Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 99 (2022) 108833

Journal of Nutritional Biochemistry

RESEARCH PAPER

A high extra-virgin olive oil diet induces changes in metabolic pathways of experimental mammary tumors

Maite Garcia-Guasch[a], Lourdes Navarro[a], Vanessa Rivero[a], Irmgard Costa[b], Eduard Escrich[a], Raquel Moral[a,*]

[a] Department of Cell Biology, Physiology and Immunology, Physiology Unit, Faculty of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain
[b] Department of Pathology, Corporació Parc Taulí-UDIAT, Sabadell, Barcelona, Spain

Received 31 July 2020; received in revised form 22 February 2021; accepted 6 July 2021

Abstract

Breast cancer is the most common malignancy in women worldwide, and environmental factors, especially diet, have a role in the etiology of this disease. This work aimed to investigate the influence of high fat diets (rich in corn oil or extra virgin olive oil -EVOO-) and the timing of dietary intervention (from weaning or after induction) on tumor metabolism in a seven,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer model in rat. The effects of lipids (oils and fatty acids) have also been investigated in MCF-7 cells. The results have confirmed different effects on tumor progression depending on the type of lipid. Molecular analysis at mRNA, protein and activity level of enzymes of the main metabolic pathways have also shown differences among groups. Thus, the animals fed with the EVOO-enriched diet developed tumors with less degree of clinical and morphological malignancy and showed modified glucose and mitochondrial metabolism when compared to the animals fed with the corn oil-enriched diet. Paradoxically, no clear influence on lipid metabolism by the high fat diets was observed. Considering previous studies on proliferation and apoptosis in the same samples, the results suggest that metabolic changes have a role in the molecular context that results in the modulation of different signaling pathways. Moreover, metabolic characteristics, without the context of other pathways, may not reflect tumor malignancy. The time of dietary intervention plays also a role, suggesting the importance of metabolic plasticity and the relation with mammary gland status when the tumor is induced.

Keywords: Dietary lipids; Extra virgin olive oil; Breast cancer; Metabolism; Carcinogenesis; Glucose metabolism.

1. Introduction

Breast cancer is the most common cancer in women worldwide with high incidence (24.2%), prevalence (30.1%) and mortality (15%) rates [1]. This disease is a multifactorial neoplastic process in which genetic, epigenetic and endocrine factors are involved. Moreover, the environment also has a role in its etiology, being diet one of the most important factors [2]. In this sense, the Mediterranean diet is known as a healthy eating pattern associated with an improvement in health status and with a protective effect in the development of chronic diseases, including breast cancer [3,4,5]. This diet is characterized by high consumption of vegetal products resulting in high intake of monounsaturated fat, complex carbohydrates, fiber, antioxidant substances, polyphenols and other micronutrients, fish, and olive oil as the principal source of fat [6].

Some epidemiological [2,4,5] and especially experimental studies have reported an influence of dietary lipids on breast cancer risk that depends on the type and the quantity of fat, and the timing of dietary intervention [7]. In general, saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) show a positive association with breast cancer risk, whereas n-3 PUFA, as well as the ratio n-3/n-6 PUFA, decrease such risk [8,9,10]. Concerning monounsaturated fatty acids (MUFA), epidemiological evidence remains unclear [10] while experimental studies have reported from a weak-promoting to a protective effect on mammary carcinogenesis [7,11,12]. Several mechanisms underlying such

Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; EVOO, extra-virgin olive oil; FA, fatty acids; HCO, high corn oil; HOO, high extra-virgin olive oil; LF, low-fat; MUFA, monounsaturated fatty acids; PPP, pentose phosphate pathway; PUFA, polyunsaturated fatty acids; TCA, tricarboxylic acid.

* Corresponding author at: Department of Cell Biology, Physiology and Immunology, Physiology Unit, Faculty of Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: 34-93-581-13-41.

E-mail addresses: mariateresa.garcia.guasch@uab.cat (M. Garcia-Guasch), Lourdes.Navarro@uab.cat (L. Navarro), Vanessa.Rivero@uab.cat (V. Rivero), icosta@tauli.cat (I. Costa), Eduard.Escrich@uab.cat (E. Escrich), Raquel.Moral@uab.cat (R. Moral).

https://doi.org/10.1016/j.jnutbio.2021.108833

effects of lipids on breast cancer have been investigated [8]. We have previously reported that dietary lipids influence clinical and morphological features of experimental mammary tumors through different complex molecular mechanisms, including modifications in expression profiles of metabolism genes [7].

Cancer cells accumulate alterations in the main metabolic pathways that sustain elevated rates of proliferation. Such metabolic reprogramming is considered a cancer hallmark [13]. The first metabolic alteration in cancer described was the Warburg Effect, a shift of glucose metabolism to its fermentation into lactate in aerobic conditions. This effect is thought to confer an advantage by producing more building blocks for cell growth and assuring NADPH production for the antioxidant defense [14,15]. Another important metabolic change affects glutamine availability, a key nitrogen and carbon supplier in several reactions [16]. Tumor cells also present increased lipid synthesis which is closely coupled to glucose metabolism and may balance lipogenesis, lipid uptake and lipolysis to maintain lipid homeostasis [17].

In previous works, we have reported different effects of high fat diets on mammary carcinogenesis. A diet high in corn oil had a clear promoting effect while a diet high in extra virgin olive oil (EVOO) had a weak influence [7,11,12]. Extra virgin olive oil and seed oils composition differ on their fatty acid profile and the minor compounds. EVOO is rich in MUFA (oleic acid represents 60-85% of total fatty acids, depending on the oil variety). Moreover, EVOO is rich in minor bioactive compounds (more than 230 have been identified), including polyphenols such as hydroxytyrosol, secoiridoids or flavonoids. Corn oil is rich in n-6 PUFA (50-60% of total fatty acids) and contains some minor compounds such as sterols and tocopherols. The differential effects that high corn oil and high EVOO diets exert on mammary carcinogenesis could be mediated, at least in part, by affecting the expression of different genes involved in proliferation, apoptosis and metabolism. Thus, this work aims to investigate the influence of such diets on tumor metabolism in the rat model of 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer. To this end, we analyzed the main metabolic pathways to gain insight into the role of dietary lipids in breast cancer development and to provide scientific knowledge to establish healthy dietary advice for the population.

2. Materials and methods

2.1. Animals and experimental design

Animals received humane care under a protocol approved following the legislation applicable in this country. Female Sprague-Dawley rats (Charles River Lab, L'Arbresle Cedex, France) at 23 days of age were distributed in five groups depending on the diet administered and the timing of dietary intervention (n=20 each group), and were maintained under standard conditions (Fig. 1). Animals were fed with a control low fat diet (LF), with a high corn oil diet from weaning (HCO) or after induction (LF-HCO), or with a high extra virgin olive oil diet from weaning (HOO) or after induction (LF-HOO). Mammary tumors were induced by oral gavage with a single dose (5 mg) of 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma-Aldrich, St. Louis, MO, USA) at 53 days of age. Rats were euthanized at 236-256 days by decapitation. Tumors were removed, the three diameters were measured, and volume was calculated ($V = 4/3\pi\ 4/3\pi$ [d1/2] x [d2/2] x [d3/2]). A portion of each tumor was fixed in 4% formalin for anatomopathological analysis, and the rest was flash-frozen and stored at -80°C for molecular analysis.

The morphological degree of tumor malignancy was characterized by applying the Scarf-Bloom-Richardson grading method (SBR3, scoring 1-3), and the modified method adapted to rat mammary adenocarcinomas (SBR11, scoring 3-11) that we have previously described [18]. The highest categories indicated the most morphologically aggressive tumors.

2.2. Diets

Three semi-synthetic diets were designed, as described before [19,20,21]: 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). Carbohydrates in the form of dextrose were 67.9% w/w in LF and 45.9% in both high fat diets. The diets also contained protein in the form of casein (18% w/w in the 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 characteristics of lipids, minor compounds and energy composition of diets are given in Supplemental Table 1. Diets were prepared weekly and stored under nitrogen in the dark at 4°C.

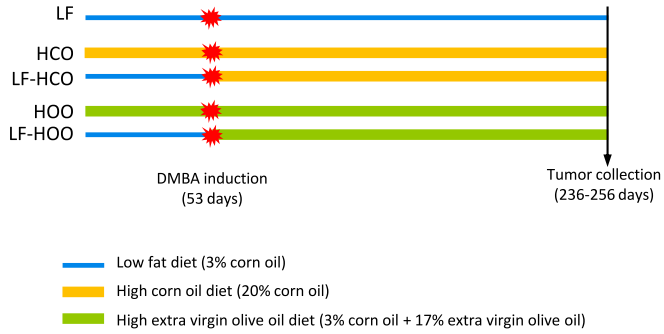

Fig. 1. Experimental design.
Female Sprague-Dawley rats were fed the low fat control diet (LF), the high corn oil diet from weaning (HCO) or from induction (LF-HCO), and the high extra-virgin olive oil diet from weaning (HOO) or from induction (LF-HOO), n = 20 animals / group. Animals were induced with 5 mg of 7,12-dimethylbenz[α]anthracene (DMBA) at 53 days of age and euthanized at 236-256 days.

2.3. RNA extraction, reverse transcription and real-time PCR

Total RNA from tumor samples was isolated using the RNeasy Extraction Kit (QiaGen, Hilden, Germany) and quantified with Nanodrop 1,000 (ThermoFisher Scientific Inc, Waltham, MA, USA). Two micrograms of total RNA were reverse-transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA, USA). For the real-time PCR, 25 ng of cDNA were amplified with the TaqMan methodology in the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Specific Gene TaqMan Assays for glut1, hk2, pfkl, pfkm, g6pdh, pgd, gls and Hprt as the housekeeping control gene were obtained from Applied Biosystems. PCRs were performed at 10min at 95°C followed by 15s at 95°C and 60s at 60°C for 40 cycles. Cycle thresholds (Ct) for each sample were obtained and $2-\Delta Ct$ calculated.

2.4. Protein extraction

Total protein was extracted homogenizing tumor samples in an extraction buffer (50 mM Tris–HCl pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 50 μM NaF, 100 μM Na3VO4, 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich), 10 mM β-mercaptoethanol and 1% Triton X-100). For cytosolic, mitochondrial and membrane extracts samples were fractioned by cold centrifugations at 105.000g for one hour following standard protocols. Protein quantification was determined by Lowry method using the commercial DC Protein Assay Kit (Bio-Rad).

2.5. Western blot

The different protein extracts (15-20 ug) were subjected to SDS-PAGE electrophoresis on an acrylamide gel (Mini-Protean TGX Stain Free Gels, Bio-Rad) and transferred to a PVDF membrane with the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 5%BSA TBS-Tween 0.1% or 5% skimmed milk TBS-Tween 0.1% for 1h at room temperature and incubated with the specific primary antibody overnight at +4°C. Primary antibodies used were anti-Glut1 (1:1,000), anti-PFKL (1:500), anti-ALDOA (1:1,000), anti-G6PDH (1:3,000), anti-PGD (1:5,000), anti-ASCT2 (1:3,000), anti-ACC (1:1,000), anti-pACC (1:3,000), anti-FASN (1:20,000), anti-SCD (1:2,000), anti-AMPK (1:2,000), anti-pAMPK (1:2,000) from cell signaling; anti-LDHA (1:30,000), anti-CPT1a (1:1,000), anti-ACDL (1:3,000), anti-UCP2 (1:5,000), anti-ACLY (1:5,000) from abcam; and anti-GLS (1:1,000) from Thermofisher scientific. After incubation with the secondary antibody (anti-rabbit, anti-mouse or anti-goat, Sigma-Aldrich) for 1h at room temperature, the membranes were incubated with Luminata Forte Western HRP Substrate (EMD Millipore) luminogen for 3-5 minutes. Proteins were visualized using the Chemidoc XRS+ hardware associated with Image Lab Software 5.1-Beta (Bio-Rad). Densitometric values were normalized with the total protein loaded [22] and relativized to an internal control sample loaded in duplicates in all blots [7].

Table 1
Clinical and anatomopaghological characteristics of tumors

	LF	HCO	LF-HCO	HOO	LF-HOO
Carcinogenesis parameters (clinical malignancy)					
Tumor-bearing animals (%)	80[a]	100[b]	100[b]	75[a]	85[a,b]
Tumor yield (total number of tumors)	47[a]	100[b]	87[b,c]	58[a,c]	82[b,c]
Anatomopathological degree (morphological malignancy)					
n	47	98	84	50	81
SBR3, number of tumors 1/2/3	27/14/6 [a]	23/43/32[b,c]	17/29/38 [b]	19/22/9 [a]	34/28/19 [a,c]
degree 1 (%)	*57.4*	*23.4*	*20.3*	*38.0*	*42.0*
degree 2 (%)	*29.8*	*43.9*	*34.5*	*44.0*	*34.6*
degree 3 (%)	*12.8*	*32.7*	*45.2*	*18.0*	*23.4*
adapted SBR11, median	5 [a]	7 [b,c]	8 [c]	7 [a,b,c]	6.5 [a,b]
Characteristics of tumors included in the molecular study					
n	14	26	24	21	25
SBR3, number of tumors 1/2/3	10/3/1[a]	8/10/8 [b,c]	3/9/12 [c]	8/11/2 [a,b,c]	7/14/4[b]
degree 1 (%)	*71.4*	*30.8*	*12.5*	*38.1*	*28.0*
degree 2 (%)	*21.4*	*38.4*	*37.5*	*52.4*	56.0
degree 3 (%)	*7.2*	*30.8*	*50.0*	*9.5*	*16.0*
adapted SBR11, median	5 [a]	7.5 [b,c]	8.5 [b]	6 [a,c]	7 [c]
Tumor volume, median (cm3)	0.60[a]	1.80[b,c]	3.89[c]	1.99[a,b,c]	2.31[a,b]

Values within a row with different superscript letters are significantly different ($P<.05$). Quantitative data (adapted SBR11, tumor volume): Mann-Whitney U test. Qualitative data (tumor-bearing animals, tumor yield, distribution of tumors according to SBR1, SBR2, SBR3): chi-squared test.

2.6. Enzymatic activity assays

For determination of citrate synthase and isocitrate dehydrogenase activity, we used the Citrate Synthase Activity Assay Kit (MAK193, Sigma Aldrich) and Isocitrate Dehydrogenase Activity Assay Kit (MAK062, Sigma Aldrich). Ten mg of tumor tissue were homogenized in 100uL of ice-cold extraction buffer, kept on ice for 10 minutes and centrifuged at 10,000xg for 5 minutes. Supernatants were transferred to fresh tubes and protein concentrations were determined. Activity assays were assessed according to the manufacturer's instructions. The enzymatic activities were determined with coupled enzymes reactions in which resultant products are proportional to activity and are measured spectrophotometrically at 412 nm.

2.7. Cell culture treatment

MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and grown in EMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 0.01 mg/mL of insulin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and tested for mycoplasma periodically.

MCF-7 cells were seeded 48 hours prior to treatments in p100 plates with EMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 0.01 mg/mL of insulin. For fatty acid treatments, oleic or linoleic acid-albumin from bovine serum (Sigma Aldrich) were used at 0mM (control) or 1 mM concentration. All the solutions contained 0.1 mg/mL of BSA. For oils treatments, extra virgin olive oil (EVOO) or corn oil solutions were prepared at 0% (control) or 0.1% oil solubilized in DMSO (0.3%). The EMEM medium was supplemented with 5% FBS and 0.01 mg/mL of insulin. All treatments (fatty acid and oils) were carried out for 24h, 48h, 72h and 96h, and protein was extracted following the standard protein extraction protocols.

2.8. Statistical analysis

Statistical analyses were performed using R Deducer. The statistical test was determined depending on the sample distribution (Shapiro – Wilk test) and the equality of variances (Levene's test). Quantitative parametrical data were analyzed with one-way ANOVA test (t-test equal variance). Non-parametrical data were analyzed with Kruskal Wallis test – wilcoxon method. The chi-squared test was used for the qualitative data (carcinogenesis parameters and distribution of mammary adenocarcinomas according to the degree of morphological malignancy). The level of significance was established at $P<.05$.

3. Results

3.1. Clinical and morphological malignancy of tumors are influenced by dietary lipids

Carcinogenesis parameters and the morphological degree of tumor aggressiveness are shown in Table 1. The groups fed the high corn oil diet had a higher incidence (percentage of tumor-bearing animals) and tumor yield (total number of tumors) than the control LF group. The HCO group had also higher incidence and tumor yield than the HOO group. Morphological degree of malignancy was also clearly higher in the groups fed the high corn oil diet (as shown by the percentage of tumors with high degree of SBR3, and the SBR11 median), especially in the LF-HCO group, whereas the groups fed the EVOO diet displayed intermediate values. Moreover, the subgroup of tumors used in the molecular analyses presented in this work also showed a higher degree of malignancy in the groups fed the high corn oil diet, especially in the LF-HCO one (significantly higher degree in comparison to LF and EVOO groups). Tumor volume was significantly higher in the high corn oil diet groups versus LF, and in LF-HCO versus LF-HOO.

3.2. Dietary lipids influence the mRNA expression of enzymes of the main metabolic pathways

The expression levels of metabolism-related genes were analyzed by RT-PCR and are shown in Fig. 2. In general, tumors from rats fed with the high fat diets showed an increase in mRNA expression of glucose uptake and glycolysis-related genes ($P<.01$, Fig. 2A) and pentose phosphate pathway (PPP) genes ($P<.001$, Fig. 2B) in comparison to tumors from the low fat-fed rats. Gene expression patterns also revealed differences due to the type of dietary lipid. Higher levels of hk2, pfkl and pfkm mRNA were observed in the LF-HCO group compared to the LF-HOO group, while g6pdh and pgd expression was increased in HOO versus HCO. We also observed differences related to the time of dietary intervention, with higher levels of glut1, hk2, pfkl and pgd genes

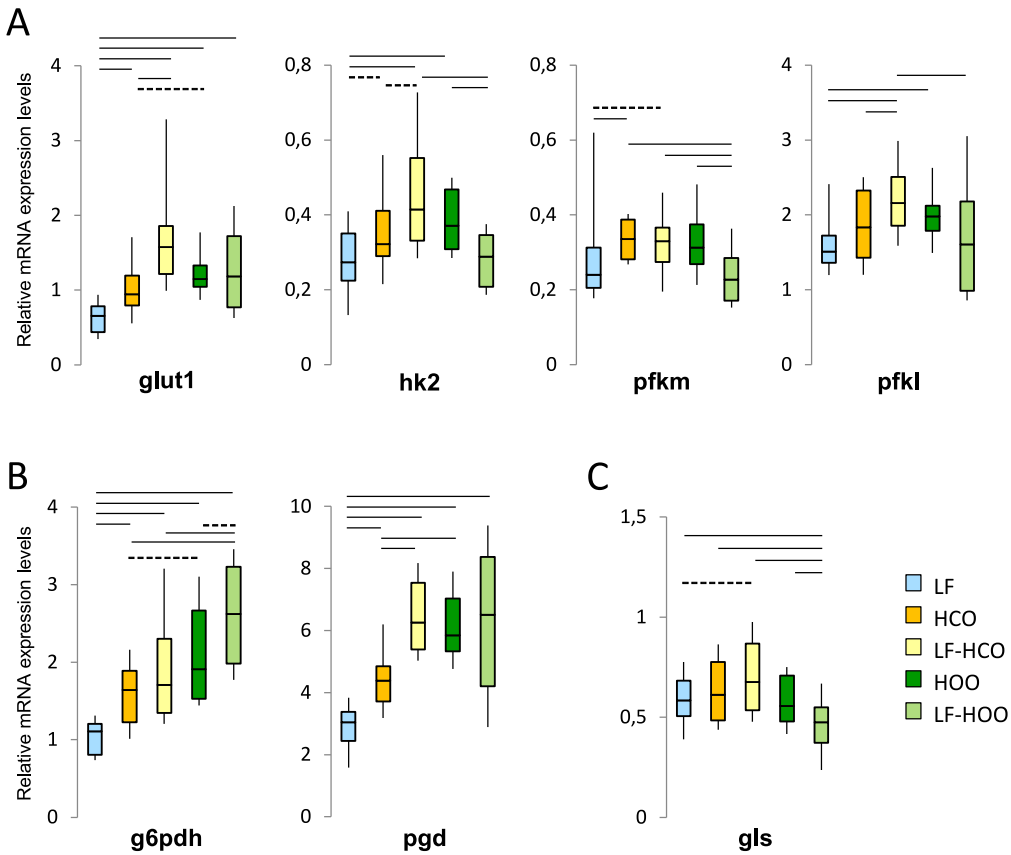

Fig. 2. Effect of diets on relative mRNA expression levels in mammary tumors. (A) Glucose transport and glycolysis-related genes. (B) Pentose phosphate pathway (PPP)-related genes. (C) Glutaminolysis-related gene. Boxplots (median, box 25 and 75 percentile, whisker 10 and 90 percentile). Solid lines connecting groups indicate statistically significant differences ($P<.05$), dashed lines indicate differences close to significance ($P<.1$), Kruskal Wallis test.

in LF-HCO compared to the group fed with the same diet from weaning (HCO). The mRNA levels of hk2 and pfkm were also different between LF-HOO and HOO.

The study of glutaminolysis (Fig. 2C) revealed a decrease in gls mRNA in the LF-HOO group in comparison to all other experimental groups.

3.3. Dietary lipids have an effect on glucose metabolism, glutaminolysis and UCP2 protein expression but not on lipid metabolism-related enzymes

The enzymes of the main metabolic pathways were analyzed by western blot. Fig. 3 depicts the results of enzymes with a role in glucose transport and glycolysis (Fig. 3A), Warburg effect (Fig. 3B) and pentose phosphate pathway -PPP- (Fig. 3C). Changes in protein expression by the effect of dietary lipids were observed in the glucose transporter Glut 1, the glycolysis key enzyme PFKL, and the PPP enzymes G6PDH and PGD, with significant higher protein levels in the HOO group compared to the HCO group. The same trend was observed in PGD when comparing LF-HOO and LF-HCO groups.

In relation to lipid metabolism, the expression levels of the main proteins of fatty acid transport (FATP1), β-oxidation (CPT1a, ACADL) and de novo synthesis (ACLY, FASN, pACC and ACC, SCD) have been analyzed (Fig. 4A). In general, no differences have been found by the effect of the experimental diets.

For the study of glutaminolysis, we have analyzed the expression of the glutamine transporter ASCT2 and GLS enzyme (Fig. 4B). Expression of GLS was increased in HCO and HOO groups in comparison to the LF group.

Analysis of UCP2 was carried out in mitochondrial fractions (Fig. 4C). Results showed higher UCP2 protein levels in the LF-HOO group compared to the control and LF-HCO groups.

3.4. The diet rich in EVOO increases the activity of the main tricarboxylic acid (TCA) cycle enzymes

The results of the enzymatic activity of citrate synthase (CS) and isocitrate dehydrogenase isoenzymes (IDH1-3) are shown in Fig. 5. An increase in CS activity was obtained in both high EVOO diet groups in comparison to LF and high corn oil diet groups. IDH1/2 activity was significantly increased in the LF-HOO group. No differences were observed in IDH3 activity.

3.5. In vitro effects of dietary lipids

MCF-7 cells were treated with fatty acids (oleic acid and linoleic acid) and oils (EVOO and corn oil) for 24, 48, 72 and 96 hours. We analyzed the proteins found as differentially expressed in the DMBA-induced tumors (PFKL, GAPDH, PGD, UCP2) in the fatty acid- and oil-treated cells (supplemental Figure 1). Results showed heterogeneous data depending on lipid, concentration and time of exposures, with no clear trends along time (*e.g.* decreased PFKL by oleic acid at 48h but increased levels at 72h). Proteins related to lipid metabolism in oil-treated cells showed an effect of EVOO increasing the lipid transporter CD36 and decreasing FASN.

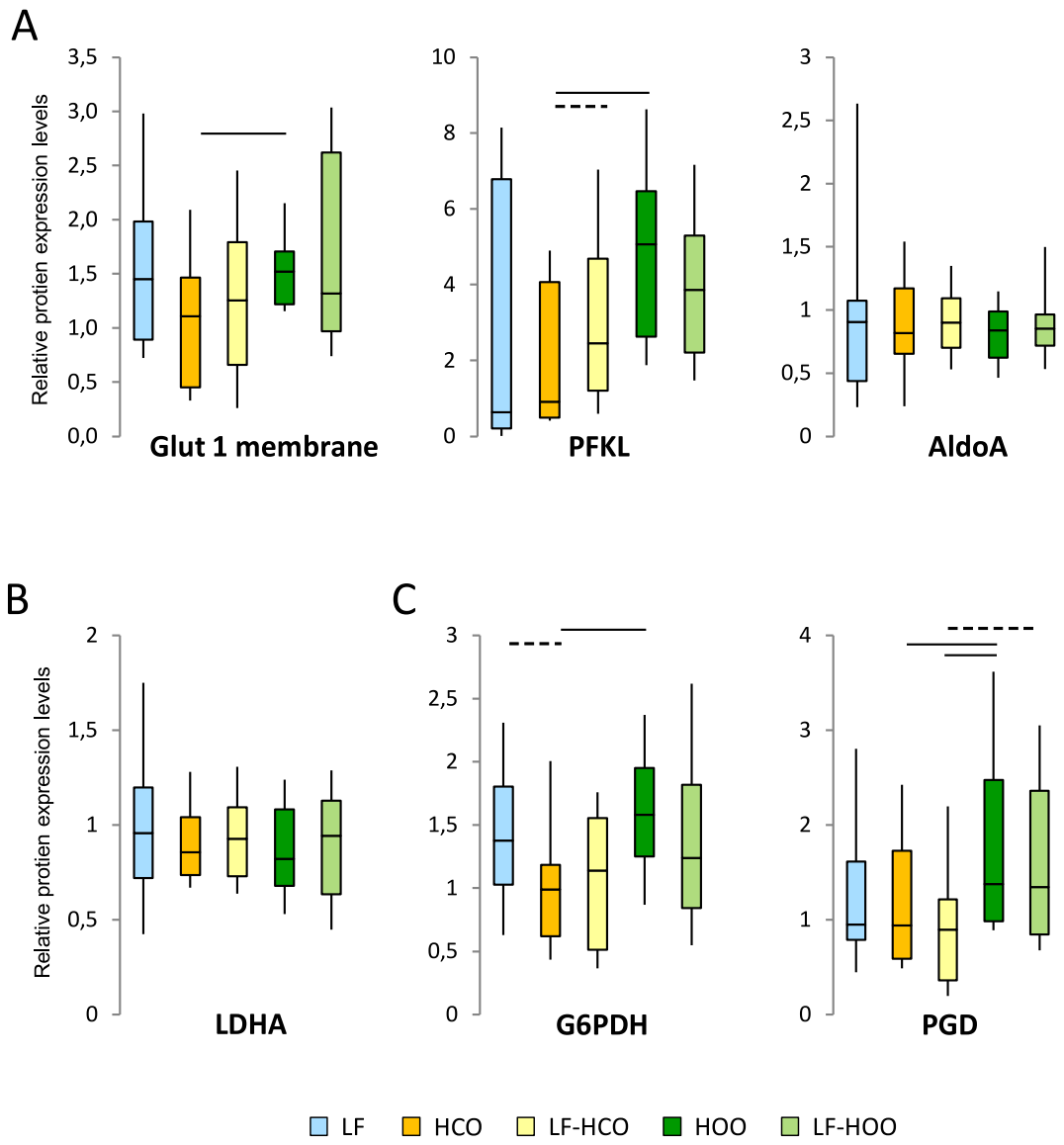

Fig. 3. Effect of diets on protein expression of main carbohydrate metabolic pathways in mammary tumors. (A) Glucose transport and glycolysis related-enzymes. (B) Warburg effect related-enzyme. (C) PPP related-enzymes. Boxplots (median, box 25 and 75 percentile, whisker 10 and 90 percentile). Solid lines connecting groups indicate statistically significant differences ($P<.05$), dashed lines indicate differences close to significance ($P<.1$), Kruskal Wallis test.

4. Discussion

In this work, we have studied the effect of high fat diets on metabolic enzymes (at mRNA, protein or activity level) in a carcinogen-induced breast cancer model, as a mechanism of the influence of diets on the progression of this disease. The results obtained show an effect of the high EVOO diet on glucose and oxidative metabolism and suggest a complex interconnection among metabolism and signaling pathways.

We have previously observed a strong promoter effect of the diet high in corn oil while a weak effect of the diet high in extra virgin olive oil, both in the clinical and morphological manifestation of mammary tumorigenesis [7]. In this study, we have also observed, in the subgroup of tumors where molecular analyses have been carried out, a differential effect of dietary lipids on the anatomopathological degree of malignancy. Tumor phenotype was characterized by both the Scarff–Bloom–Richardson (SBR) grading method (SBR3) and the one adapted to this rat model (SBR11) [23]. Results showed that tumors from rats fed with the high fat diets, especially from the high corn oil diet groups, displayed morphological characteristics of higher degree of malignancy. These results suggest that diets rich in fat may have an unspecific promoting effect on tumorigenesis, but for the same amount of fat there is a different specific effect depending on the main type of dietary lipid.

To get insight into the molecular mechanisms by which dietary lipids influence mammary carcinogenesis we previously performed transcriptomic analyses in tumors from the different experimental groups. Gene expression profiles and enriched functional categories indicated an effect of high fat diets on tumor metabolism, increasing the expression of glycolytic enzymes [7]. In this work, we have validated and extended those results, analyzing the effect of these two different high fat diets on key metabolism pathways. At mRNA level, we found changes in glycolysis and PPP enzymes in the high fat diet groups related to the control, but these differences were not significant at the protein level. Taking in mind that the

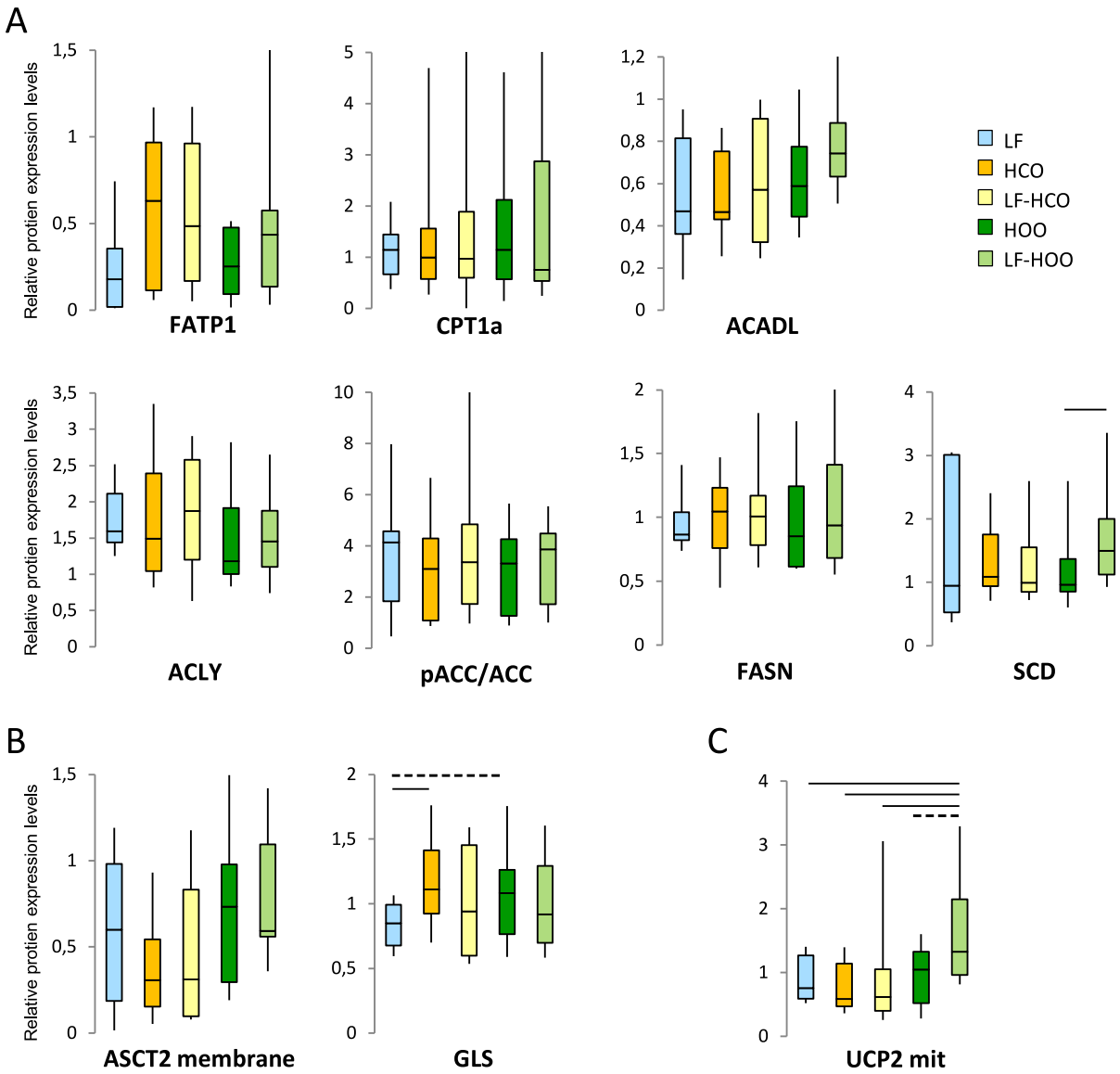

Fig. 4. Effect of diets on the expression of proteins related to lipid metabolism and glutaminolysis, and UCP2, in mammary tumors. (A) Lipid metabolism-related proteins. (B) Glutaminolysis-related proteins. (C) UCP2. Boxplots (median, box 25 and 75 percentile, whisker 10 and 90 percentile). Solid lines connecting groups indicate statistically significant differences ($P<.05$), dashed lines indicate differences close to significance ($P<.1$), Kruskal Wallis test.

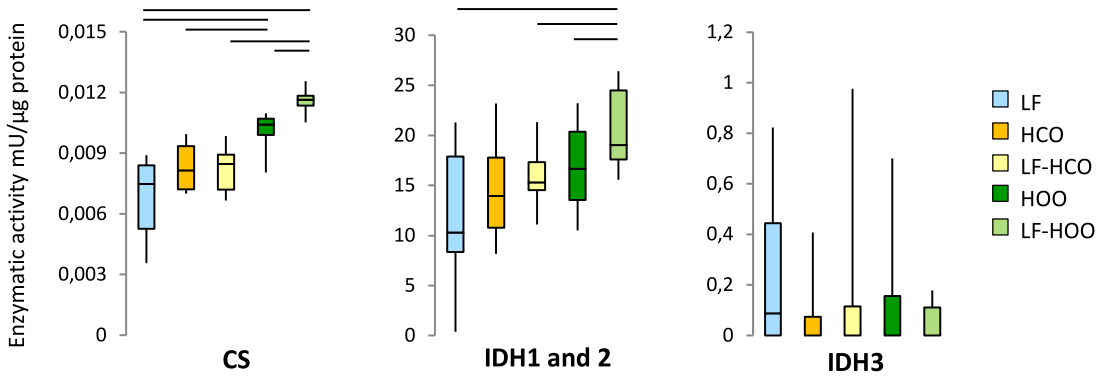

Fig. 5. Effect of diets on the activity of TCA cycle enzymes in mammary tumors. Enzymatic activity of citrate synthase (CS) and isocitrate dehydrogenase (IDH1, IDH2 and IDH3). Boxplots (median, box 25 and 75 percentile, whisker 10 and 90 percentile). Solid lines connecting groups indicate statistically significant differences ($P<.05$), dashed lines indicate differences close to significance ($P<.1$), Kruskal Wallis test.

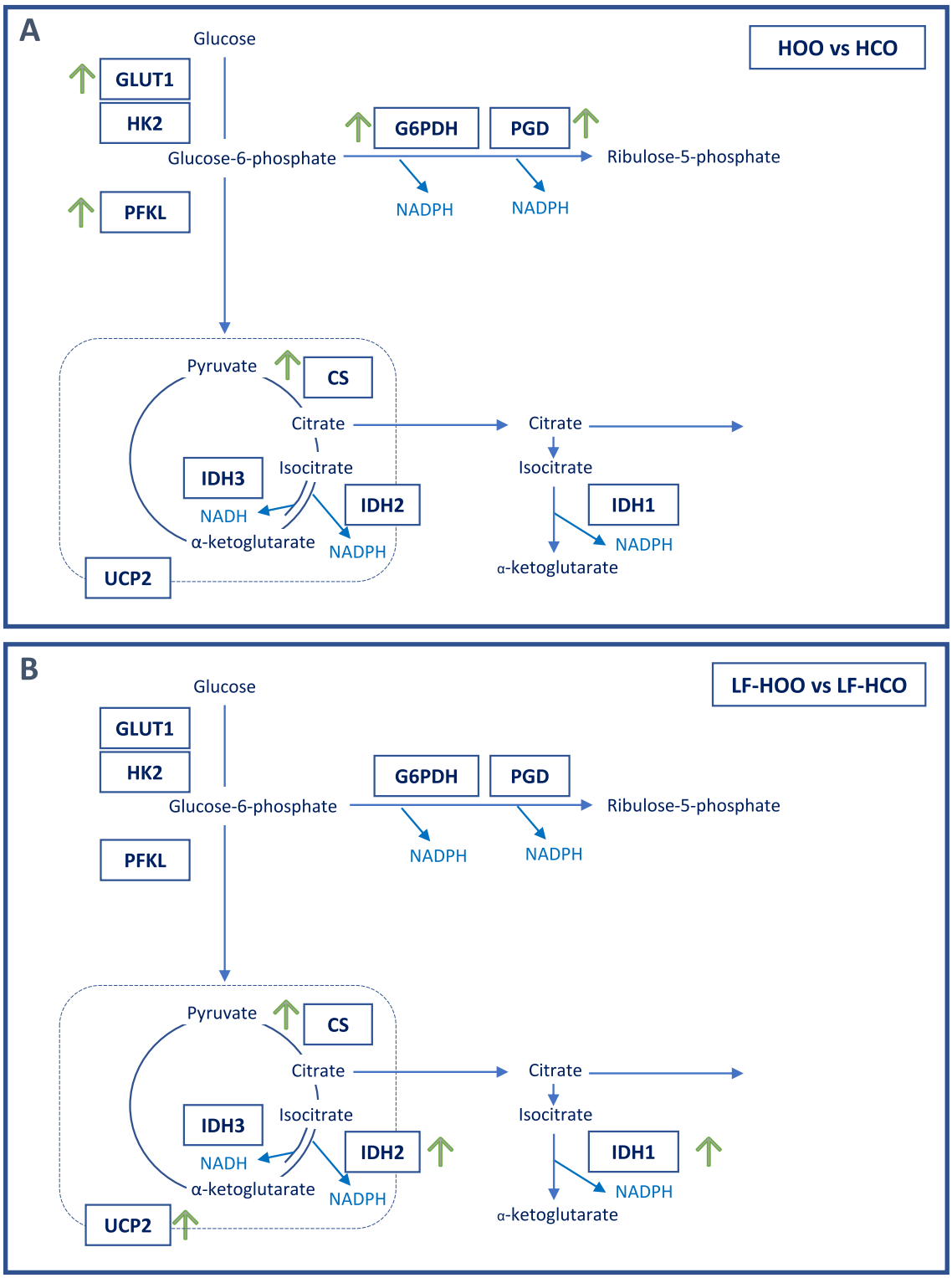

Fig. 6. Effects of the EVOO-rich diet on tumor metabolic pathways. (A) Effect of the administration of the EVOO-rich diet from weaning (comparison of HOO versus HCO). (B) Effect of the administration of the EVOO-rich diet after induction (comparison of LF-HOO versus LF-HCO). Green arrows represent significant higher levels of protein expression or enzymatic activity in the EVOO-rich diet group. Color version of figure is available online.

two high fat diets were isocaloric and that the type of oil was the only difference in their composition, we compared among high fat diet groups. As summed up in Fig. 6, our results suggest that the EVOO-enriched diet provided from weaning (observed in the HOO group) increased the mRNA and protein levels of enzymes with a role in glucose uptake (Glut1), glycolysis (PFKL) and PPP (G6PDH, PGD) and increased the activity of the TCA cycle enzyme citrate synthase (CS). The group fed with the same diet after induction (LF-HOO) showed increased UCP2 protein levels and enzymatic activity of TCA cycle enzymes (CS and IDH).

No differences were found among groups when beta-oxidation, lipid synthesis or protein metabolism were studied. On the other hand, results obtained in MCF-7 cells treated with fatty acid or oils showed subtle and heterogeneous effects on the expression of metabolism proteins. Although the *in vitro* results do not show clear trends in many proteins, treatment with oils suggests changes such as in the lipid transporter CD36 or an increase in FASN (a key protein in fatty acid synthesis) by the effect of corn oil. Therefore, the tumor cells would respond to the exogenous source of lipids, as also suggested by the results of gene expression in tumors. This effect of lipids, although subtle, can be of great importance in the early stages of carcinogenesis. The chronic effect of the dietary intervention may be different in established tumors, such as those analyzed in this work, as many characteristics have already been selected and cannot be separated from the context of other cellular processes. Thus, although caution must be applied when extrapolating in vitro and in vivo effects, both n-6 PUFA and n-9 MUFA may affect tumor metabolism depending on dose or time variables.

There is evidence of the modulation of metabolism genes by dietary lipids in tissues like liver but reported data is less conclusive for tumor tissues [24]. Tumor cells undergo uncontrolled growth that has an impact on the regulation of metabolic pathways, either as a cause or as a consequence [14]. Nowadays, it is known that tumor cells use glycolysis, oxidative phosphorylation, or both, depending on the cell conditions. Moreover, cancer cells can shift their metabolic profile during tumorigenesis, tumor progression and metastasis to provide the requirements needed for cell growth and survival. Actually, metabolic changes and adaptation to maintain cell growth is considered a cancer hallmark [14,25]. Upregulation of glycolysis, PPP, the Warburg effect or IDH1/2 activity in malignant cells may be related to ROS detoxification and increased redox potential via NADPH generation [26,27]. Also, the function of UCP2 (an uncoupling protein family located in the mitochondrial membrane) is thought to be an adaptive response limiting ROS in breast cancer cells and avoiding ROS-mediated apoptosis [28]. Hence, according to reported data in the literature, the metabolic profiles we found in tumors from rats fed the EVOO-enriched diet (in comparison to those from the groups fed the high corn oil diet), may reflect an adaptative response to sustain cell growth or prevent cell death, but this is not in accordance with the clinical and morphological behavior of tumors that we have observed. Thus, anatomopathological and clinical parameters clearly showed a lower degree of aggressiveness in the groups fed the EVOO diet in comparison to the ones fed the high corn oil diet. This apparently contradictory fact may be explained by the interconnection of signaling pathways required for cell growth and survival. Therefore, the results obtained can be part of a complex and interconnected cell net. In this sense, we have previously reported that the EVOO-rich diet influences the molecular context in tumor cells resulting in increased apoptosis and decreased proliferation when compared to the effects of the high corn oil diet [29]. Moreover, in the same experimental model presented here we observed an increase in proliferation proteins by the effect of the high corn oil diet (activated p21Ras, phospho-AKT, phospho-Erk or Myc), while we found higher levels of pro-apoptotic proteins by the effect of the EVOO diet (FADD, Bid, Bax or p53 -unpublished results-).

We have also observed that the groups fed with the EVOO-enriched diet present differences in metabolic profiles depending on the time of the dietary intervention. The LF-HOO group showed higher activity of TCA cycle and UCP2 protein expression. We previously reported an increase in hepatic UCP2 mRNA by the effect of this high EVOO diet [30], in accordance with other authors reporting increased UCP2 by EVOO or oleic acid in different tissues [31]. Although UCP2 has been related to avoiding ROS-mediated apoptosis in cancer [32], our previous results showed higher levels of p53 and pro-apoptotic proteins expression in such LF-HOO group. The tumor suppressor p53 is involved in key cellular processes such as cell cycle arrest, cellular senescence, apoptosis or cell oxidative balance, in addition to the control of metabolism by limiting glycolysis and promoting mitochondrial respiration [33]. On the other hand, the same level of oxidative stress may have different consequences depending on the cell context, e.g. evading or inducing apoptosis depending on the activated signaling pathways [34]. Thus, our results suggest that UCP2 is increased in the LF-HOO group as cell protection from oxidative stress, concomitantly with an increase in apoptotic pathways that would lead to cell death, which would be consistent with the lower morphological malignancy of tumors in this group. Other studies have also reported an effect of EVOO components (polyphenols and oleic acids) on apoptosis in cancer [35]. In tongue squamous cell carcinomas, oleic acid treatment induced apoptosis and autophagy [32], and hydroxytyrosol regulated proliferation and apoptosis-related pathways in DMBA-induced mammary tumors [36].

Results less conclusive were obtained from the HOO group. This group showed an increase in glycolysis and PPP, which has been related to sustained cell growth and a decrease in apoptosis [37]. This metabolic profile favors NADPH generation which may lead to inhibit caspase activation [38]. Thus, an increase in NADPH could be a cause that apoptosis is not increased in this group fed with EVOO. On the other hand, the HOO group showed less activation of proliferation pathways when compared to HCO [29] as well as a lower clinical and anatomopathological degree of malignancy. Therefore, the metabolic profile is not a direct reflection of tumor malignancy since it is interconnected with the whole cell network. More studies are required to deep in and clarify the importance of cell metabolic profiles and its relation to tumor malignancy.

In summary, in this work we have studied the effect of dietary fats on breast cancer tumorigenesis pointing out the importance of the type of lipid. Thus, high fat diets lead to a more malignant phenotype when compared with a low fat diet, but the degree of malignancy is clearly associated with the type of fat. Tumors from animals fed with the EVOO-enriched diet showed a lower degree of malignancy and changes in metabolic pathways, in comparison to those from rats fed with the corn oil-enriched diet. The results obtained, together with previous studies in these same samples, suggest that these metabolic changes have a role in the molecular context that results in the modulation of different signaling pathways in the tumors from the animals fed with EVOO. Therefore, the metabolic characteristics, without the context of other pathways such as proliferation or apoptosis, may not reflect tumor malignancy. Moreover, we have also observed that the time of dietary intervention has a role in metabolic plasticity and that may be related to the status of the mammary gland when the tumor is induced. Future studies in metabolomics will be conducted to elucidate the effects of dietary lipids on the molecular basis and interconnection of different pathways. In vitro analysis with EVOO minor compounds will also get insight into the effect of that oil on tumor metabolism. In conclusion, our study provides new data on the link between dietary lipids and tumor biology, and highlight the importance of dietary habits from early life in the risk of this disease.

Author Contributions

Maite Garcia-Guasch: Investigation, Methodology, Analysis, Visualization, Writing - Original Draft; Lourdes Navarro: Investigation; Vanessa Rivero: Investigation; Irmgard Costa: Methodology, Investigation; Eduard Escrich: Conceptualization, Methodology, Funding acquisition, Resources; Raquel Moral: Conceptualization, Supervision, Analysis, Writing - Review & Editing.

Declaration of Competing Interests

No conflicts of interest.

Funding

This work was supported by grants from “Plan Nacional de I+D+I” (AGL2006-07691); “Fundación Patrimonio Comunal Olivarero (FPCO)” (FPCO2008-165.396; FPCO2013-CF611.084); “Agencia para el Aceite de Oliva del Ministerio de Agricultura, Alimentación y Medio Ambiente” (AAO2008-165.471); “Organización Interprofesional del Aceite de Oliva Español (OIAOE)” (OIP2009-CD165.646), “Departaments de Salut i d’Agricultura, Alimentació i Acció Rural de la Generalitat de Catalunya” (GC2010-165.000), FPCO and OIAOE (FPCO-OIP2016-CF614.087).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108833.

References

[1] IARC Global Cancer Observatory. GLOBOCAN; 2018. Available at https://gco.iarc.fr .
[2] Kotepui M. Diet and risk of breast cancer. Contemp Oncol (Pozn) 2016;20:13–19. doi:10.5114/wo.2014.40560.
[3] Turati F, Carioli G, Bravi F, Ferraroni M, Serraino D, Montella M, et al. Mediterranean diet and breast cancer risk. Nutrients 2018;10:1–11. doi:10.3390/nu10030326.
[4] Li Y, Hu BQ, Wu XJ, Qi XW, Jiang J, Cui X, et al. Adherence to Mediterranean diet and the risk of breast cancer: A meta-analysis. Transl Cancer Res 2018;7:1290–7. doi:10.21037/tcr.2018.10.13.
[5] Buckland G, Travier N, Cottet V, González CA, Luján-Barroso L, Agudo A, et al. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. Int J Cancer 2013;132:2918–27. doi:10.1002/ijc.27958.
[6] Willett WC, Sacks F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, et al. Mediterranean diet pyramid: A cultural model for healthy eating. Am J Clin Nutr 1995;61:1402S–1406S. doi:10.1093/ajcn/61.6.1402S.
[7] Escrich R, Costa I, Moreno M, Cubedo M, Vela E, Escrich E, et al. 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. J Nutr Biochem 2019;64:218–27. doi:10.1016/j.jnutbio.2018.11.001.
[8] Yang B, Ren XL, Fu YQ, Gao JL, Li D. Ratio of n-3/n-6 PUFAs and risk of breast cancer: A meta-analysis of 274135 adult females from 11 independent prospective studies. BMC Cancer 2014;14:1–14. doi:10.1186/1471-2407-14-105.
[9] Huerta-Yépez S, Tirado-Rodriguez AB, Hankinson O. Role of diets rich in omega-3 and omega-6 in the development of cancer. Bol Med Hosp Infant Mex 2016;73:446–56. doi:10.1016/j.bmhimx.2016.11.001.
[10] Binukumar B, Mathew A. Dietary fat and risk of breast cancer. World J Surg Oncol 2005. doi:10.1186/1477-7819-3-45.
[11] Escrich E, Solanas M, Moral R. Olive oil, and other dietary lipids in cancer: experimental approaches. In: Quiles JL, Ramirez-Tortosa MC, Yaqoob P, editors. Olive oil and health. Oxford: CABI Publishing; 2006. p. 317–74.
[12] Escrich E, Solanas M, Moral R, Escrich R. Modulatory Effects and Molecular Mechanisms of Olive Oil and Other Dietary Lipids in Breast Cancer. Curr Pharm Des 2011;17:813–30. doi:10.2174/138161211795428902.
[13] Ward PS, Thompson CB. Metabolic Reprogramming: A Cancer Hallmark Even Warburg Did Not Anticipate. Cancer Cell 2012;21:297–308. doi:10.1016/j.ccr.2012.02.014.
[14] Kroemer G, Pouyssegur J. Tumor Cell Metabolism: Cancer's Achilles' Heel. Cancer Cell 2008;13:472–82. doi:10.1016/j.ccr.2008.05.005.
[15] Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci 2016;41:287. doi:10.1016/j.tibs.2016.01.004.
[16] Michalak KP, Mackowska-Kedziora A, Sobolewski B, Wozniak P. Key Roles of Glutamine Pathways in Reprogramming the Cancer Metabolism. Oxid Med Cell Longev 2015;2015. doi:10.1155/2015/964321.
[17] Zaidi N, Lupien L, Kuemmerle NB, Kinlaw WB, Swinnen JV, Smans K. Lipogenesis and lipolysis: the pathways exploited by the cancer cells to acquire fatty acids. Prog Lipid Res 2013;52:585–9. doi:10.1016/j.plipres.2013.08.005.
[18] Costa I, Solanas M, Escrich E. Histopathologic characterization of mammary neoplastic lesions induced with 7,12 dimethylbenz(α)anthracene in the rat: A comparative analysis with human breast tumors. Arch Pathol Lab Med 2002;126:915–27. doi:10.5858/2002-126-0915-HCOMNL.
[19] Escrich E, Solanas M, Segura R. Experimental diets for the study of lipid influence on the induced mammary carcinoma in rats: I - Diet definition. In Vivo 1994;8:1099–105.
[20] Escrich E, Solanas M, Ruiz De Villa M, Ribalta T, Muntane J, Segura R. Experimental diets for the study of lipid influence on the induced mammary carcinoma in rats: II-suitability of the diets. In Vivo 1994;8:1107–11.
[21] Solanas M, Hurtado A, Costa I, Moral R, Menéndez JA, Colomer R, et al. Effects of a high olive oil diet on the clinical behaviour and histopathological features of rat DMBA-induced mammary tumors compared with a high corn oil diet. Int J Oncol 2002;21:745–53. doi:10.3892/ijo.21.4.745.
[22] Gürtler A, Kunz N, Gomolka M, Hornhardt S, Friedl AA, McDonald K, et al. Stain-Free technology as a normalization tool in Western blot analysis. Anal Biochem 2013;433:105–11. doi:10.1016/j.ab.2012.10.010.
[23] Moral R, Escrich R, Solanas M, Vela E, Costa I, De Villa MCR, et al. Diets high in corn oil or extra-virgin olive oil provided from weaning advance sexual maturation and differentially modify susceptibility to mammary carcinogenesis in female rats. Nutr Cancer 2011;63:410–20. doi:10.1080/01635581.2011.535956.
[24] Jump DB. Fatty Acid Regulation of Gene Transcription. Crit Rev Clin Lab Sci 2004;41:41–78. doi:10.1080/10408360490278341.
[25] Bishal Paudel B, Quaranta V. Metabolic plasticity meets gene regulation. Proc Natl Acad Sci U S A 2019;116:3370–2. doi:10.1073/pnas.1900169116.
[26] Jin L, Zhou Y. Crucial role of the pentose phosphate pathway in malignant tumors. Oncol Lett 2019;17:4213–21. doi:10.3892/ol.2019.10112.
[27] Ghanbari Movahed Z, Rastegari-Pouyani M, Mohammadi M hossein, Mansouri K. Cancer cells change their glucose metabolism to overcome increased ROS: One step from cancer cell to cancer stem cell? Biomed Pharmacother 2019;112:108690. doi:10.1016/j.biopha.2019.108690.
[28] Vozza A, Parisi G, De Leonardis F, Lasorsa FM, Castegna A, Amorese D, et al. UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. Proc Natl Acad Sci U S A 2014;111:960–5. doi:10.1073/pnas.1317400111.
[29] Solanas M, Grau L, Moral R, Vela E, Escrich R, Escrich E. Dietary olive oil and corn oil differentially affect experimental breast cancer through distinct modulation of the p21Ras signaling and the proliferation-apoptosis balance. Carcinogenesis 2010;31:871–9. doi:10.1093/carcin/bgp243.
[30] Moral R, Escrich R, Solanas M, Vela E, Ruiz de Villa MC, Escrich E. Diets high in corn oil or extra-virgin olive oil differentially modify the gene expression profile of the mammary gland and influence experimental breast cancer susceptibility. Eur J Nutr 2016;55:1397–409. doi:10.1007/s00394-015-0958-2.
[31] Gonçalves-de-Albuquerque CF, Medeiros-de-Moraes IM, De Oliveira FMJ, Burth P, Bozza PT, Faria MVC, et al. Omega-9 Oleic Acid Induces Fatty Acid Oxidation and Decreases Organ Dysfunction and Mortality in Experimental Sepsis. PLoS One 2016;11:1–18. doi:10.1371/journal.pone.0153607.
[32] Jiang L, Wang W, He Q, Wu Y, Lu Z, Sun J, et al. Oleic acid induces apoptosis and autophagy in the treatment of Tongue Squamous cell carcinomas. Sci Rep 2017;7:1–11. doi:10.1038/s41598-017-11842-5.
[33] Matsuura K, Canfield K, Feng W, Kurokawa M. Metabolic Regulation of Apoptosis in Cancer. Int Rev Cell Mol Biol 2016;327:43–87. doi:10.1016/bs.ircmb.2016.06.006.
[34] Redza-Dutordoir M, DA Averill-Bates. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 2016;1863:2977–92. doi:10.1016/j.bbamcr.2016.09.012.
[35] Keating E, Martel F. Antimetabolic Effects of Polyphenols in Breast Cancer Cells: Focus on Glucose Uptake and Metabolism. Front Nutr 2018;5. doi:10.3389/fnut.2018.00025.
[36] El-azem N, Pulido-Moran M, Ramirez-Tortosa CL, Quiles JL, Cara FE, Sanchez-Rovira P, et al. Modulation by hydroxytyrosol of oxidative stress and antitumor activities of paclitaxel in breast cancer. Eur J Nutr 2019;58:1203–11. doi:10.1007/s00394-018-1638-9.
[37] Majors BS, Betenbaugh MJ, Chiang GG. Links between metabolism and apoptosis in mammalian cells: Applications for anti-apoptosis engineering. Metab Eng 2007;9:317–26. doi:10.1016/j.ymben.2007.05.003.
[38] Yi CH, Vakifahmetoglu-Norberg H, Yuan J. Integration of apoptosis and metabolism. Cold Spring Harb Symp Quant Biol 2011;76:375–87. doi:10.1101/sqb.2011.76.010777.