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A high-corn-oil diet strongly stimulates mammary carcinogenesis, while a high-extra-virgin-olive-oil diet has a weak effect, through changes in metabolism, immune system function and proliferation/apoptosis pathways☆,☆☆

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Abstract

Breast cancer is the most common malignancy in women worldwide, and dietary lipids are important environmental factors influencing its etiology. We have investigated the effects, and the mechanisms associated, of high-fat diets on 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors. Animals were fed a low-fat, a high-corn-oil (HCO) or a high-extra-virgin-olive-oil (HOO) diet from weaning or after induction. The HCO diet had a clear stimulating effect on mammary carcinogenesis, especially when dietary intervention started after induction, whereas the tumors from HOO diet groups exhibited clinical and morphological characteristics similar to those from low-fat controls. Transcriptomic and further protein and immunohistochemical analyses of tumors also indicated different modulatory effects of high-fat diets affecting relevant biological functions: metabolism, immunosurveillance and proliferation/apoptosis pathways. Thus, the results suggested different metabolic adaptations with increased glycolysis by effect of HOO diet. Moreover, leukocyte tumor infiltration and inflammation mediators showed increased cytotoxic T cells and decreased TGFβ1 expression by the HOO diet, while the HCO one increased arginase expression and IL-1α plasma levels. Furthermore, the study of proteins controlling proliferation/apoptosis pathways (Sema3A, Stat5, Smad1, Casp3) suggested an increase in proliferation by the HCO diet and an increase of apoptosis by the diet rich in olive oil. In conclusion, the HCO diet clearly stimulated mammary carcinogenesis, especially in the promotion phase, and induced molecular changes suggesting increased tumor proliferation/apoptosis balance and a proinflammatory microenvironment. The HOO diet, despite being high fat, had a weaker effect on tumorigenesis probably related to metabolic adaptations, enhanced immunosurveillance and increased apoptosis.
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Keywords: Dietary lipids; Breast cancer; Olive oil; Transcriptome; Immune system; Apoptosis; Metabolism

1. Introduction

Breast cancer was the most common cancer overall in 2015, with 2.4 million cases worldwide, and a leading cause of women death [1]. This neoplasia is multifactorial, and there is substantial contribution of environmental factors, diet being one of the most important [2]. Although there are some controversial data, epidemiological and especially experimental data have shown the effects of dietary lipids on breast cancer risk [2–5]. Thus, their influence on malignant transformation depends on the quantity and type of fat and timing

Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; EVOO, extra-virgin olive oil; FA, fatty acids; GO, gene ontology; HCO, high corn oil; HOO, high extra-virgin olive oil; LF, low fat; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

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of dietary intervention, among other factors. In this regard, experimental data have revealed that, in general, n-6 polyunsaturated fatty acids (PUFA) and animal saturated fatty acids clearly promote breast cancer, whereas n-3 PUFA would inhibit tumor growth and metastasis [3,4]. In relation to monounsaturated fatty acids (MUFA), evidence is still inconclusive, with data ranging from protective to weak promoting effects on experimental breast carcinogenesis [3,4]. Epidemiological data indicate that women in the Mediterranean European countries have lower rates of breast cancer than in other Western countries [5,6]. Mediterranean diet is characterized by the consumption of olive oil (rich in the MUFA oleic acid) as the main source of fat [7].

Although there is increasing experimental evidence of the influence of diet on the development of neoplastic diseases, fewer data support that dietary factors may influence not only the clinical manifestation of the disease but also the histopathologic degree of malignancy, which has important prognostic value [8]. Moreover, the molecular mechanisms by which dietary lipids may influence mammary gland transformation are not well elucidated, but modifications in hormonal status, structure and function of cell membranes, signaling pathways or gene expression have been reported [3,4]. It has long been known that dietary lipids can modulate the expression of metabolism genes in tissues like liver, but scarce evidence exists on their effects on cancer-related genes [9]. We have previously demonstrated that the stimulating effect of a corn-oil-enriched diet on experimental mammary carcinogenesis can be mediated, at least in part, by transcriptomic changes affecting the expression of differentiation genes [10]. In this work, we aimed to investigate the effects of different high-fat diets, i.e., high in a seed oil (rich in n-6 PUFA) or in extra-virgin olive oil (EVOO, rich in oleic acid and bioactive compounds) and administered from prepuberty or in adulthood, on clinical and anatomopathological characteristics of experimental mammary tumors. Moreover, to gain insight into the molecular mechanisms underlying such effects, we investigated transcriptomic changes in tumors suggesting changes in metabolism, immune system function and proliferation/apoptosis pathways.

2. Methods

2.1. Diets

Three semisynthetic diets were designed: a low-fat diet (LF, 3% corn oil w/w), a high-corn-oil-diet (HCO, 20% corn oil) and a high-olive-oil-diet (HOO, 3% corn oil+17% EVOO) (Supplementary Table 1). Carbohydrates in the form of dextrose were 67.9% w/w in LF and 45.9% in the high-fat diets. They also contained protein in the form of casein (18% w/w in LF diet and 23% in the high-fat diets) and 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 methionine (0.51% w/w in LF and 0.66% in the high-fat diets), choline (1800 mg/kg diet) and folic acid (5 mg/kg diet). The definition, preparation and suitability of the experimental diets were previously described [11–13], as well as the specific composition of the oils used [14]. Diets were prepared weekly and stored under nitrogen in the dark at 4°C.

2.2. 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 were purchased from Charles River Lab (L'Arbresle Cedex, France) at 23 days of age and maintained under standard conditions. Animals were distributed according to the type and timing of dietary intervention, thus diets influencing initiation and promotion, or only promotion of the carcinogenesis. From weaning, animals were fed a low-fat diet (group LF, $n=60$), a high-corn-oil diet (group HCO, $n=20$) or a high-extra-virgin-olive-oil diet (group HOO, $n=20$) (Fig. 1). At 53 days of age, mammary tumors were induced 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. After induction, 40 rats from LF group were changed to high-fat diets (groups LF-HCO and LF-HOO, $n=20$ each). Body weights were measured weekly throughout the assay, and nose-to-anus length was measured at days 36, 51, 100 and the end of the assay to calculate body mass index (g/cm^2). On days 236–256, rats were euthanized by decapitation. Plasma was obtained and maintained at −80°C until used. Tumors were removed and measured, a portion of each was fixed in 4% buffered formalin, and the rest was flash-frozen and stored at −80°C. Only confirmed mammary adenocarcinomas have been included in this study.

2.3. Characterization of tumor malignant phenotype and tumor growth

Tumor phenotype was characterized determining the degree of morphological aggressiveness by applying the modified Scarff–Bloom–Richardson grading method adapted to rat mammary adenocarcinomas that we previously described [15]. This global histopathologic grade of adenocarcinomas adds together the differentiation pattern, nuclear pleomorphism and mitotic index and scores 3–5 points (grade I or well differentiated), 6–8 points (grade II or moderately differentiated) and 9–11 points (grade III or poorly differentiated). Moreover, though most rat mammary carcinomas are known to be nodular and very clearly bounded, stromal response to the invasion was classified as absent (−) or as positive (+) when occasional prominent fibroblasts were seen around neoplastic nests. Significant cellular and stromal reaction was classified as ++ or +++ depending on the extent and intensity of the response, the latter when a truly desmoplastic reaction was present. Microscopic tumor necrosis was also evaluated as absent, focal or extensive.

On the other hand, tumor growth was characterized with several carcinogenesis parameters: latency (average time from carcinogen administration to first palpable malignant tumor), tumor incidence (percentage of rats bearing at least one tumor), tumor yield (cumulative total number of tumors) and total tumor volume (volume of all the tumors of the group).

2.4. RNA extraction and microarrays analysis

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.

Six mammary adenocarcinomas of each group were chosen for whole-genome gene expression profiling. Briefly, GeneChip Rat Exon 1.0 ST Array filters containing probesets for 850,000 exons were obtained from Affymetrix (Santa Clara, CA, USA). Three micrograms of RNA (integrity number >8) was labeled with One-Cycle Target Labeling and Control Reagent (Affymetrix), hybridized to chips (Hybridization oven 640, Affymetrix) and scanned using GeneChip Scanner 3000 7G (Affymetrix) in the Microarrays Service from Vall d'Hebron Research Institute.

Images obtained were processed with Microarray Analysis Suite 5.0 (Affymetrix). Raw expression values were preprocessed using the RMA method [16]. Data were also nonspecific filtered to remove low-signal and low-variability genes. These normalized and filtered values were the basis for all the analysis. Microarray data have been deposited at ArrayExpress under accession number E-MTAB-3541.

The selection of differentially expressed genes between conditions was based on a linear model analysis [17]. Different statistics tests (fold change, t or P values) were used to classify genes into ranks depending on its differential expression. In order to deal with the

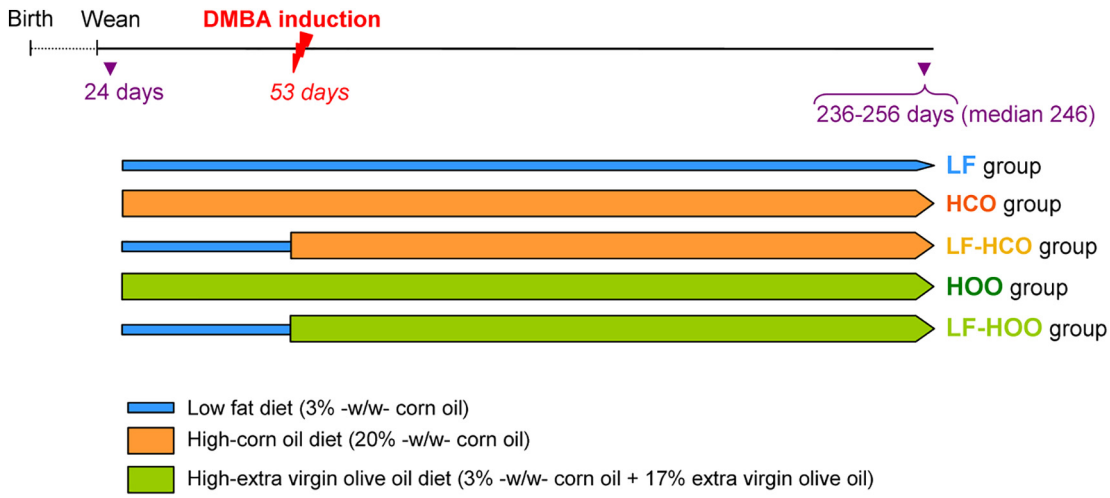

Fig. 1. Experimental design. Female Sprague–Dawley rats were fed the low-fat control diet (LF), the high-corn-oil diet from weaning (HCO) or from induction (LF-HCO), and the high-EVOO diet from weaning (HOO) or from induction (LF-HOO); n=20 animals/group. Animals were induced with 5 mg of DMBA at 53 days of age and euthanized at 236–256 days.

multiple testing issues, P values were adjusted to obtain strong control over the false discovery rate using the Benjamini and Hochberg method [18].

We performed analysis of biological significance based on overrepresentation tests aiming to establish if genes found as differentially expressed were concentrated in some Gene Ontology (GO) categories. Enrichment tests were performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System and Genecodis and FatiGO from Babelomics tool, where Fisher's Exact Test for 2×2 contingency tables and multiple test correction were applied to find significant overrepresentation of GO terms.

2.5. Reverse transcription and real-time PCR

Two micrograms of total RNA was 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 Scd, Fabp3, Notch4, Pfkl, Il18, Il2Rg, Sema3a, Jak2, Smad1, Stat5a, Cdkn2b, Arg1, Nos2, TGFβ1 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.

2.6. Protein extraction and Western blot

Tumor samples were homogenized in a buffer containing 50 mM Tris–HCl pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 50 μM NaF, 100 μM Na_3VO_4, 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich), 10 mM β-mercaptoethanol and 1% Triton X-100. Protein extracts (10–20 μg) were subjected to SDS-PAGE on a 7.5% acrylamide using the Mini-Protean TGX Stain Free Gels and transferred to PVDF membrane with Trans-Blot Turbo Transfer Sytstem (Bio-Rad). Primary antibodies and dilutions used were anti-GLUT1 (Cell Signaling Technology, 1:1000), anti-PFKL (Cell Signaling Technology, 1:500), anti-ALDOA (Cell Signaling Technology, 1:1000), anti-NOTCH4 (Abcam, Cambridge, 1:5000), anti-Semaphorin 3A (Abcam, 1:3000), anti-SMAD 1/5/9 (Abcam, 1:1000), anti-Phospho-SMAD 1/5/9 (Cell Signaling Technology, 1:500), anti-STAT5A/B (Cell Signaling Technology) (1:3000), anti-CASP3 (Cell Signaling Technology, 1:2000) and anti-PCNA (Cell Signaling Technology, 1:5000). After incubation with secondary antibody (Sigma-Aldrich) and Luminata Forte Western HRP Substrate (EMD Millipore) luminogen, proteins were visualized using the Chemidoc XRS+ hardware associated with Image Lab Software 5.1-Beta (Bio-Rad). This Bio-Rad system normalizes the densitometric values of the bands with the total protein loaded in each line, correcting the load by comparing with the first lane of the blot [19]. Thus, in order for the blots to be comparable, we used an "internal control" of pooled samples loaded in duplicate in all the blots. Original Western blots of proteins shown in the "Results" section are presented in Supplementary Fig. 1.

2.7. Immunohistochemistry and quantification of immunoreactivity

Formalin-fixed and paraffin-embedded tumor sections were deparaffinized, rehydrated and stained using the Vectastain Elite ABC system (Vector Laboratories Inc.). Heat-induced antigen retrieval was performed using EDTA (for CD3 detection) or citrate buffer (for CD8 and CD68 detection). For staining of immune cells, anti-CD3 (Abcam), anti-CD8 (Millipore) and anti-CD68 (Abcam) antibodies were used at a 1:100 dilution.

For quantification of CD3 and CD8 immunostaining, 10 photographs per tumor at ×400 magnification were taken, and CD-3 and CD8-positive cells were manually counted. The total number of cells was quantified by Image J software, and results were expressed as the percentage of positive cells with respect to the total number of cells in the sample. For CD68, due to the difficulty to define individual positive cells, a semiquantitative score system was used by two independent investigators, classifying the staining as low, medium or high.

2.8. Circulating levels of cytokines

Determination of plasma levels of 27 cytokines was performed with the LuminexMAP technology (Rat Cytokine/Chemokine Magnetic Bead Panel; Milliplex MAP Kit, Millipore) by immunoassay on the surface of fluorescent-coated magnetic beads following manufacturer's instructions. Cytokines analyzed were: GCSF, GMCSF (=CSF2), leptin, EGF, VEGF, IL1α, IL1β, IL2, IL4, IL5, IL6, IL10, IL12 p70, IL13, IL17α, IL18, IFNγ, MCP1 (=Ccl2), MIP1α (=Ccl3), RANTES (=Ccl5), eotaxin (=Ccl11), GRO-KC(=Cxcl1), MIP2 (=Cxcl2), LIX (=Cxcl5), IP10 (=Cxcl10), fractalkine (=Cx3cl1) and TNFα. Samples were run in duplicate.

2.9. Hormone levels

Plasma levels of 17-β-estradiol and progesterone were determined using Estradiol Concentration ELISA Kit and Progesterone Concentration ELISA Kit according to the manufacturer's directions (GenWay Biotech, Inc.). Plasma prolactin levels were measured by radioimmunoassay using ^{125}I-prolactin (NEN) as the tracer, rat prolactin (rat PRL-RP-3) as the standard and an antibody rose against rat prolactin (anti-rPRL-S-9) (Dr. AF Parlow, NIDDK National Hormone and Peptide Program, CA, USA).

2.10. Metabolic parameters

Nonfasting plasma glucose and insulin levels were obtained at the end of the assay from animals fed *ad libitum* as an appropriate and acceptable method based on rodent habits, the stress caused by fasting and the interference with other parameters [20]. Plasma levels of glucose were determined using an automated clinical analyzer in the Biochemistry and Molecular Genetics, Biomedical Diagnostic Center (CDB), Hospital Clínic. Insulin levels were determined with an insulin enzyme-linked immunosorbent assay kit from Millipore.

2.11. Statistical analysis

Statistical analyses for histopathological, clinical and molecular analyses other than microarrays were performed using SPSS software 17.0. We used parametric or no-parametric statistics depending on the distribution of each variable studied, determined by the Kolmogorov–Smirnov test, and the equality of variances, determined by Levene's test. Body weight throughout the assay was analyzed using nonlinear mixed models. Parametric quantitative data were analyzed with ANOVA followed by Tukey's test and depicted as mean+S.E.M. Nonparametric quantitative data were analyzed with Mann–Whitney *U* test and depicted as median, and mean+S.E.M. is presented in Supplementary Fig. 2. The χ^2 test was used for the qualitative data. The level of significance was established at $P<.05$.

3. Results

3.1. High-fat diets differentially influence tumor malignant phenotype and tumor growth

Diagnosis of mammary lesions revealed a high percentage of malignant tumors among all the neoplasias collected (Fig. 2a). Thus, the percentages of adenocarcinomas in relation to all mammary tumors (benign and malignant) were: control LF 88.5% (46/52), HCO 84.8% (100/118), LF-HCO 79.8% (87/109), HOO 80.6% (58/72) and LF-HOO 81.2% (82/101) (Supplementary Table 2).

In order to analyze the tumor aggressiveness, we studied the differentiation pattern, nuclear pleomorphism and mitotic activity index of adenocarcinomas. Applying all these parameters, we scored the modified Scarff–Bloom–Richardson overall grade adapted to rat mammary carcinomas [15]. Groups of animals fed with high-corn-oil diet presented the most aggressive tumors, with significant differences in comparison to control and LF-HOO groups (Fig. 2b). Thus, while more than 57% of the carcinomas from control showed low aggressive grade (scores 3–5), fewer tumors in the high-corn-oil-fed groups exhibited such grade (23% in HCO and 20.1% in LF-HCO), whereas groups fed the EVOO diet showed intermediate values (36.5% in HOO and 41.7% in LF-HOO). On the contrary, the percentage of high-grade aggressive tumors (scores 9–11) in groups fed the corn-oil-enriched diet was 33% (HCO) and 39% (LF-HCO), in contrast to the 12.8% in the control group, and also the intermediate percentages of HOO (21.2%) and LF-HOO (24%). Moreover, tumors from HCO diet groups showed the highest stromal reaction (24.7% tumors from HCO group and 23.5% from LF-HCO showed intense reaction) and tumor necrosis (in 10.6% of those from LF-HCO was extensive). Tumors from LF-HCO also displayed the highest mitotic index (Fig. 2c).

The results of carcinogenesis parameters are detailed in Table 1. Latency time was shorter in high-corn-oil groups, although differences did not reach statistical significance. Tumor incidence was maximum (100%) in both HCO diet groups, significantly higher than control and HOO. Tumor yield was higher in the corn-oil-enriched diet groups and LF-HOO *versus* the control. HCO, with the largest number of tumors, was also statistically different to HOO. Finally, total tumor volume was higher in all high-fat diet groups, especially in LF-HCO. This quantitative parameter cannot be statistically analyzed since there is one single value per group.

3.2. High-fat diets differentially modify the gene expression profile of the experimental tumors

In order to find genes modulated by dietary lipids, we compared gene expression profiles of tumors from high-fat diet groups with those from control group. Lists of differentially expressed sequences were further compared to identify genes modulated in common (co-modulated by effect of the high-fat diets) or unique to each specific group. Venn diagram in Fig. 3a depicts the number of overlapping and specific genes. In general, there were a low number of common genes. LF-HCO was the group with the largest number of specific genes.

Analyses of biological significance (functional clustering) of all the genes found as differentially expressed in each group are shown in Supplementary Table 3 (GO level 3). Some functional categories were significantly overrepresented in the clusters of down-modulated genes in several groups: metabolism, response to stimuli or proliferation and cell cycle. Moreover, immune system categories were specifically overrepresented among the down-modulated genes in LF-HCO group.

Functional analysis was also performed dividing the clusters of genes in commonly modulated and specific for each group. Only genes specific for LF-HCO and HOO groups showed significantly enriched categories. In particular, the genes down-modulated in LF-HCO group were mainly related to the immune system, response to stress and apoptosis (Fig. 3b–c), whereas the up-modulated genes had a role in metabolism (Fig. 3d–e). Metabolism, cell homeostasis and proliferation were overrepresented in the up-modulated genes in HOO group (Fig. 3f–g).

3.3. Validation of gene expression profiles and protein expression analyses

We selected for real-time PCR validation genes regulated in common (Scd, Fabp3 and Notch4) or specific for each condition and related to overrepresented functions. Gene expression results were similar by arrays and real-time PCR when we compared the same six samples in each group (Supplementary Fig. 3). When real-time PCR analyses were extended to 17–22 samples per group, a high variability among tumors was observed.

Extended analysis is detailed in Fig. 4. In relation to the common genes, Scd was down-modulated in high-fat diet groups, significantly in EVOO groups, while Fabp3 and Notch4 were significantly up-modulated in HCO and EVOO groups. Another metabolism gene, phosphofructokinase (Pfkl), was up-modulated in LF-HCO and HOO groups. Expression of genes related to immune system showed great variability with few significant differences between groups. The apoptosis-related gene Sema3a was significantly up-modulated in HOO group. Regarding the genes with a role in proliferation and cell cycle control, we found differences in the expression of Jak2 (up-modulated in HCO), Smad1 (down-modulated in high-EVOO diet groups) and Cdkn2b (up-modulated in LF-HOO), while a not significant decrease in Stat5a levels was also observed in EVOO groups.

In order to get insight into the overrepresented functions, expression of metabolism and proliferation/apoptosis proteins was analyzed by

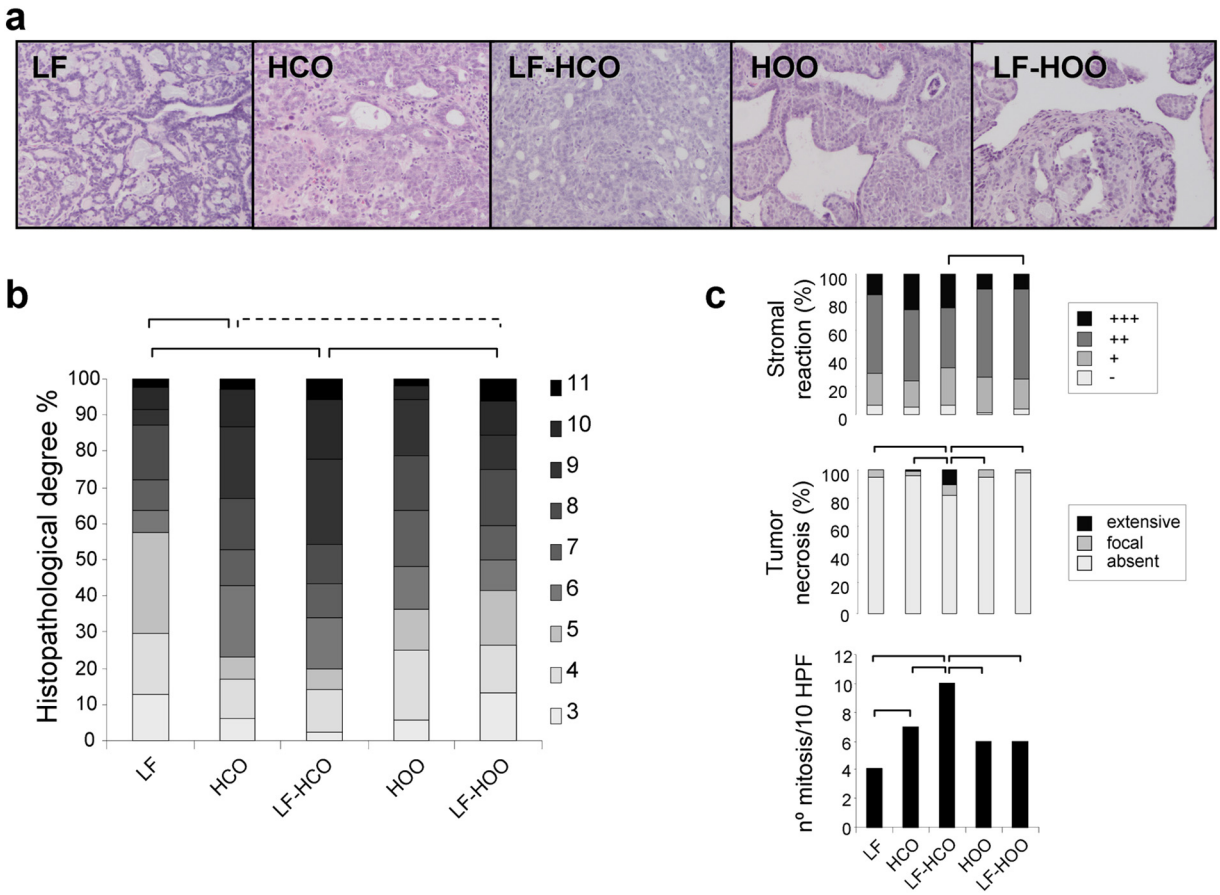

Fig. 2. Effects of high-fat diets on the histopathological features of DMBA-induced mammary adenocarcinomas. (a) Representative images of adenocarcinomas from the different experimental groups (200× magnification). (b) Distribution of mammary adenocarcinomas of each group according to the modified Scarff–Bloom–Richardson index, which includes differentiation pattern, nuclear pleomorphism and mitotic index (solid line: $P<.05$, dashed line: $P<.1$, χ^2 test). (c) Stromal reaction (− absent, + mild, ++ intense, +++ truly desmoplastic), necrosis and number of mitosis in 10 high-power fields (400× magnification); solid line: $P<.05$, nonparametric Mann–Whitney U test. $n=46$ (LF), 100 (HCO), 87 (LF-HCO), 58 (HOO) and 82 (LF-HOO).

Western blot. In relation to metabolism, validation of the glycolytic enzyme phosphofruktokinase (PFKL) was performed, and the analysis was extended to other proteins whose mRNA was found in the microarrays analysis as modulated by the high-fat diets: Glut1 (Slc2a1) and Aldolase A (AldoA). Results showed an increase of Glut1 and PFKL levels in tumors from HOO diet groups compared with those from HCO diet groups, while no differences in AldoA were found (Fig. 5a).

Notch4, Sema3a, Stat5A/B and Smad1 as well as Caspase 3 and PCNA were also investigated. No differences in Notch4 and PCNA were observed (results not shown). The 90-kDa full-length isoform of Sema3a was barely detected, but the 65-kDa cleaved fragment was decreased, in tumors from EVOO groups. No differences were observed in the total levels of Smad1/5/9 and StatA/B, but the activated form of Smad (phopho-Smad 1/5/9) was increased in HCO *versus* HOO. On the other hand, total Caspase 3 (Casp3) and its activated 17-kDa cleaved form were significantly increased in the LF-HOO group (Fig. 5b).

3.4. High-fat diets differentially modulate tumor infiltration of cytotoxic lymphocytes and immunosuppressive mechanisms

Since no clear trend was observed in the expression of immune system genes, to further investigate the role of immunological mechanisms on the effects of the high-fat diets in this experimental model, we first evaluated tumor infiltration of leukocytes (Fig. 6a). Infiltration of T lymphocytes (CD3-positive cells) was found to be similar between the different experimental groups. However, increased cytotoxic T lymphocytes (CD8-positive cells) infiltrate was observed in the LF-HOO group compared to the LF group. On the other hand, tumor-associated macrophages (CD68-positive cells) infiltration was not influenced by the high-fat diets. Since these macrophages and other populations such as myeloid-derived suppressor cells can suppress T cell proliferation and activity through production of arginase (Arg1), inducible nitric oxide synthase (Nos2) and transforming growth factor β 1 (Tgfβ1), gene expression of these molecules

Table 1
Carcinogenesis parameters

	LF	HCO	LF-HCO	HOO	LF-HOO
Latency time (days, median)	97	71.5	68	89	89
Incidence (% of tumor-bearing animals)	80 (16/20)^{a}	100 (20/20)^{b}	100 (20/20)^{b}	75 (15/20)^{a}	85 (17/20)^{ab}
Tumor yield (total number of tumors)	46^{a}	100^{b}	87^{bc}	58^{ac}	82^{bc}
Total tumor volume (cm^3)	69.3	105.0	188.2	121.9	100.6

Values within a row with different superscript letters are significantly different by Mann–Whitney U test ($P<.05$).

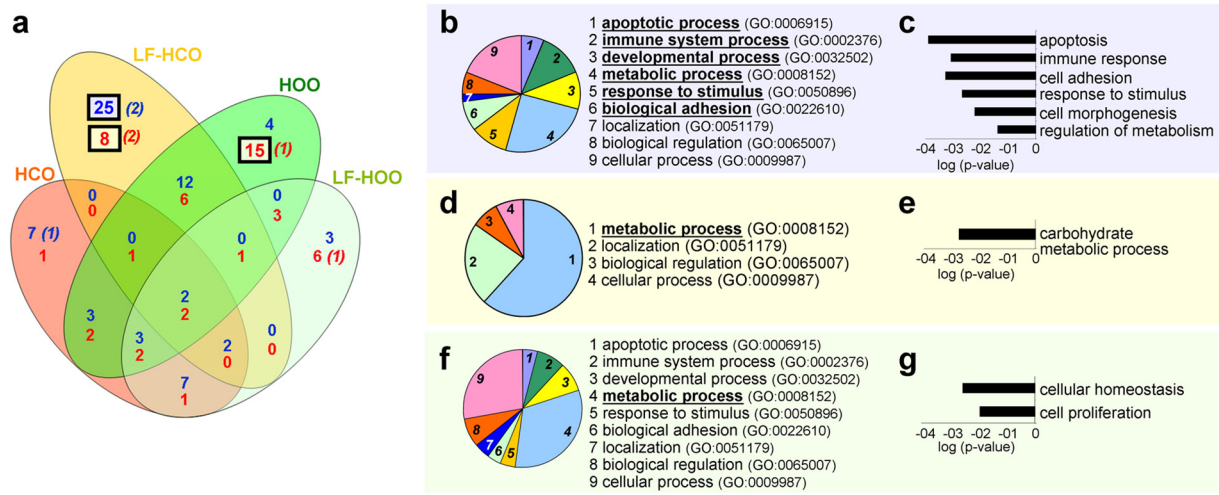

Fig. 3. Effects of high-fat diets on gene expression profiles. (a): Number of sequences differentially expressed (Benjamini and Hochberg false discovery rate method) in adenocarcinomas (n=6/group) from animals fed the high-fat diets compared to control. Sequences differentially expressed were then compared between high-fat diet groups. Venn diagram depicts the number of specific and overlapping genes. Blue numbers indicate down-regulated genes, and red numbers indicate up-regulated genes. Italic numbers in parentheses indicate not annotated sequences (unknown genes). Numbers inside boxes highlight the lists of genes where overrepresented functional categories have been found. (b, d, f) Pie charts represent GO term for biological functions in genes down-regulated in LF-HCO (b), up-regulated in LF-HCO (d) and up-regulated in HOO (f) through PANTHER Classification System. Bold underlined terms indicate significantly overrepresented functions (Fisher's Exact Test). (c, e, g) GO term enrichment for biological functions in genes down-regulated in LF-HCO (c), up-regulated in LF-HCO (e) and up-regulated in HOO (g) through FatiGO-Babelomics software (Fisher's exact test).

was evaluated in total extracts of tumor samples from the experimental groups. No differences were found in Nos2. The LF-HOO group showed lower expression of Arg1compared to both HCO groups and lower expression of Tgfβ1 compared to all other groups (Fig. 6b).

3.5. Dietary lipids influence circulating levels of inflammatory cytokines

Inflammation is a well-known player on the cancer process; thus, circulating levels of different inflammation-related cytokines were evaluated by immunoassay. We first carried out a screening in a panel of 27 analytes in 6 samples per group from which we selected 7 cytokines for further analyses (MCP1=Ccl2, IL18, GRO-KC=Cxcl1, VEGF, fraktalkine=Cx3cl1, IL1α and leptin; n=17–19 per group). Results are shown in Fig. 6c. IL1α plasma levels were higher in the LF-HCO group compared with the other high-fat diet groups. Leptin levels were increased in all the high-fat diet groups in comparison to the low-fat control and especially in HCO which also had higher levels than the LF-HOO group. No statistically significant differences were found in the other cytokines.

3.6. Dietary lipids effects in other systemic factors

Body weight throughout the study, as well as the body weight change compared to control, was increased in the rats fed with the HCO diet from weaning but not if dietary intervention started after induction (group LF-HCO). Body mass index was also higher in the HCO group at the end of the assay (Supplementary Fig. 4). The HOO diet did not change body weight or mass.

In addition, there were no significant differences between groups in the metabolic parameters plasma glucose or insulin (Supplementary Fig. 5). There were also no significant differences in the plasma levels of the hormones estradiol, progesterone or prolactin (Supplementary Fig. 6).

4. Discussion

In this work, we have observed a different influence of high-fat diets, depending on the type of fat and timing of dietary intervention, on anatomopathological and clinical characteristics of experimental mammary tumors. The diet rich in n-6 PUFA, especially when administered after puberty onwards, clearly stimulated mammary carcinogenesis and promoted tumors to a high degree of morphological malignancy. The diet rich in EVOO oil exerted a weaker influence, and tumors developed were more similar to those from low-fat controls. Gene expression profiles of tumors and validation analysis also indicated different effects of the high-fat diets, likely being one of the molecular mechanisms of their influence on mammary carcinogenesis and suggesting a differential influence of these diets on basic cellular processes: metabolism, immune system function and proliferation/apoptosis pathways.

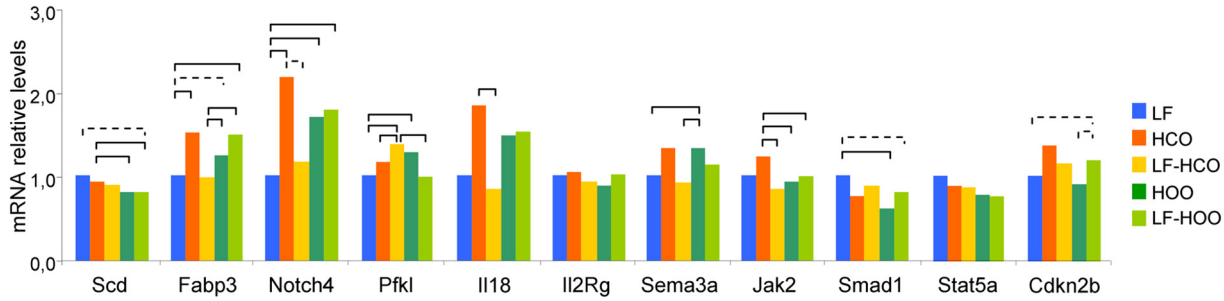

Fig. 4. Real-time PCR analysis of genes found in the microarrays as modulated by the high-fat diets. Data represent relative gene expression (median) in the different experimental groups (n=17–22/group). Solid lines connecting groups indicate statistically significant differences (P<.05); dashed lines indicate differences close to significance (P<.1), Mann–Whitney U test.

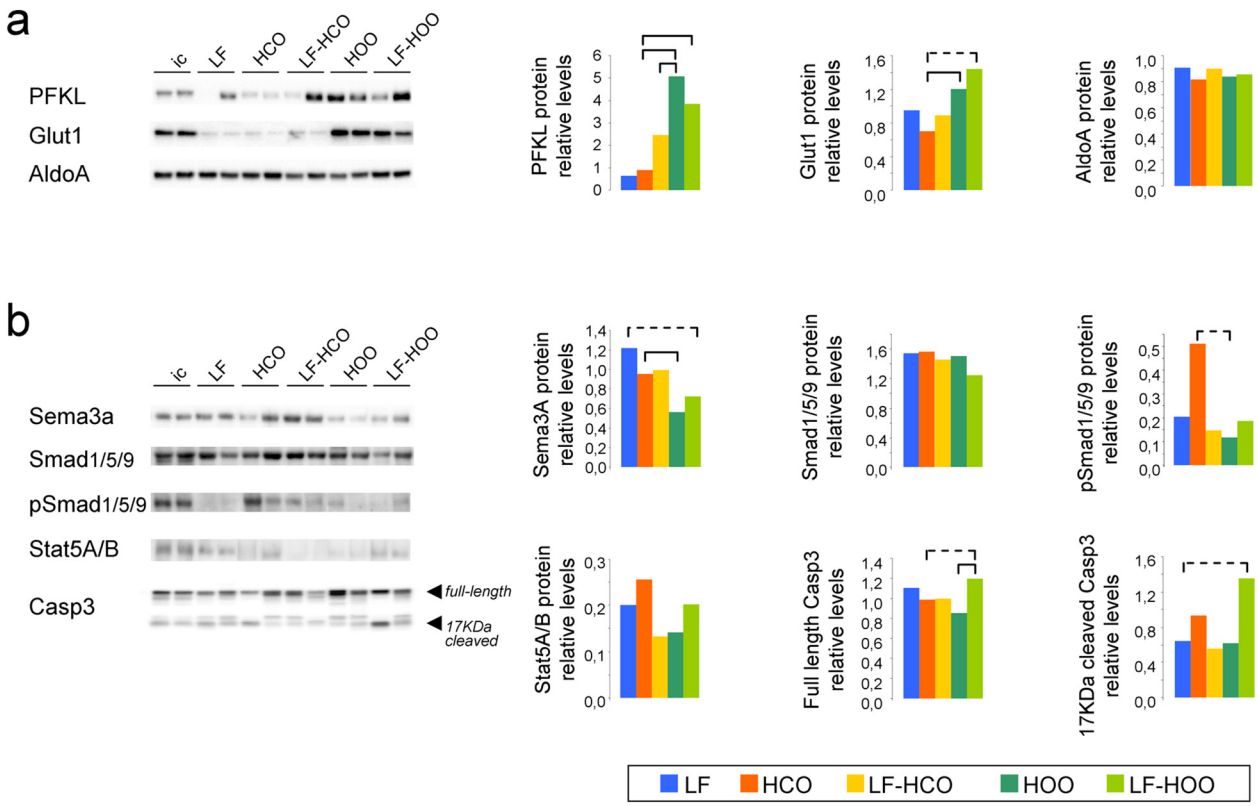

Fig. 5. Effects of high-fat diets on the expression of proteins related with metabolism and with proliferation/apoptosis pathways. (a) Representative Western blots and relative protein levels of glycolytic enzymes (PFKL, AldoA) and the glucose transporter Glut1 in the different experimental groups. (b) Representative Western blots and relative levels of proteins related to proliferation and apoptosis pathways. Bars represent medians. Solid lines connecting groups indicate statistically significant differences ($P<.05$); dashed lines indicate differences close to significance ($P<.1$), Mann–Whitney U test, $n=8–20$/group. ic refers to an internal control of pooled samples.

Histopathologic evaluation of the mammary lesions developed in the DMBA-induced animals showed the predominance of malignant adenocarcinomas and confirmed their similarity with human tumors [15]. Benign and malignant tumors arise from different epithelial structures, and benign lesions are highly influenced by hormones such as prolactin [21]. Although these high-fat diets have shown to influence the puberty onset [14], which would affect the number of differentiated structures at the moment of induction, there were no significant differences between groups in the percentage of the malignant tumors in relation to all mammary tumors, as well as no differences in hormone levels by effect of high-fat diets.

We characterized the tumor malignant phenotype determining the tumor growth and the degree of morphological aggressiveness by applying the modified Scarff–Bloom–Richardson grading method that we previously described [15]. Results revealed that adenocarcinomas from animals fed with the high-corn-oil diet, especially from LF-HCO group, displayed morphologic characteristics of high malignancy, i.e., high pattern and nuclear grade, mitotic activity, stromal reaction and necrotic areas, as well the overall histopathologic grade. Likewise, the study of clinical parameters of the carcinogenesis showed a clear stimulatory effect of the high-corn-oil diet since HCO and LF-HCO groups had the earliest onset of tumor appearance and the highest incidence and total malignant mammary tumors. However, the high-EVOO diet exerted a weaker stimulating effect on mammary carcinogenesis since tumors from animals fed such diet showed intermediate values between control and the high-corn-oil groups in the anatomopathological and clinical parameters.

The stimulating effect that n-6 PUFA-enriched diet exerted on mammary carcinogenesis was stronger during the promotion phase, given that tumors showed characteristics of higher malignancy when the dietary intervention started after induction (group LF-HCO). The differential effect that this diet may exert depending on the timing of dietary exposure could be related to several factors. On the one hand, the administration of this diet from weaning advanced the puberty onset [14], thus modifying the window of susceptibility of the mammary gland [21] and therefore the progression of the carcinogenesis. Puberty is a particularly sensitive life stage in which dietary factors can have a great influence [14,22]. On the other hand, we found an effect of this diet on the initiation of carcinogenesis through the modulation of detoxification enzymes [23,24]. In addition, the HCO diet has a clear promoter effect, already observed in previous works [8,13,14,25,26] and in other experimental models [27–29]. The combination of all these effects would produce a greater initiation of cancer in the HCO group than in the LF-HCO, but of slower progression (more tumors but with a slight increase in the latency time and of smaller volume, number of mitosis and necrosis).

The effects of the high-fat diets through the caloric density should also be taken into consideration. Animals fed with the HCO diet from weaning did gain significant body weight and mass in the long term, which is in accordance with the highest leptin levels in the HCO group. Also interesting are the consequences that such diet may have to the metabolic conditions associated to obesity. In this regard, there were no significant differences in plasma glucose or insulin levels between groups despite the increased body weight in HCO group. Several known risk factors (such as early sexual maturation or obesity) can be underlying mechanisms of the high-fat diet effects of tumorigenesis. Nevertheless, the HCO diet had a clear promoting effect on carcinogenesis in normal-weight animals (group LF-HCO).

On the other hand, few studies have investigated the effects of EVOO on mammary carcinogenesis. We have previously observed from a weak protective [3,13,25] to a weak promoting [14,26] effect of the high-EVOO diet on experimental breast cancer. In any case, all the

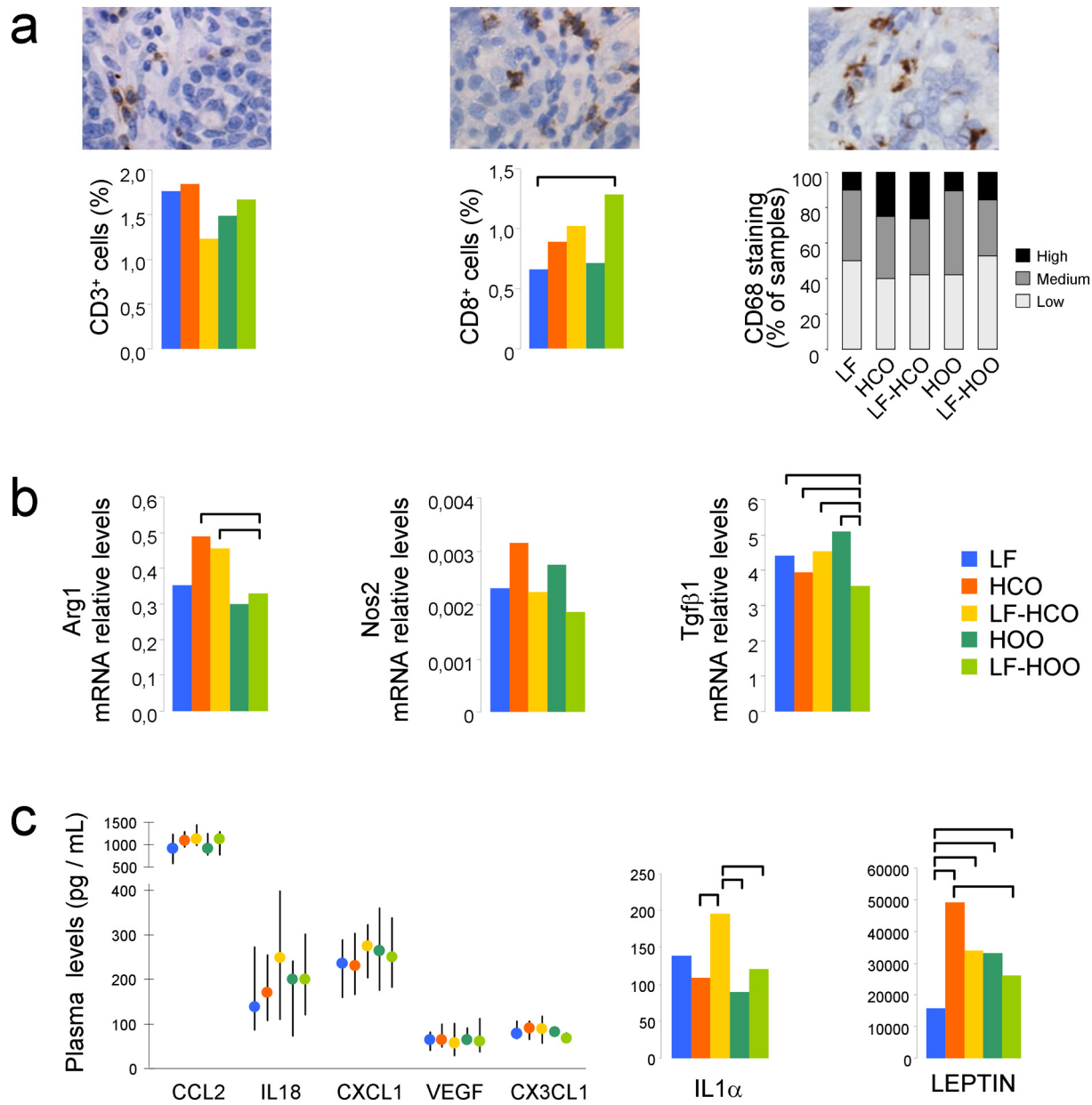

Fig. 6. Effects of high-fat diets on immune system function. (a) Representative images (magnification ×400) and quantification of CD3, CD8 and CD68 immunostaining in the different experimental groups, n=18–20/group. (b) Gene expression of the immunosuppressive mediators arginase 1 (Arg1), nitric oxide synthase 2 (Nos2) and transforming growth factor β 1 (Tgfβ1), n=16–31/group. (c) Circulating levels of inflammation-related cytokines in plasma, n=17–19/group. Bars represent medians, except for CD68. For CCL2, IL18, CXCL1, VEGF and CX3CL1, dots represent medians and whiskers 25th–75th percentiles. Solid lines connecting groups indicate statistically significant differences (P<.05), Mann–Whitney U test.

studies indicated that the experimental tumors from animals fed with this diet, despite being influenced by a high intake of total fat, showed clinical and histopathological characteristics of low aggressiveness, similar to those from control group. Considering the unspecific stimulating effect on carcinogenesis through caloric density [30] and that both experimental high-fat diets were isocaloric, the results suggest a beneficial effect of the EVOO that partially counteracts the deleterious effect of an excessive intake of fat. Differences observed among the assays can be related to the different timing of the dietary intervention, as well as to distinct varieties of oil used, since it has been reported that the influence of olive oil on carcinogenesis depends on the proportions of oleic acid and minor bioactive components [31].

Several molecular mechanisms have been proposed in relation to the effects of dietary fat on carcinogenesis. There is evidence that dietary fatty acids (FA) modulate biological functions through the regulation of gene expression [9,32]. Hence, we analyzed the influence of these high-fat diets on the gene expression profile of mammary adenocarcinomas. Although the number of differentially expressed genes was low, probably due to the intrinsic high variability among tumors, transcriptomic analysis showed different effects of corn oil and EVOO. Biological significance analysis and functional clustering of differentially expressed genes revealed different enriched categories depending on the type of diet administered. The high-n-6-PUFA diet down-modulated genes related to cell death and immune response, whereas the high-EVOO diet up-modulated genes with a role in cell death and proliferation. Moreover, when we divided clusters of genes in commonly modulated (influenced by both kinds of high-fat diets) or specific for each group, among the genes only down-modulated in LF-HCO, apoptosis and immune system were also significantly overrepresented categories. Metabolic processes were also enriched categories by both high-fat diets.

Further expression analyses by real-time PCR have also suggested metabolic adaptation to the increased intake of lipids in all high-fat diets groups, up-regulating the expression of the fatty acid binding protein Fabp3 and down-regulating that of the desaturase enzyme Scd, especially in the EVOO groups. Extensive evidence has been published regarding metabolic dysfunction of cancer cells, but there are little data about how a high intake of FA probably determines the metabolic adaptation of tumor cells. Transformed cells have a highly increased need for FA, and it has been mostly considered that the main source of FA in tumor cells was *de novo* synthesis [33]. However, our expression results point out the importance of the extracellular source of lipids. Besides the adaptation to the high amount of unsaturated FA, our results indicate other metabolic modifications that depend on the type of FA administered. Expression and Western blot analyses suggested enhanced glycolytic pathways by effect of the high-EVOO diet in comparison with the high-corn-oil diet, rising PFKL levels (a key point control of glycolytic pathway) and the glucose transporter Glut1. Nevertheless, recently, the intratumoral compartmentalization of metabolic features and the distinct relevance if enzymes are expressed by cancer epithelial cells or cancer-associated fibroblasts [34] have been pointed out. Further studies are needed to elucidate the significance of the effects of high-fat diets in the context of microenvironment and dynamic interplay among cell types.

On the other hand, several genes related to proliferation/apoptosis pathways were detected as differentially modulated by the high-fat diets, although the variability among tumors resulted in subtle differences, which were later analyzed at the protein level. Sema3a protein has been related to apoptosis, migration and regulation of the tumor microenvironment including the immune cells [35]. While the full-length mRNA was up-regulated in the HOO group, a proteolytic 65-kDa isoform was significantly decreased. Although disparate data have been published, this isoform was originally reported as a fragment with reduced functional activity [36]. Thus, the interpretation of the increased mRNA but decreased cleaved protein by effect of EVOO is unclear but suggests modifications in apoptosis and/or immunosurveillance. In relation to proliferation genes, different trends have also been observed in expression analysis in concordance with the clinical and morphological features of tumors. Smad1 mRNA (a promoter of proliferation and migration [37]) was decreased in both high-olive-oil groups. While little differences were observed in Smad1/5/9 protein levels, the active protein (phosphoSMAD1/5/9) was significantly increased in HCO compared to HOO, which was in accordance with the higher degree of malignancy of tumors from HCO group. Some differences were also observed in the total levels of Stat5A/B proteins, but they did not reach significance. We further determined key proteins in apoptosis (Caspase 3 and its activated cleaved form), finding increased levels of activated Caspase 3 in tumors from LF-HOO which suggested an increase in apoptosis in such tumors.

In relation to immune system, it has been reported that the expression of immune genes reflects leukocytes infiltration in tumor tissue [38]. However, although microarrays indicated down-modulation of Il18 and Il2Rg in LF-HCO group, extended real-time PCR analysis did not confirm such regulation. Since it is likely that subtle modifications in leukocyte infiltration may not be detected in whole tumor extracts, immunohistochemical analyses were conducted. Thus, we observed an increase in the infiltration of cytotoxic T lymphocytes (CD8) in tumors from LF-HOO group. Other authors have also suggested an effect of an EVOO diet on production of CD8 lymphocytes and their infiltration into the colon mucosa of colon-tumor-bearing rats [39], and immunomodulatory effects on B lymphocytes [40] and natural killer cells [41]. In addition, we also evaluated tumor-infiltrating macrophages, a cell population that plays a key role in the generation and maintenance of an immunosuppressive microenvironment within the tumor. The groups fed the HCO diet exhibit increased staining for CD68, the marker of macrophages, but differences between groups were not statistically significant. Nevertheless, other studied markers [42] did suggest differences in the immunosuppressive context of tumors by effect of the dietary intervention, resulting in higher levels of arginase in HCO groups, while in the LF-HOO group, Tgfβ1 was down-modulated. Moreover, plasma levels of the proinflammatory and protumorigenic [43] cytokine IL-1α and those of Leptin were increased in HCO group in comparison with animals fed the high-EVOO diet. Taken together, these results suggest that there is a modulation of the immune system by dietary intervention that would be one of the mechanisms on the differential effects of these high-fat diets on the tumor progression.

Finally, results of microarrays profiling and validation by real-time PCR highlighted the molecular heterogeneity among tumors and within a single tumor. Beyond the limitations of each technique, both methodologies showed concordant results in the same samples, while intertumor variability was manifested when analyses were extended to a higher number of samples. Thus, each tumor has a unique and also heterogeneous molecular context with its single combination of genomic and epigenomic features, where environmental factors may exert subtle but relevant influence. Taking all the molecular results together and considering the effects of these diets on the anatomopathological and clinical characteristics of the mammary tumors, a scenario is plausible in which tumors from animals fed the high-EVOO diet show modulation of pathways conducting to Casp3 mediated apoptosis, perhaps enhanced by Sema3a activity (which could also participate in immunosurveillance). On the other hand, a high intake of corn oil would induce more active proliferation pathways, e.g., increasing the active form of Smad1, and a more inflammatory phenotype.

In conclusion, this work reports several effects of high-fat diets on experimental breast carcinogenesis depending on the type and time of dietary intervention, showing a clear stimulating influence of corn-oil-enriched diet which is manifested on the morphological and clinical characteristics of tumors. The high-EVOO diet, despite having the unspecific promoting effect that all high-fat diets exert, seemed to have some beneficial influence eliciting a progression of tumors more similar to that of the controls. Different and complex molecular mechanisms would drive such effects, including modifications in gene expression profiles and changes in metabolic pathways, alteration in immunosurveillance and modifications in proliferation/apoptosis balance. Thus, these results point out the relevance that dietetic habits from childhood may have on the susceptibility to malignant transformation and the progression of the disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2018.11.001.

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