difference being lower levels in the HEVOO group at the end of the assay, Figure 5E). The systemic levels detected in blood were in general

higher in the EVOO groups at 36 and 100 days, and in all high-fat diet groups at the end of the assay (Figure 5F).

Discussion

In this work we aimed to get insight into the molecular mechanisms of the influence of lipids on mammary carcinogenesis, investigating the effects of a diet high in corn oil (rich in n-6 PUFA) and a diet high in EVOO (rich in MUFA and bioactive compounds) on the oxidative stress.
We studied enzymatic and non-enzymatic antioxidant status of the mammary glands, at different ages, and tumors. Enzymatic antioxidant capacity was determined by analysis of mRNA levels and activity of the enzymes that are in first line of antioxidant defense: two isoforms of SOD (the cytosolic SOD1 and the mitochondrial SOD2), two isoforms of GPx (the cytosolic GPx1 and the one that reduces phospholipid hydroperoxides within membranes -GPx4-) and catalase. In addition to their importance as scavengers of free radicals, it has been described that these enzymes can be modified by dietary lipids [19]. On the other hand, we determined the levels of glutathione, the most important non-enzymatic antioxidant system. Glutathione is the main regulator of intracellular redox homeostasis, and acts as a cofactor of different antioxidant enzymes, besides acting directly on free radicals. In this sense, the ratio oxidized/reduced (GSSG/GSH) is considered an indicator of the oxidative stress levels [20]. Our study was carried out in the mammary glands at different times of the development: 24 days (post-weaning), 36 days (around puberty onset), 51 days (just before the induction with DMBA, when the mammary gland is highly susceptible to carcinogenic transformation), and in the induced gland (100 days and the end of the assay).

The results obtained in glands throughout the study suggested an acute effect of the high-fat diets, in which the antioxidant capacity decrease after a short period of dietary intervention (at 36 days, after two weeks on the experimental diets), followed by a phase of adaptation of the body (values more similar to control). Thus, such acute effect has been observed at 36 days of age in the lower expression and activity of antioxidant enzymes (mainly SOD and CAT), the accumulation of glutathione and the ratio of oxidized/reduced glutathione (GSSG/GSH). This indicator of oxidative stress would be increased by the effect of high-fat diets, significantly in the group fed the diet rich in corn oil. After two more weeks of dietary intervention (51 days of age) the results of the three dietary conditions (LF, HCO and HEVOO groups) became more similar (e.g. SOD and GSSG/GSH). These results suggest, on the one hand, that changes in dietary patters may have an impact on health and the risk of disease, even if such changes are maintained only for a short period of time, and on the other hand the capacity of the organism to adapt to this changing exposure.

Regarding the chronic effect of the high-fat diets, i.e., after a longer period of administration (from 51 days of age onwards), it would probably be added to the effect of the carcinogenic process itself, resulting in much more heterogeneous data in the induced mammary gland (100 and 246 days). In any case, the mechanisms controlling the production of reactive oxygen species in normal mammary cells are complex [21] and different factors may be at the base of the variability of the results obtained: cellular heterogeneity, different developmental state at the time of induction [22, 23], or methodological issues.

In addition to the effect of diets, the profile of expression and activity of antioxidant enzymes suggested an influence of age on the antioxidant capacity, which decreased from puberty to maturity. According to this, an advance on maturation can also result in changes in the

antioxidant capacity. Actually, we have previously demonstrated that the high corn oil diet increased the body weight and advanced the puberty onset, while the isocaloric high EVOO diet did not show such effect on body weight and mass [22].

On the other hand, data obtained in tumors did not show a clear influence of diets on oxidative stress levels after cancer is well established (246 days). Nevertheless, results in tumors may suggest also an effect of dietary lipids increasing oxidation (higher levels of oxidized glutathione GSSG) and a response of the tissue increasing the levels of the total glutathione and thus the reduced form, although the differences in the ratio were not statistically significant. However, the same level of oxidative stress can have different consequences depending on the cellular context, since an increase in such stress can became an advantage of selection of the cancer cells (assuming a greater source of mutation) if cells can evade apoptosis, or trigger cell death programs if apoptosis is functional [8, 10]. In this sense, we have previously observed an effect of the EVOO diet on apoptosis, concomitantly with a potential protective effect on tumor malignancy in comparison with the high corn oil diet [13, 24].

In addition to the antioxidant capacity on target tissues, we also investigate the effect of age and diet on other markers of systemic stress, such as isoprostanes in serum and lipofuscin in liver. Both molecules are final products of the chain of reactions that takes place during the peroxidation of fatty acids. F_2-isoprostanes (F_2-isoP) are formed by the non-enzymatic oxidation of arachidonic acid and are used as biomarkers of oxidative stress since they are released to blood [25]. Although differences did not reach statistical significance, F_2-IsoP levels also suggest an acute effect of diets (higher levels in the high-fat diet groups at 36 days) followed by an adaptation to the chronic intake (similar values among groups at 51 days). It has been reported that heterogeneous results in this parameter are related to several factors, such as the health status

or methodological issues [25]. Thus, our highly variable results after induction (100 and 246 days) can be influenced by the different progression of the disease in the animals. In relation to lipofuscin, this pigment is formed by oxidized components, mainly the final product of lipid peroxidation [26] and has been considered a marker of sustained oxidative stress [27]. The results obtained suggested a higher chronic oxidative stress in the group fed the diet rich in n-6 PUFA for the longer time (HCO group) [27].

Finally, a decrease in antioxidant capacity elicit by cancer may increase the levels of genomic damage induced by oxidative stress. Thus, to investigate the genomic damage we analyzed the levels of 8-oxo-dG in mammary gland and tumor, and single- and double-stand DNA breaks by comet assay in mammary gland and blood. Although DNA fragmentation is not specific of oxidative stress, 8-oxo-dG is the final product of guanine oxidation and the most frequent premutagenic lesion in high oxidative stress conditions [28]. In general results were discordant with other parameters, which suggest that they may reflect different pathways of oxidative stress. Nevertheless, in tumors 8-oxo-dG levels were higher in the LF-HCO group, which interestingly is the group with the highest level of clinical and morphological malignancy [13].

In conclusion, our study suggests a short-term effect of the high intake of lipids on oxidative stress after two weeks of dietary intervention (reflected in changes in antioxidant enzymes, oxidized/reduced ratio of glutathione and DNA damage in mammary gland, and isoprostanes in blood), followed by an adaptation to the chronic consumption. In older tumor-bearing animals the effects of a high corn oil diet and a high EVOO diet are heterogeneous but also suggest an increase in oxidative stress by both high-fat diets, especially the one rich in n-6 PUFA according to the higher lipid peroxidation in liver and the higher DNA damage in tumor. Taking into

consideration the differential effect that these diets have on mammary carcinogenesis (clearly stimulating in the case of the high corn oil diet and similar to the low-fat one in the case of the diet rich in EVOO), other studies in these same tumors suggest different consequences in the proliferation/apoptosis balance due to the effect of these two isocaloric high-fat diets. From the public health point of view, the study highlights the impact that a change in dietary patterns, even for a short period of time, can have on health and the disease risk. Moreover, the results also point out the importance of dietetic habits and healthy choices from early ages on future risk of diseases, including cancer.

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Figures:

Figure 1. Experimental design.

Female rats were fed the low-fat control diet (LF), the high-corn oil diet from weaning (HCO) or from induction (LF-HCO), and the high-extra virgin olive oil diet from weaning (HEVOO) or from induction (LF-HEVOO). Animals were induced with 5 mg of dimethylbenz[α]anthracene (DMBA) at 53 days of age. Samples were collected at 24, 36, 51, 100 and the end of assay.

Figure 2. Antioxidant enzymes in mammary gland at different ages.

A. Expression (mRNA levels) of the antioxidant enzymes SOD (isoforms 1 and 2), GPx (isoforms 1 and 4), and Catalase. B. Total enzyme activity of SOD, GPx and Catalase. Data presented are medians. Non-parametric Mann-Whitney's U test (36 and 51 days n = 6/group; 100 days n = 5/group, 246 days n = 20/group). 24 days, post-weaning group, not fed with the experimental diets (n = 6). *: $p<0.05$ in comparison to LF control.

Figure 3: Oxidized (GSSG) and reduced (GSH) glutathione and the ratio GSSG/GSH in mammary gland at different ages.

Data presented are medians. Non-parametric Mann-Whitney's U test (36 and 51 days n = 6/group; 100 days n = 5/group, 246 days n = 20/group). Lines connecting groups indicate differences statistically significant ($p<0.05$).

Figure 4: Antioxidant enzymes and glutathione in mammary tumors.

A. Expression (mRNA levels) of the antioxidant enzymes SOD (isoforms 1 and 2), GPx (isoforms 1 and 4), and Catalase. B. Total enzyme activity of SOD, GPx and Catalase. C. Oxidized (GSSG) and reduced (GSH) glutathione and the ratio GSSG/GSH. Data presented are

medians. Non-parametric Mann-Whitney's U test, n = 18-20/group. Lines connecting groups indicate differences statistically significant ($p<0.05$).

Figure 5: Lipid peroxidation and DNA damage.

A. Levels of free isoprostanes (F_2-IsoP) in serum samples at different ages. B. Lipofuscin detected by Schmorl staining in liver at the end of the assay (246 days). C. Levels of 8-oxo-dG in mammary gland at different ages. D. Levels of 8-oxo-dG in tumors at the end of the assay. E. Comet assay in mammary gland at different ages (inset: representative image). F. Comet assay in blood at different ages (inset: representative). Data presented are medians. Non-parametric Mann-Whitney's U test (36 and 51 days n = 6/group; 100 days n = 5/group, 246 days n = 18-20/group). 24 days, post-weaning group, not fed with the experimental diets (n = 6). *: $p<0.05$ in comparison to LF control. #: $p<0.05$ in comparison to HCO group. Lines connecting groups: $p<0.05$. 10E6 dG: 10^6 deoxiguanosine.

Figure 1

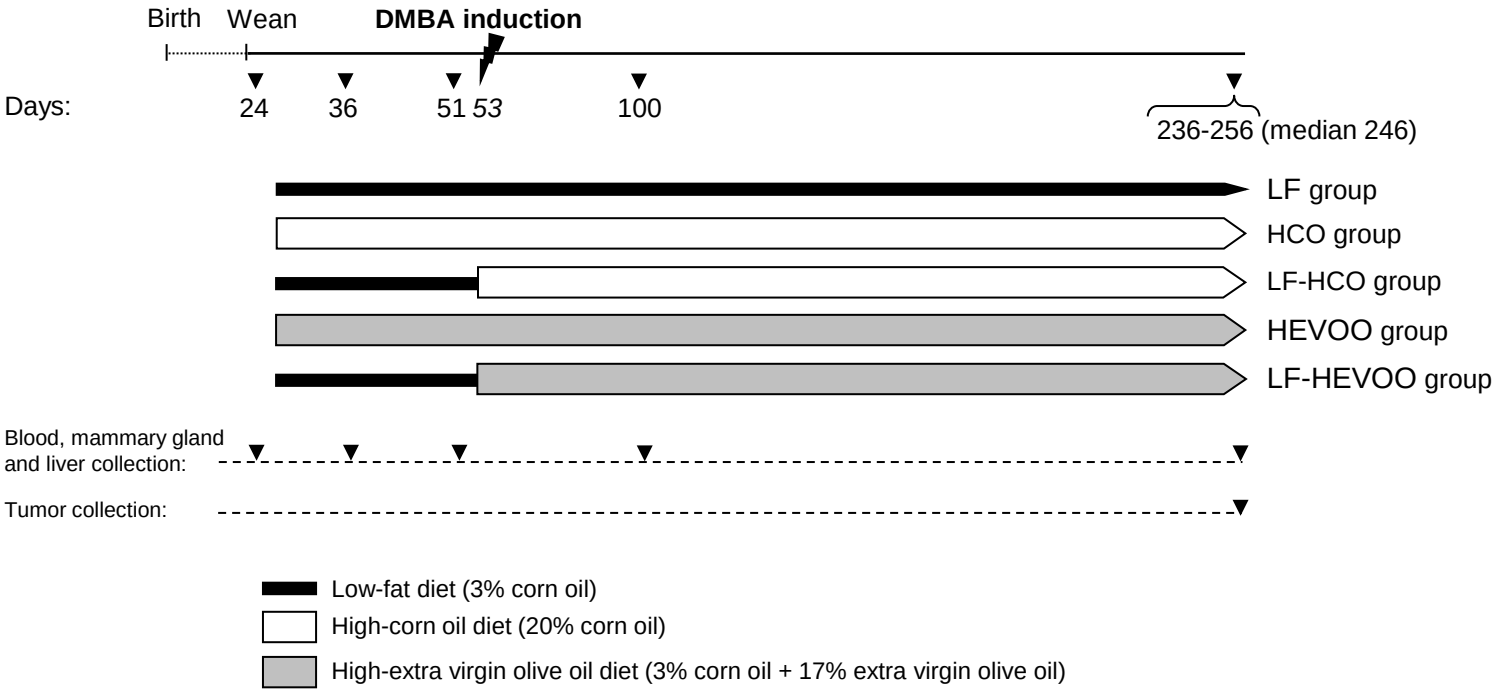

Birth
Wean
DMBA induction
Days:
24
36
51
53
100
236-256 (median 246)
LF group
HCO group
LF-HCO group
HEVOO group
LF-HEVOO group
Blood, mammary gland and liver collection:
Tumor collection:
Low-fat diet (3% corn oil)
High-corn oil diet (20% corn oil)
High-extra virgin olive oil diet (3% corn oil + 17% extra virgin olive oil)

Figure 2

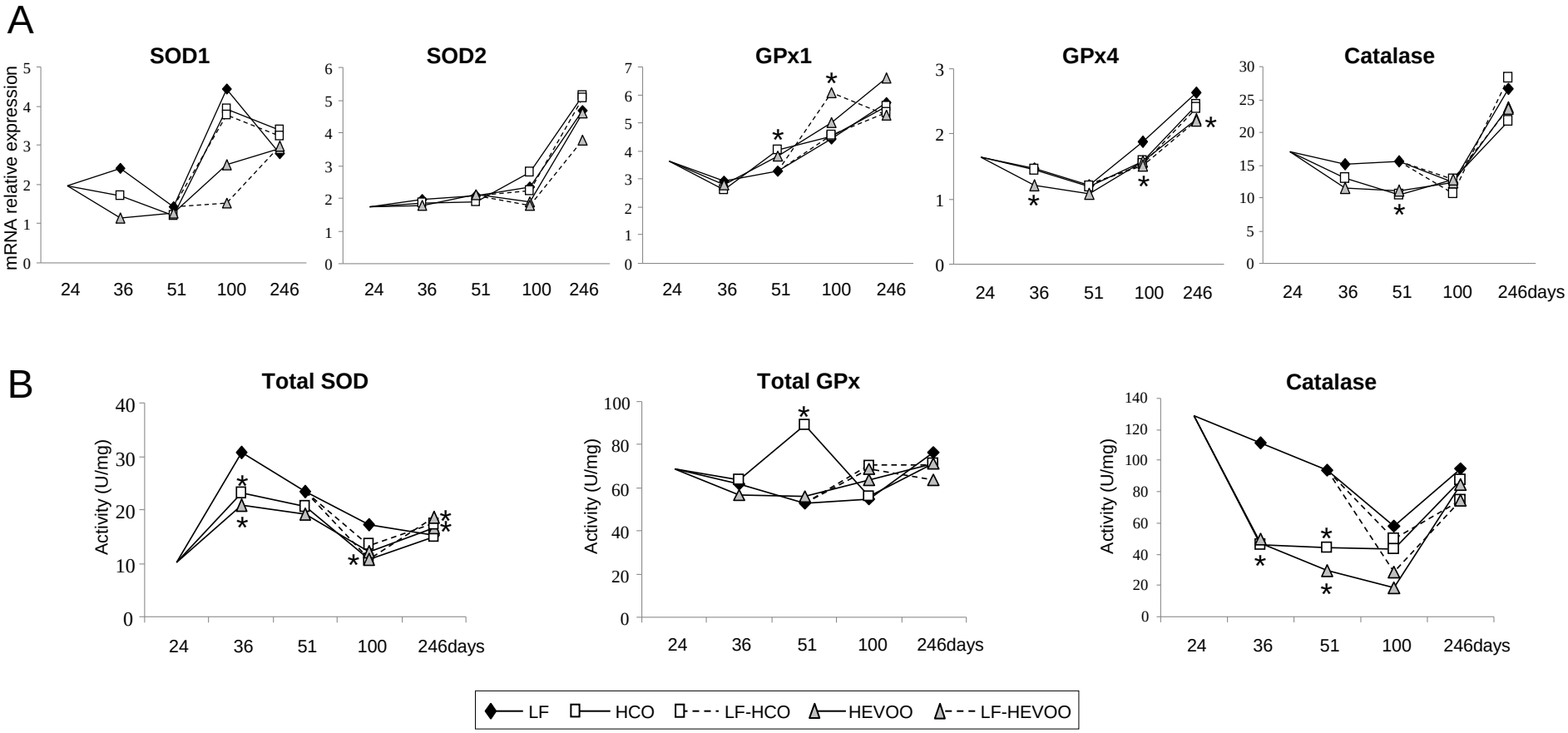

A
SOD1
SOD2
GPx1
GPx4
Catalase
mRNA relative expression
24 36 51 100 246
24 36 51 100 246
24 36 51 100 246
24 36 51 100 246
24 36 51 100 246days
B
Total SOD
Total GPx
Catalase
Activity (U/mg)
24 36 51 100 246days
24 36 51 100 246days
24 36 51 100 246days
LF
HCO
LF-HCO
HEVOO
LF-HEVOO

Figure 3

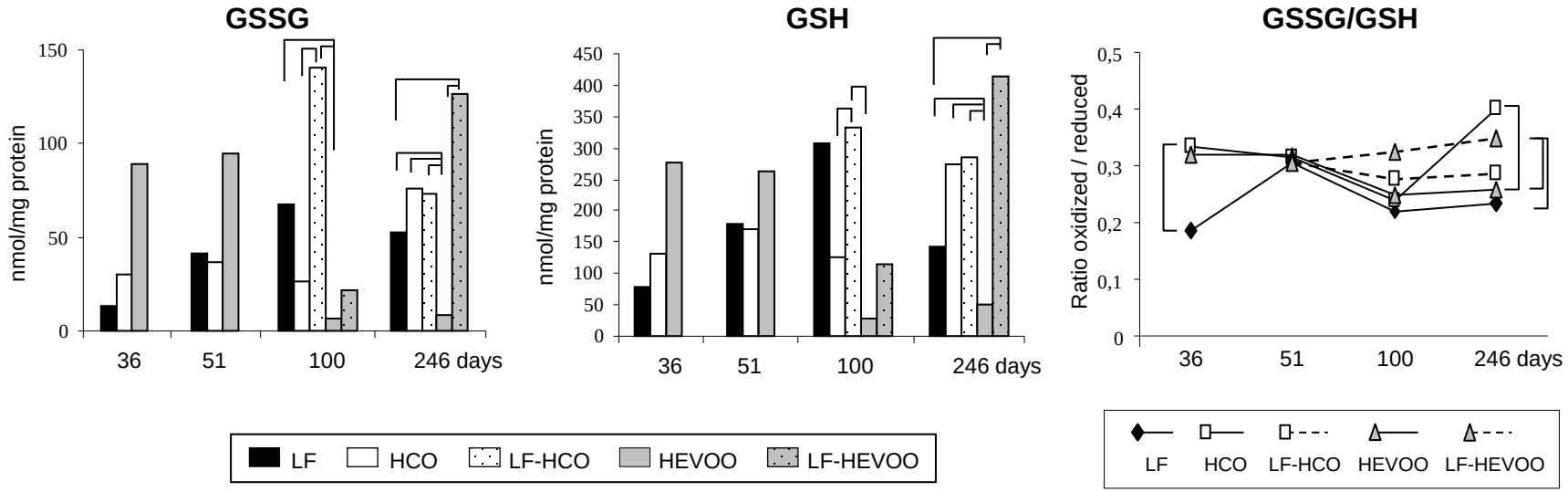

GSSG
nmol/mg protein
150
100
50
0
36
51
100
246 days
GSH
nmol/mg protein
450
400
350
300
250
200
150
100
50
0
36
51
100
246 days
GSSG/GSH
Ratio oxidized / reduced
0,5
0,4
0,3
0,2
0,1
0
36
51
100
246 days
LF
HCO
LF-HCO
HEVOO
LF-HEVOO
LF
HCO
LF-HCO
HEVOO
LF-HEVOO

Figure 4

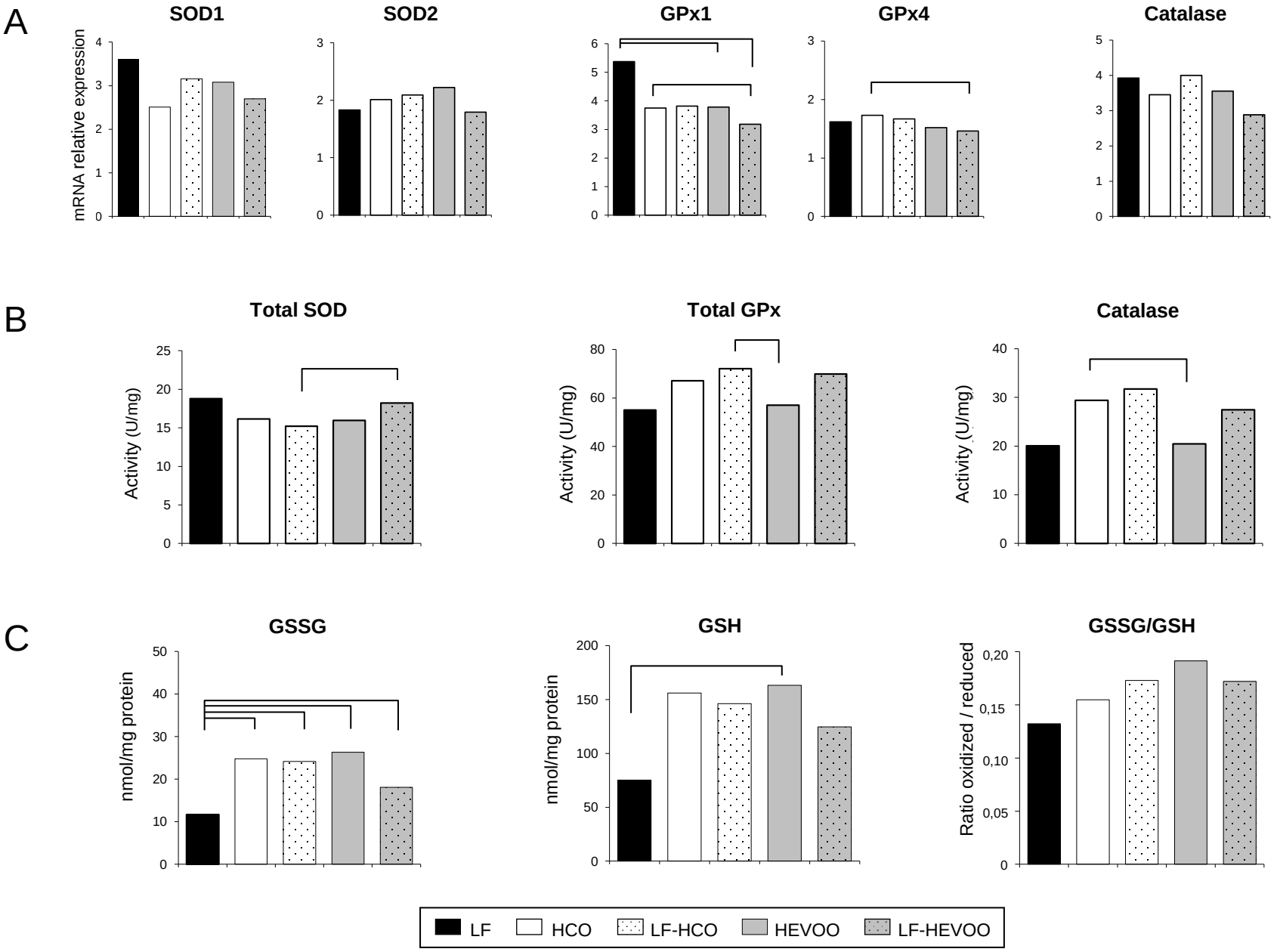
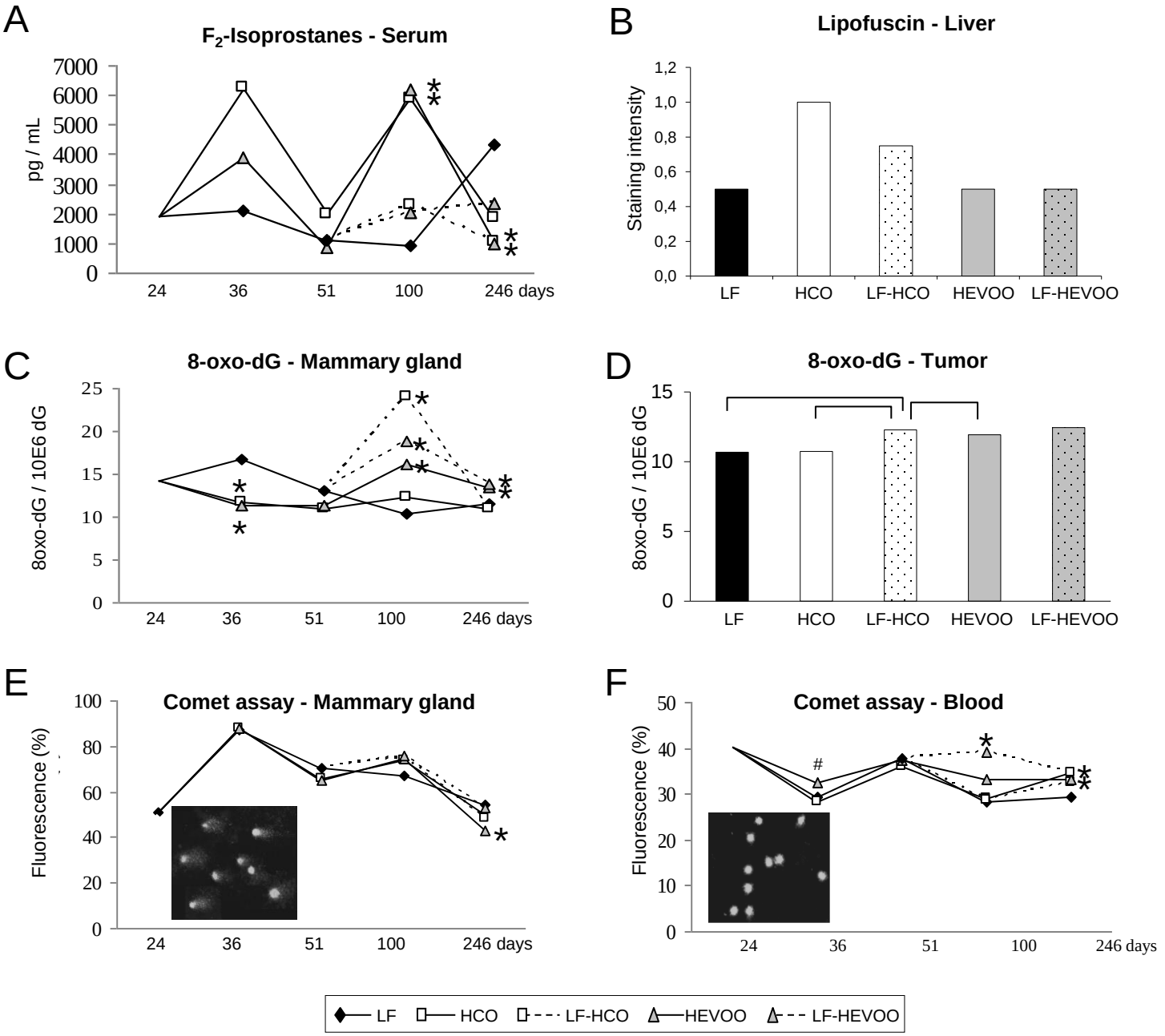

A
SOD1
SOD2
GPx1
GPx4
Catalase
mRNA relative expression
B
Total SOD
Total GPx
Catalase
Activity (U/mg)
C
GSSG
GSH
GSSG/GSH
nmol/mg protein
Ratio oxidized / reduced
LF
HCO
LF-HCO
HEVOO
LF-HEVOO

Figure 5


A
F2-Isoprostanes - Serum
pg / mL
7000
6000
5000
4000
3000
2000
1000
0
24
36
51
100
246 days
B
Lipofuscin - Liver
Staining intensity
1,2
1,0
0,8
0,6
0,4
0,2
0,0
LF
HCO
LF-HCO
HEVOO
LF-HEVOO
C
8-oxo-dG - Mammary gland
8oxo-dG / 10E6 dG
25
20
15
10
5
0
24
36
51
100
246 days
D
8-oxo-dG - Tumor
8oxo-dG / 10E6 dG
15
10
5
0
LF
HCO
LF-HCO
HEVOO
LF-HEVOO
E
Comet assay - Mammary gland
Fluorescence (%)
100
80
60
40
20
0
24
36
51
100
246 days
F
Comet assay - Blood
Fluorescence (%)
50
40
30
20
10
0
#
24
36
51
100
246 days
LF
HCO
LF-HCO
HEVOO
LF-HEVOO