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Interplay Between Western Diet and Mammary Cancer: Data from a Chemically-induced Model in Wistar Rats

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Abstract. *Background/Aim: This study aimed to investigate the influence of Western diet on mammary cancer in Wistar female rats, focusing on systemic responses and tumor development. Materials and Methods: Twenty-eight Wistar female rats were acclimatized and divided into four experimental groups (n=7 each): Western diet (WD), Western diet with N-methyl-N-nitrosourea (MNU) administration (WD+MNU), standard diet (CTR), and standard diet with MNU administration (CTR+MNU). MNU was administered intraperitoneally at 50 mg/kg at seven weeks of age to induce mammary cancer. The 20-week experiment involved monitoring animal weight, food and water intake. At the end of the study, rats were euthanized, and blood samples and organs were collected for hematological and plasma biochemical analysis, oxidative stress, and histo-pathological and immunobiological evaluations of the tumors. Results: No significant differences were found in body weight, composition, or organ weights, but the WD group showed reduced food and water intake and lower cholesterol levels. Leptin and adiponectin levels were higher in the WD+MNU group, suggestive of changes in appetite regulation. Histopathological analysis showed malignant tumors in both MNU-induced groups. However, WD groups had fewer tumors compared to the CTR+MNU group. Conclusion: WD led to higher feed efficiency and increased visceral adipose tissue but*

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decreased systemic cholesterol and triglyceride levels. While this diet resulted in lower tumor incidence, the volume and weight of the tumors were higher. Additionally, the WD decreased ERα and progesterone receptor immunoexpression, while Ki-67 immunoexpression was elevated.

Breast cancer is one of the most common forms of cancer affecting women worldwide (1). The incidence of this type of cancer varies globally, with higher rates observed in developed countries (2). In 2022, 2.3 million new cases of breast cancer were diagnosed, placing this disease among the most frequently diagnosed and the leading cause of cancer-related deaths among women globally (66,103 deaths in 2022) (3, 4). The precise etiology of breast cancer remains elusive, although a number of risk factors have been identified, including genetic mutations, a family history of breast cancer, age, and lifestyle factors, such as diet and lack of physical activity (5, 6).

In the field of breast cancer research, animal models represent a crucial resource for investigating disease mechanisms and developing new and more effective therapeutic approaches to improve the lifestyle and quality of life of oncological patients (7, 8). Among the various models available, the Wistar female rats stand out for their extensive use and significant contributions to breast cancer research (9).

MNU (*N*-methyl-*N*-nitrosourea) is a chemical carcinogen commonly used in research to induce tumors in experimental animal models. The mammary tumors induced by MNU in these models closely resemble human breast cancer due to their histopathological features, hormone receptor statuses, and molecular characteristics. Due to these pathophysiological similarities to women breast cancer, MNU-induced tumors serve as a valuable resource for diagnosis purposes, biopathological studies and to evaluate responses to various lifestyles (10).

The adoption of a healthy lifestyle has been demonstrated to significantly reduce the risk of developing breast cancer (11, 12). Key factors include maintaining a balanced diet, regularly engaging in physical activity to regulate hormones, boosting the immune system, and keeping a healthy weight (13). These practices are crucial in reducing the risk of breast cancer. Furthermore, healthy lifestyle can also positively impact treatment outcomes and enhance quality of life (12, 14).

A WD comprises pre-packaged foods, refined grains, red and processed meats, sugary drinks, candies, sweets and fried foods. This diet is characterized by excessive calorie intake, and has attracted attention because of its association with adverse health outcomes (15). These include weight gain and metabolic changes, such as, insulin resistance, increased blood sugar levels, elevated triglycerides, and higher cholesterol levels. Such diets are typically high in calories, poor in nutrients, rich in sugar and unhealthy fats. They have been identified as significant contributors to obesity, metabolic syndrome, and an increased risk of diseases, including cancer (16, 17).

The interactions between high-fat diets and breast cancer are a subject of active investigation. Studies have indicated a correlation between obesity, excessive calorie consumption, and an increased risk of breast cancer. This relationship may be mediated by hormonal imbalances, chronic inflammation, and disrupted insulin signaling (18). Visceral adipose tissue is a significant site for estrogen production, particularly after menopause (19). In obese individuals, the higher amount of fat tissue leads to increased estrogen levels, which are associated with the development and progression of hormone-receptor-positive breast cancer. Estrogen can stimulate the growth of breast cancer cells (20).

This research assessed how different diets, specifically a high-fat diet influences the development and progression of mammary cancer in a controlled animal model.

Materials and Methods

Animals and experimental design. Twenty-eight female Wistar rats (Rattus norvegicus), aged 4 weeks, were obtained from Envigo RMS Spain S.L. (Barcelona, Spain). The animals were housed in the facilities of the University of Trás-os-Montes and Alto Douro, under controlled conditions of humidity (50±10%), temperature (23±2˚C), air system filtration (10-20 ventilations/hour) and a 12h:12h light (8 a.m.): dark (8 p.m.) cycle. The Western diet (WD) groups were fed a high-fat diet comprising 60% of total calories derived from fat (Kcal from: protein 18.3%, carbohydrate 21.4% and fat 60.3%) (MD.06414, Envigo), while the control groups received a standard diet (CTR) (Kcal from: protein 20%, carbohydrate 67% and fat 13%) (2014 Teklad Global Rodent diet, Envigo). Tap water and food were provided *ad libitum*. All biosecurity standards for studies using animal models were respected (European Directive 2010/60/EU and National Decree-Law 113/2013). The experimental protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Alimentação e Veterinária) approval no. 04583, and by an Ethics Review Committee – Animal Welfare and Ethics Review Body ref. 834-e-CITAB-2020.

Experimental protocol. Following one-week of acclimatization to the animal facilities, the animals were randomly assigned to four experimental groups (n=7 *per* group): Western diet (WD), Western diet with *N*-methyl-*N*-nitrosourea (MNU) administration (WD+MNU), standard diet (CTR), and standard diet with MNU administration (CTR+MNU). At the fifth week of age, the animals began to be fed their corresponding diets and remained acclimatized until the administration of MNU. At seven weeks of age, animals from the MNU-induced groups received an intraperitoneal injection of the carcinogen MNU (50 mg/kg) (Fluorochem, Hadfield, UK) dissolved in saline solution (NaCl 0.9%, B. Braun, Melsungen, Germany). The solution was administered within one hour of preparation, while the non-induced groups were administered with the vehicle (0.5 ml NaCl 0.9%, B. Braun) intraperitoneally (Figure 1). Following the MNU or vehicle administration, the health status of the animals was evaluated daily, and the palpation of mammary glands was performed by two researchers twice a week. The time of appearance of the first tumor (latency period) and the total number of tumors in each group were recorded. The animal welfare

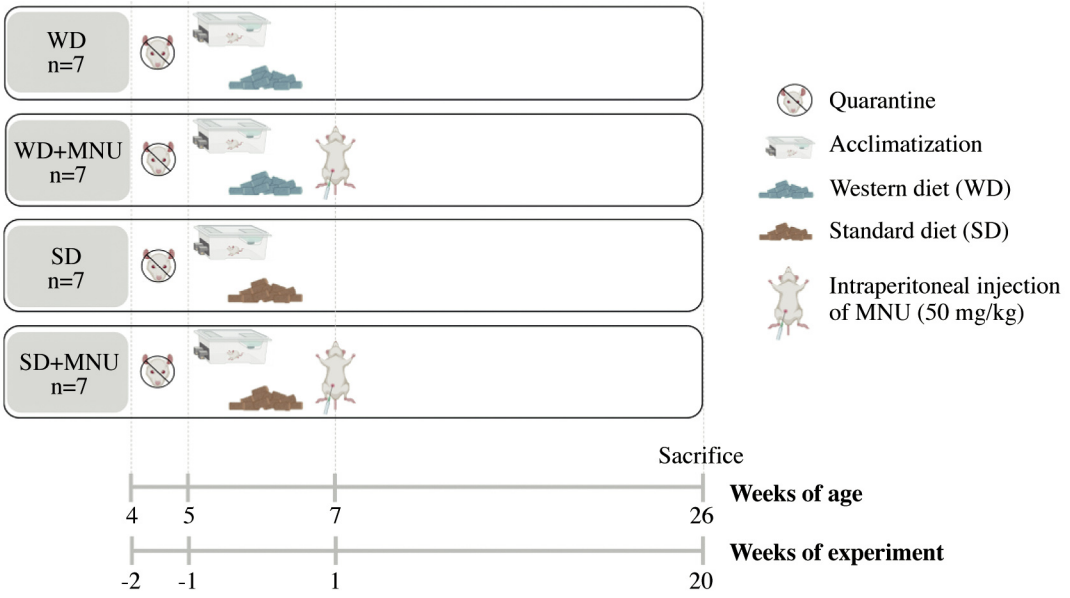

Figure 1. *Experimental protocol scheme performed in each group: WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer-induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=7).*

was evaluated, once a week, using a previously established table of humane endpoints by Faustino-Rocha *et al*., 2019 (21). We evaluated aspects, including general appearance (body condition, body weight, food and water intake, posture, coat and grooming, mucosal, eyes, ears and whiskers), behavior (response to external stimuli) and clinical signs, such as hydration status (assessed by gently lifting the skin on the animals' backs, noting that the skin does not immediately snap back due to reduced turgor), respiratory rate (counting breaths *per* minute), heart rate (counting heartbeats *per* minute), body temperature (measured with a thermometer), and the location and macroscopic appearance of mammary tumors.

Body condition and feed efficiency. The body weight (BW) of the animals, as well as the food and water weights were recorded on a weekly basis to estimate BW variations, and food and water consumption. At the end of the experimental protocol, ponderal weight gain was calculated by subtracting the initial BW from the final BW, dividing by the initial BW and multiplying by 100 (22, 23). The Lee index was calculated as the cube root of the final BW divided by the naso-anal length of the animal multiplied by 100 (24). Body mass index was calculated from the ratio of the final BW to the naso-anal length squared (23, 25). Additionally, the specific rate of body mass gain was calculated from the ratio of the difference between the final BW and the initial BW, to the initial BW (23). Feed efficiency was determined by dividing the difference between the final BW and the initial BW by the total amount of food eaten during the experiment (24, 26). The formulas used are referenced in Table I.

Sacrifice and necropsy of animals. At the end of the 20-week experimental period, all animals underwent a 12-hour fasting period before being euthanized via intraperitoneal injection of ketamine (75 mg/kg, Imalgene 1000, Merial SA, Lyon, France) and xylazine (10 mg/kg, Rompun 20%, Bayer Healthcare S.A., Kiel, Germany), followed by exsanguination through cardiac puncture. Blood samples obtained from exsanguination were collected into lithium-heparin tubes and centrifuged for 15 min at 1,500 ×g (Heraeus Labofuge 400R, Thermo Fisher Scientific, Waltham, MA, USA). The plasma obtained was stored at −80˚C for subsequent biochemical determinations. Following euthanasia, all animals were scalped, and the skin was carefully observed under a light to detect the presence of mammary tumors (27). The number of tumors was recorded, and they were excised and weighed. Finally, tumor weight was subtracted from the BW to determine the accurate BW. Tumor volume was calculated as previously described by Faustino-Rocha *et al*. (28). After being weighed, mammary tumors, internal organs (heart, lungs, kidneys, spleen, liver) and visceral adipose tissue were immediately fixed in 10% phosphate-buffered formaldehyde.

Blood samples analysis. The following biochemical parameters were determined from the collected blood derived plasma samples: albumin, cholesterol, glucose, triglycerides, and urea using an autoanalyzer (Prestige 24i, PZ Cormay S.A., Łomianki, Poland). Total proteins were measured using of an optical refractometer (URC-PN, ATAGO Co., Ltd, Tokyo, Japan) For microhematocrit analysis, two heparinized capillary tubes were filled with blood from each animal. Then, blood was centrifuged using a Pro-Vet centrifuge (Centurion Scientific Limited, West Sussex, UK) at 13,500 × *g* for 5 min. The microhematocrit value was measured using a microhematocrit reader. Plasma samples for the determination of C-reactive protein (CRP), adiponectin and leptin, were diluted at a ratio of 1:20 in TBS (10 mM Tris, pH 8.0, 0.15 M NaCl) and loaded onto a nitrocellulose membrane using the HYBRI-SLOT® Manifold (Whatman Biometra, Marlborough, MA,

Table I. *Formulas used to evaluate the diets and animal conditions in the experimental work.*

Parameter	Formula	Reference
Ponderal gain (%)	$\frac{\textit{Final body weight (g)} - \textit{Initial body weight (g)}}{\textit{Initial body weight (g)}} \times 100$	(57)
Lee index	$\frac{\sqrt[3]{\textit{Final body weight (g)}}}{\textit{Nasoanal length (cm)}} \times 100$	(24)
Body mass index	$\frac{\textit{Final body weight (g)}}{\textit{Nasoanal lenght}^2\ (\textit{cm}^2)}$	(23, 25)
Specific rate of body mass gain (g/kg)	$\frac{\textit{Final body weight (g)} - \textit{Initial body weight (g)}}{\textit{Initial body weight (g)}}$	(23)
Feed efficiency (%)	$\frac{\textit{Final body weight (g)} - \textit{Initial body weight (g)}}{\textit{Total amount of food eaten (g)}} \times 100$	(26)

USA), previously activated in a 10% methanol solution. All analyses were repeated six times on each membrane, using two membranes, for a total of 12 replicates *per* group. After sample application under vacuum, membranes were stained with Ponceau S for protein loading control. Subsequently, the membranes were incubated with a 5% (w/v) non-fat milk blocking solution in TBS-T (TBS and 0.5% Tween 20) for 90 min at room temperature. The primary antibodies: anti-leptin (rabbit, ab16227, Abcam, Cambridge, MA, USA), anti-adiponectin (mouse, ab22554, Abcam), and anti-CRP (rabbit, ab32412, Abcam) were incubated overnight in a dilution of 1:1,000. After washing, with TBS-T three times, 10 min each, to remove the unbound antibodies, and then incubated with specific anti-rabbit or anti-mouse peroxidase secondary antibodies (anti-mouse, 1:1,000, NA931V, GE Healthcare, Chicago, IL, USA, and anti-rabbit, 1:1,000, NA934V, GE Healthcare) for 90 min at room temperature. Finally, the membranes were exposed to Chemiluminescence ECL reagent, and images were captured and analyzed using the ChemiDoc XR System (Bio-Rad, Advansta, Hercules CA, USA) and Image Lab software (Hercules, CA, USA), respectively. The optical densities obtained were expressed in arbitrary units.

Oxidative stress. The activity of superoxide dismutase (SOD) and catalase (CAT) were evaluated as markers of oxidative stress. Each liver lobe sample and a portion of the kidney was thawed and homogenized using a Potter homogenizer in cold phosphate-buffered saline (100 mM-EDTA 1 mM, pH 7.4). The samples were homogenized in an ice bath using an ultrasound processor (4×20 s, intermittent 20 s). After homogenization, the samples were centrifuged at 2,000 × *g* for 10 min. The resulting supernatant underwent a second centrifugation at 1,200 × *g* for 10 min, and the last supernatant was collected in an Eppendorf tube for further analysis.

The enzymatic activity results were normalized to the protein content of the samples, which was determined using a BioTek Gen5™ (Powerwave XS2, BioTek Instruments, Inc. Winooski, VT, USA) based on the absorbance measurements at 280 nm.

Histopathology and immunohistochemistry. All mammary tumors underwent standard histological processing. Three μm thickness paraffin sections were cut and stained with hematoxylin and eosin (H&E). The histological evaluation of mammary tumors was based on criteria established by Russo and Russo under a light microscope by an experienced pathologist (29).

The NovoLink Polymer Detection System (Leica Biosystems, Newcastle, UK) was used for the immunohistochemical detection of estrogen receptor alpha (ERα, clone 6F11, Novocastra, Newcastle, UK), progesterone receptor (clone SP2, Abcam), and Ki-67 (clone SP6, Abcam).

To evaluate immunoexpression, a minimum of 1000 neoplastic cells *per* mammary tumor were assessed, and the proportion of immunopositivity cells was calculated using the ImmunoRatio plugin in the ImageJ program [National Institute of Health (NIH), Bethesda, MD, USA]. The images, at a magnification of 400× objective (Nikon E600 diagnostic microscope, Tokyo, Japan), were analyzed using automated cell counting. Results were presented as percentage of immunopositive cells.

Statistical analysis. The data were statistically analyzed using SPSS version 26 (IBM, Chicago, IL, USA). Continuous data were compared among groups using analysis of variance (two-way ANOVA) for independent samples, followed by *post-hoc* Tukey test for multiple comparisons. The association between the number of tumors, the histological tumors and groups was examined using the Chi-square test. The immunohistochemistry assessment and the tumor volumes were statistically evaluated using a *t*-test. The Pearson correlation was utilized to assess the relationship between the ponderal gain weight and the feed efficiency. Statistical significance was determined at $p<0.05$.

Table II. *Initial and final body weight, ponderal weight gain, tumor weight and accurate body weight in all experimental groups (mean±SD).*

Group	Initial body weight (g)	Final body weight (g)	Ponderal weight gain (%)	Tumor weight (g)	Accurate body weight (g)
WD	153.19±8.53	290.28±25.36*	90.30±22.69	-	290.28±25.36
WD+MNU	152.80±10.78	284.56±24.22*	86.07±20.46**	4.94±6.29	283.15±24.01
CTR	137.47±11.99	278.71±10.47*	104.28±22.07	-	278.71±10.47
CTR+MNU	124.30±7.8	276.16±30.90*	120.47±16.82	4.61±0.01	274.32±31.18

*$p<0.001$ when compared with initial body weight. **$p<0.05$ when compared with CTR+MNU group. WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5).

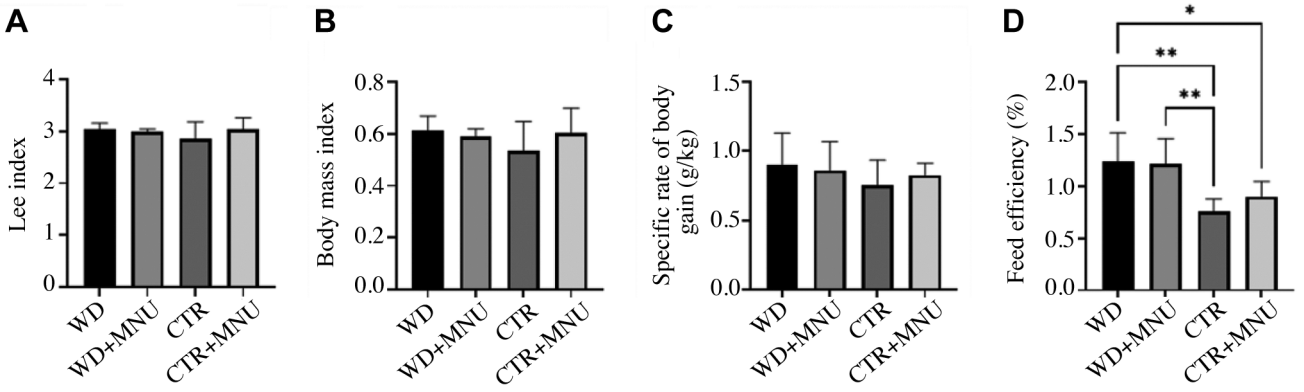

Figure 2. *Lee index (A), body mass index (B), specific rate of body gain (C) and feed efficiency (D) in all experimental groups. *p<0.05; **p<0.005. WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5).*

Results

Animals. Two animals from the CTR+MNU group displayed changes in specific humane endpoint parameters, including unresponsiveness to external stimuli, tumor ulceration, decreased body weight, anemia, and lack of grooming. Considering the critical limit for animal welfare assessment was exceeded, these animals were humanely euthanized prior to the conclusion of the experiment at the 12^{th} and 17^{th} weeks, respectively. The data obtained from these animals were excluded from the study. The remaining animals that reached the end of the experiment did not exhibit any signs of distress or suffering based on the monitored humane endpoint parameters.

Body condition and tumor weight. Although the assignment of animals to cages was randomized, the initial BW was slightly higher in the animals of the WD group, when compared with those of the CTR group (Table II). A significant increase in body weight was observed from the first to the final week of the experimental protocol ($p<0.001$). Ponderal weight gain confirmed this overall increase in BW in all groups. It is worth noting that the WD+MNU group had a significantly lower ponderal weight gain when compared to the CTR+MNU group ($p<0.05$). Total tumor weight was similar between the WD+MNU and CTR+MNU groups. The accurate body weight did not show any statistically significant differences among groups, although it was slightly lower in the CTR groups when compared with the WD groups ($p>0.05$).

Statistical analysis revealed no significant differences among the groups regarding the Lee index, body mass index, and specific rate of body mass gain ($p>0.05$). However, the groups fed with the high-calorie diet (WD and WD+MNU) demonstrated a greater feed efficiency when compared to those fed with the standard diet (CTR and CTR+MNU group) ($p<0.05$) (Figure 2).

A significant positive correlation was found between final body weight and feed efficiency in the WD group (r=0.9175, $p<0.001$) (Figure 3A) and SD group (r=0.3776, p=0.018) (Figure 3B).

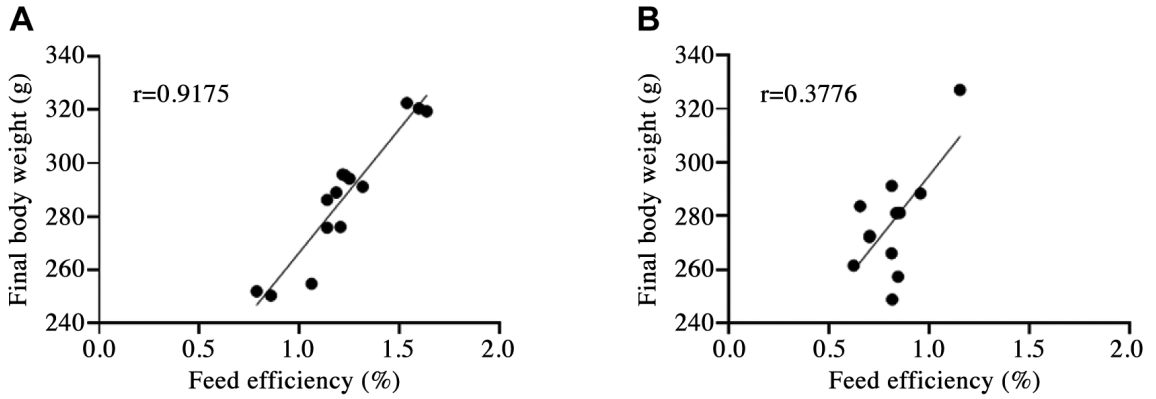

Figure 3. *Correlation between final body weight (g) and feed efficiency (%) in WD group (A) and SD group (B). Both correlations were statistically significant (A – $p<0.001$ and B – $p<0.05$).*

Table III. *Food, protein, oils and fats, kcal ingested, and drink mean consumption per day and per animal in all experimental groups (mean).*

Parameter		WD	WD+MNU	CTR	CTR+MNU
Food intake (g)	Initial	10.95	10.99	14.95	14.06
	Final	12.40	12.22	15.56	14.00
Protein ingested (g)	Initial	2.52	2.53	2.17	2.04
	Final	2.85	2.81	2.26	2.03
Oils and fats ingested (g)	Initial	4.22	3.74	0.60	0.56
	Final	4.22	4.16	0.62	0.56
Kcal ingested	Initial	55.84	56.07	43.34	40.76
	Final	63.26	62.34	45.12	40.61
Water intake (ml)	Initial	20.55	18.81	22.32	21.55
	Final	20.99	21.40	28.84	33.34

Statistical analysis was precluded by the fact that only one cage was available for each experimental group. Consequently, the calculation of the mean and standard deviation was not possible, as only a single value was available for each cage/group.

Table IV. *Absolute weight of internal organs and visceral adipose tissue in all experimental groups (mean±SD).*

Groups	Liver (g)	Right kidney (g)	Left kidney (g)	Visceral adipose tissue (g)
WD	2.18±0.16	0.38±0.03	0.36±0.04	15.67±4.29
WD+MNU	2.48±0.26	0.38±0.04	0.35±0.05	17.71±4.82*
CTR	2.57±0.33	0.36±0.06	0.35±0.06	11.37±2.39
CTR+MNU	2.76±0.70	0.40±0.08	0.36±0.07	16.53±4.03

WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5). *$p<0.05$ when compared with CTR group.

Food and drink intake. The standard diet groups (CTR and CTR+MNU) showed higher values for food and drink intake when compared to the Western diet groups (WD and WD+MNU) (Table III). The daily protein intake *per* animal remained consistently similar across all groups. However, in the groups consuming a high-calorie diet (WD groups), despite a reduction in overall food intake, there was an increase in the consumption of fats.

Organs and visceral adipose tissue weight. No significant alterations were observed in the weight of the liver and kidneys across all groups ($p>0.05$). However, the visceral

Table V. *Microhematocrit and biochemical profile for each experimental group (mean±SD).*

Parameter	WD	WD+MNU	CTR	CTR+MNU
Microhematocrit (%)	43.04±2.16	40.90±8.01	44.40±2.70	42.60±3.84
Albumin (g/l)	4.31±0.27	3.95±0.50*	4.77±0.68	4.52±0.02
Cholesterol (mg/dl)	63.63±15.34*	64.91±17.79*	104.77±29.50	90.20±6.64
Glucose (mg/dl)	245±36.71	290±46.33	264±131.96	360±116.17
Total proteins (g/dl)	6.76±0.32	6.81±0.67	7.29±0.40	7.12±0.45
Triglycerides (mg/dl)	48.87±19.18†	100.56±28.65	77.83±36.38	122.45±61.95
Urea (mg/dl)	28.63±3.91	38.36±12.76	43.73±10.16	34.13±8.60

WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5). *p<0.05 when compared with CTR group; †p<0.05 when compared with CTR+MNU group.

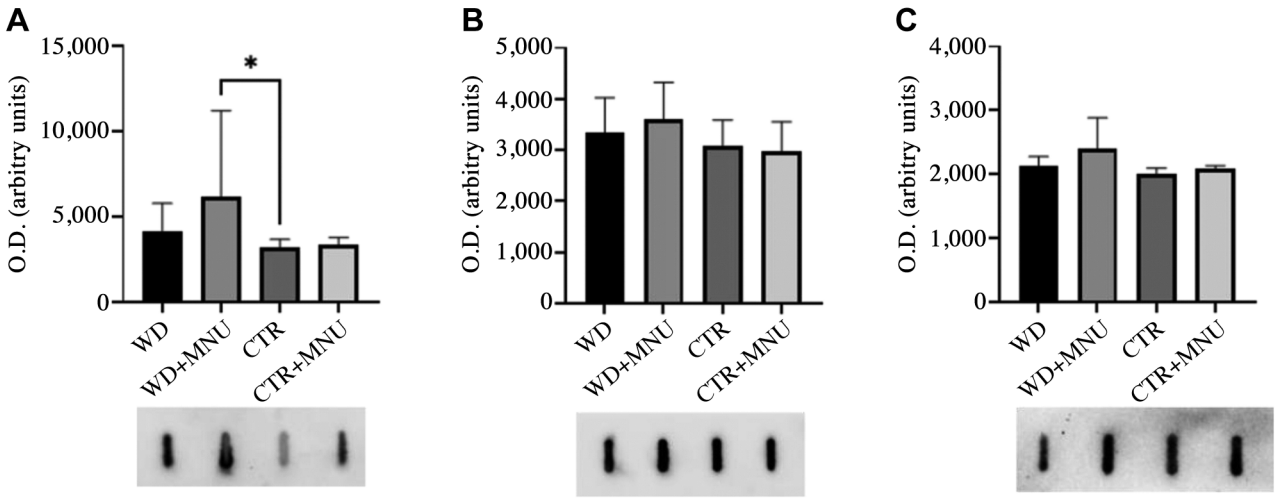

Figure 4. *Leptin (A), adiponectin (B), and C-reactive protein (CRP) (C) detected using western blot analysis in blood. Values are presented as mean±SD optical density (OD). WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5). *p<0.05.*

adipose tissue exhibited a statistically significant increase in the WD+MNU group in comparison to the CTR group (p<0.05) (Table IV).

Blood samples. Notable differences were observed in some parameters during the evaluation of the microhematocrit and biochemical profile (Table V). In general, the values for microhematocrit, glucose, total proteins, and urea showed a trend in groups fed with the standard diet (CTR and CTR+MNU) when compared with those fed with the Western diet (WD and WD+MNU) although they did not reach statistical significance (p>0.05). Albumin and cholesterol levels were significantly lower in the WD+MNU group compared to the CTR groups (p<0.05). Triglyceride levels were significantly lower the WD group when compared to CTR+MNU group (p<0.05).

The WD+MNU group exhibited the highest serum leptin levels compared to the CTR group, reaching statistical significance (p<0.05). However, it also exhibited higher levels of adiponectin and CRP when compared with the CTR groups (CTR and CTR+MNU, Figure 4) although the observed differences did not reach the level of statistical significance. A trend towards increased plasma levels of CRP was observed in WD+MNU compared to other groups, which together with the significantly lower levels of albumin observed in this group suggests a pro-inflammatory status.

Oxidative stress. The WD group exhibited the highest values for both CAT and SOD, though not statistically significant. Regarding kidney oxidative stress, the CTR+MNU group exhibited a trend towards elevated CAT values, while the CTR group exhibited a tendency to higher SOD values.

Table VI. *Oxidative stress parameters for each experimental group (mean±SD).*

Organ	Parameter	WD	WD+MNU	CTR	CTR+MNU
Liver	CAT (μmol H_2O_2 consumed/min/mg protein)	185.19±86.81	99.78±74.51	154.30±80.96	80.15±37.05
	SOD (U activity/min/mg protein)	1.97±0.78	1.80±0.59	1.94±0.9	0.92±0.73
Kidney	CAT (μmol H_2O_2 consumed/min/mg protein)	380.17±99.71	415.40±175.07	377.21±233.15	468.54±283.90
	SOD (U activity/min/mg protein)	3.32±0.99	3.07±0.80	3.45±1.30	3.40±0.83

WD is the control group fed with a Western diet (n=7); WD+MNU is the cancer induction group fed with a Western diet (n=7); CTR is the control group fed with a standard diet (n=7), and CTR+MNU is the cancer induction group fed with a standard diet (n=5). Statistically significant differences were not found ($p>0.05$).

Table VII. *The effect of Western and standard diets on the development of MNU-induced mammary tumors (tumor volume is presented as mean±SD).*

Group	Number of animals with tumors	Tumor incidence (%)	Latency period	Tumor volume (cm^3)
WD+MNU (n=7)	2	28.6	15th week	2.81±5.31
CTR+MNU (n=5)	3	60.0	14th week	2.02±2.46

These results are shown in Table VI, and no statistically significant differences were found among groups ($p>0.05$).

Mammary tumors. The incidence of mammary tumors was found to be lower in animals fed with a Western diet (WD+MNU) than those fed with a standard diet (CTR+MNU), with rates of 29% and 60%, respectively. Additionally, the WD+MNU group showed a longer latency period (15 *vs*. 14 weeks). Tumor volume was found to be higher in the WD+MNU group (2.81±5.31 cm^3) when compared with the CTR+MNU group (2.02±2.46 cm^3) ($p>0.05$). The number of animals with tumors was two out of seven in the WD+MNU group and three out of five in the CTR+MNU group (incidence of 28.6% versus 60.0%) (Table VII).

No mammary tumors were found in the animals from the WD and CTR groups, The CTR+MNU group exhibited a higher number of tumors in comparison to the WD+MNU group throughout the entire experimental assay (Figure 5). At the last week of the experimental assay, the WD+MNU group presented three tumors, while the SD+MNU group presented five.

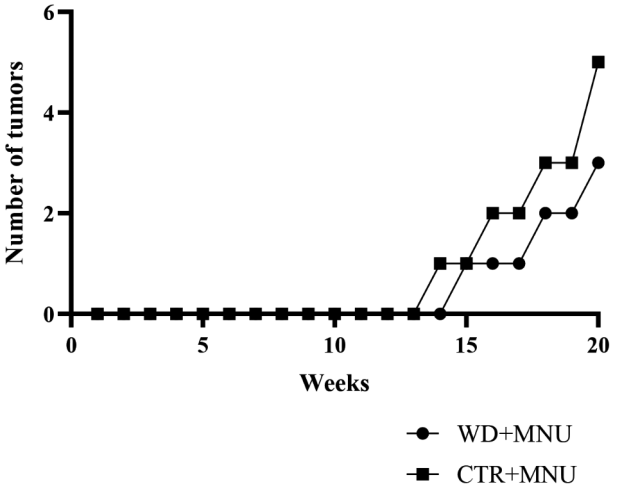

Figure 5. *Total number of palpable mammary tumors after MNU administration per week, during the assay.*

Histological and immunochemical analysis of mammary tumors. The histological evaluation of tumors using H&E staining (Figure 6A and B) revealed that all tumors were malignant. All tumors in the WD+MNU group (n=3) were classified as invasive cribriform carcinoma. The CTR+MNU (n=5) group had five invasive carcinomas, four cribriform carcinomas, and one papillary carcinoma. These values indicate that two out seven animals in the WD+MNU group developed tumors and three out five animals in the CTR+MNU group developed tumors. As reported previously, spontaneous mammary tumors were not observed in the non-induced groups (WD and CTR, Table VIII).

All mammary tumors in both the WD+MNU and CTR+MNU groups were positive for ERα (Figure 6C and D) and progesterone receptor (Figure 6E and F). The WD+MNU group exhibited a lower expression of ERα and progesterone receptor when compared to the CTR+MNU group. Inversely, the expression of Ki-67 was higher in the WD+MNU group when compared to the CTR+MNU group

Table VIII. *Histological classification of mammary tumors identified in animals exposed to MNU based on the predominant histological pattern.*

Histological classification	WD n (%)	WD+MNU n (%)	CTR n (%)	CTR+MNU n (%)
Malignant tumors				
Cribriform invasive carcinoma	0	3	0	4
Papillary invasive carcinoma	0	0	0	1
Total number of malignant tumors	0 (0%)	3 (100%)	0 (0%)	5 (100%)

Statistically significant differences were not found ($p>0.05$).

(Table IX, Figure 6G and H). Statistically significant differences among groups were not found ($p>0.05$).

Discussion

This study evaluated the effects of a WD on a rat model of mammary cancer induced by intraperitoneal injection of MNU and showed that the WD increased adipose tissue and altered the lipid profile, but did not cause obesity or significant changes in body weight. Although the WD reduced tumor incidence, the tumors were larger and had a higher Ki-67 expression, and lower immunostaining for estrogen and progesterone receptors.

Our results indicated that the BW increased in accordance with normal growth patterns, with no statistically significant differences between the dietary treatments. Despite these finding, we observed that the ponderal weight gain was lower in the WD groups than in the CTR groups, suggesting that female Wistar rats were resistant to WD-induced obesity. Consistent with our results, Ramos *et al*. (2019) fed female Sprague-Dawley rats of unknown age for seven weeks with WD and did not notice any significant changes in body weights when compared to animals fed with standard diet (30). This may be related to, at least in part, the lower food intake by WD rats. Fats can trigger satiety signals more effectively than carbohydrates or proteins. This can result in the rats feeling fuller faster and reducing their overall food intake. The indices of body composition, including the Lee index, body mass index, and specific body mass gain, did not exhibit significant differences among the groups. This indicates that the rats were resilient to the dietary interventions, suggesting that similar lean gains in lean mass were observed across all groups. Notably, the CTR group exhibited lower feed efficiency than the WD group, which aligns with the findings from other studies. For instance, in a study conducted by Skinner *et al*. (2018), Sprague-Dawley female rats aged between 22 and 29 days were fed a WD, and the results indicated that the rats exhibited higher feed efficiency when fed the WD compared to control diet group (31).

Table IX. *Immunoexpression of estrogen receptor α (ERα), progesterone receptor, and Ki-67 in MNU-induced mammary tumors (mean±SD).*

Markers	WD+MNU (n=4)	CTR+MNU (n=8)
ERα (%)	35.63±14.43	44.38±9.87
Progesterone receptor (%)	31.80±12.79	57.20±9.12
Ki-67 (%)	9.32±3.68	5.88±7.85

Statistically significant differences were not found ($p>0.05$).

There was an increase in food and drink intake from the initial to the final week of the experiment, with the standard diet groups (CTR and CTR+MNU) exhibiting higher intake. This is consistent with previous studies that have observed increased food and water intake in animals fed a standard diet. A study conducted by Ramos *et al*. (2019) also noted that Sprague-Dawley female rats fed with the standard diet consumed more food than WD groups (30). Similarly, Marques *et al*. (2015) provided a high-fat diet to Wistar and Sprague-Dawley male rats, aged seven weeks, for 17 weeks and observed high water consumption in both strains on the normal diet (32). Since the WD has more calories *per* gram, we noticed that despite the larger amount of food intake in the SD groups, the daily calorie intake was similar across all groups.

Despite the lack of differences in body weights or body condition indices, an increase in the visceral adipose tissue was observed in the WD+MNU group when compared with the CTR group ($p<0.05$, Table IV). To the best of our knowledge, this result has so far not been presented in the literature. The increased levels of visceral adipose tissue in WD+MNU is related with the increased plasma levels of leptin, since the levels of this adipokine correlates with adipose tissue mass (33). The higher levels of this adipokine may explain, at least in part, the lower food consumption observed in these animals.

Nevertheless, no alterations were observed in the levels of the other measured adipokine, adiponectin. One of the effects of this hormone is the regulation of insulin action, which may partly explain why no changes were observed in the circulating glucose levels among the groups.

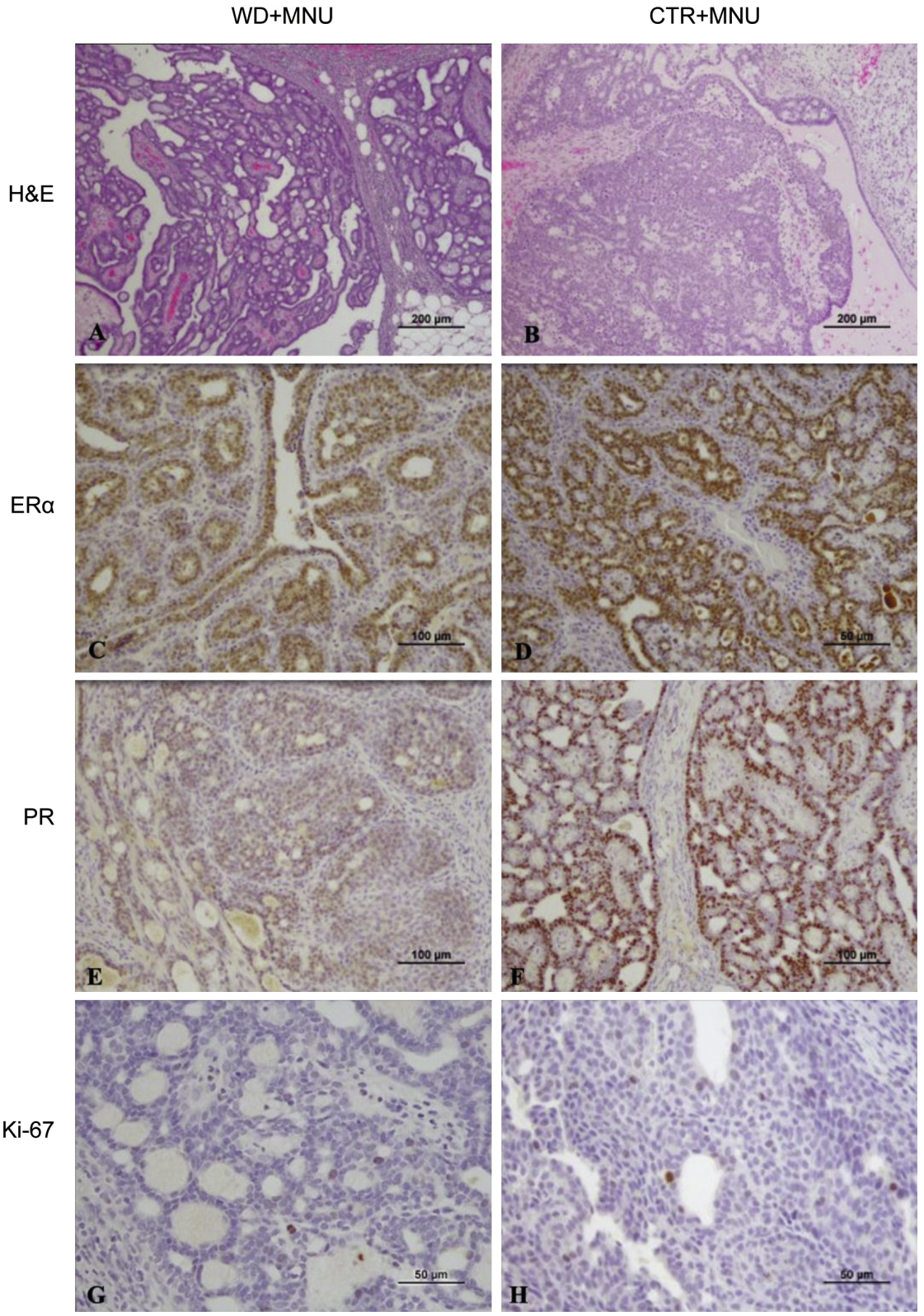

Figure 6. *H&E staining of mammary tumors in the Western diet group (WD+MNU) (A) and standard diet group (CTR+MNU) (B) group. Immunoexpression of estrogen receptor α (ERα) in WD+MNU (C) and CTR+MNU (D) groups; Progesterone receptor (PR) in WD+MNU (E) and CTR+MNU (F) groups, and Ki-67 in WD+MNU (G) and CTR+MNU (H) groups. Statistically significant differences were not observed (p>0.05).*

Moreover, we did not observe differences in liver and kidney weight between the groups, suggesting that WD and/or mammary carcinogenesis did not promote dysfunction in these organs. Kostogrys *et al*. (2015) also did not identify any significant differences in the liver and kidneys of 6-week-old male albino Wistar rats fed a WD (34). Animals fed a WD are expected to exhibit increased liver weights due to hepatic steatosis, characterized by fat accumulation in the liver. This condition is commonly observed in high-calorie diets rich in fats and sugar, which are often converted into triglycerides and stored in the liver (35). The trend towards decreased circulating triglycerides levels may indicate increased triglycerides accumulation in the liver and/or reduced very-low-density lipoproteins secretion by the liver in response to WD.

In the WD group, a significant decrease in cholesterol levels was noted, with an average of 63.63 mg/dl compared to 104.77 md/dl in the CTR group that further suggests decreased lipoprotein production by the liver. This reduction in circulating lipid levels could be related to alterations in the intestinal microbiota induced by the WD, which reduces cholesterol absorption in the gut (36, 37). In the liver, cholesterol is converted into bile acids and subsequently excreted in bile, aligning with previous findings that suggest increased bile acid production (38). Additionally, the positive acute-phase protein CRP showed no changes, while lower levels of the negative acute-phase protein albumin, which are both produced in the liver, were observed. This suggests an inflammatory phenotype and potential alterations in liver functionality. The specific components of the WD could influence albumin levels, potentially altering liver function and protein synthesis. Moreover, the presence of mammary cancer might result in systemic alterations that affect albumin metabolism due to the cancer-related inflammatory process (39, 40).

Furthermore, no signs of oxidative stress in the liver were evident, as indicated by the activity levels of the CAT and SOD. In a similar study with 18-months-old Wistar female rats, the WD did not significantly raise systemic cholesterol levels and led to a decrease in triglycerides compared to the control (41). These results collectively suggest complex interaction between diet, lipid metabolism, and systemic inflammation without clear liver dysfunction.

Leptin is a crucial mediator that links obesity to breast cancer by promoting tumor initiation, progression, growth, and metastasis (42). This connection may explain why the WD+MNU group exhibited higher leptin levels compared to the CTR groups. Marques *et al*. (2015) also found significantly increased leptin levels in the WD groups compared to the CTR group in seven-week-old Sprague-Dawley female rats fed with a WD (32).

Although we did not observe significant changes in adiponectin and CRP levels, the WD+MNU group showed increased levels of both. This increase could be due to moderate inflammation, which can trigger the production of adiponectin as a defense mechanism (43). Adiponectin is a protein primarily produced and secreted by adipocytes (fat cells), and higher levels of adiponectin are associated with increased visceral adipose tissue (44, 45). Marques *et al*. (2015) found similar results, with the WD group showing higher adiponectin levels and mesenteric adipocyte area compared to the CTR group in seven-week-old Sprague-Dawley female rats (32).

The lack of significant differences in adiponectin levels between our groups suggests that there is no inflammation originating from adipose tissue and, consequently, no systemic inflammation, as indicated by non-significant changes in CRP ($p>0.05$). This indicates that despite the increase in adiponectin in the WD+MNU group, there was no significant inflammatory response from adipose tissue.

The latency period is defined as the time elapsed between the administration of MNU and the appearance of the first tumor (46). During our experimental protocol, the animals were palpated twice a week, allowing us to accurately determine the date of the appearance of the first tumor in each group. In our study, the CTR+MNU group exhibited a shorter latency period than the WD+MNU group, with a difference of only one week, which in humans corresponds to approximately 15 to 16 years. Thordarson *et al*. (2001) observed a latency period of approximately eight weeks in animals subjected to the same conditions as those in the present study, with a standard diet (47). The incidence of tumors in our study was less than 60%, contrasting with other studies that obtained incidence rates of 100%. In a study conducted by Faustino-Rocha *et al*. (2016), a 100% incidence was achieved in 4 to 5-week-old female Sprague-Dawley rats (48). The same rate (100%) was obtained by Murray *et al*. (2009) in female Wistar-Furth rats after the 15th week of MNU injection (at 50 mg/Kg) (49). This discrepancy can be attributed to factors, such as the sensitivity of the rat strain, dietary differences, duration of the study, variability in individual responses, and the specific lot of the chemical carcinogen (50–53).

Overall, only malignant tumors were detected, indicating that MNU treatment effectively induced predominantly carcinomas. The WD may have promoted inflammation, or induced metabolic changes that influenced tumor development (15). In contrast, the standard diet's lower fat and higher carbohydrate content may have led to different metabolic profiles affecting tumor growth and progression (54). Additionally, the absence of benign tumors in both groups suggests that these tumors may have progressed more rapidly from a pre-cancerous stage.

Considering the immunoexpression of ERα, tumors in the CTR+MNU group may exhibit a slightly higher dependency on estrogen for their growth, as indicated by the higher

percentage of ERα-positive cells detected. ERα is a receptor protein that binds estrogens, which can stimulate the growth of certain breast cancers. The higher percentage of ERα-positive cells in the CTR group suggests that these tumors may rely more on estrogen for their growth. The WD diet may have created a hormonal environment conducive to the development and survival of ERα-positive tumor cells, possibly due to a higher dietary intake of compounds that interfere with estrogen signaling or differences in gut microbiome composition affecting estrogen metabolism. These results are not necessarily negative, since hormone-dependent tumors are generally less aggressive compared to triple negative tumors and respond better to hormonal therapy (49). Additionally, the WD reduced progesterone receptor expression in mammary tumors (WD+MNU) compared to control group (CTR+MNU). This could indicate that diet can influence hormone receptor status in cancer cells. Considering the immunoexpression of ERα, tumors in the control group exhibit a slightly higher dependency on estrogen for their growth, suggesting that diet and hormonal environment together impact tumor behavior and potential responsiveness to hormone-based treatments, as reported by Satpathi *et al.* (2023) (55). Ki-67 protein is expressed in actively dividing cells (56). The low immunoexpression of Ki-67 in both groups (WD+MNU and CTR+MNU) indicates that tumor proliferation is slow, which could be a characteristic of the tumor type itself.

Despite the importance of our study, it would be interesting to study the composition of the two diets, analyze their protein and amino acid availability, and compare these factors between the diets. A high-fat diet, despite having the same caloric content, often contains a much lower quantity and variety of available amino acids, which may affect tumor growth. Additionally, examining how diet influences gene expression related to cancer growth and metastasis is crucial. Investigating whether switching from a WD to a SD could alter the course of mammary cancer and understanding how diet-induced changes in the gut microbiome affect mammary cancer progression and overall health are also very important. Conducting studies in humans to correlate findings in animal models with human dietary habits and cancer rates would further enhance our understanding.

The relationship between diet and cancer is complex and influenced by factors, such as genetics, individual variation, and the specific dietary components. Our results suggest that the WD+MNU had higher feed efficiency, leading to increased visceral adipose tissue and changes in circulating lipid profile. Although the WD group showed a lower tumor incidence, the volume and weight of the tumors were higher. Furthermore, ERα and progesterone receptor immunoexpression were decreased by the WD, whereas Ki-67 immunoexpression was elevated, suggesting that WD could be associated with more aggressive carcinomas. This fact should be examined in new and longer studies.

Conclusion

Our study investigated the impact of WD on mammary cancer induced by MNU intraperitoneal injection in Wistar rats. The WD groups exhibited decreased food consumption, which may be indicative of a potential association with greater satiety in these groups. Furthermore, the WD led to increased visceral adipose tissue accumulation and lower cholesterol levels compared to CTR, contrary to the usual association with hypercholesterolemia. Additionally, the WD+MNU group had elevated leptin levels, highlighting leptin's role in obesity and mammary cancer. This group also had lower incidence of tumors but both volume and weight of the tumors were higher. Additionally, the WD led to reduced immunoexpression of ERα and progesterone receptor, while Ki-67 immunoexpression was increased. These findings offer valuable insights into the complex interactions between diet, mammary cancer induction, and various physiological parameters in Wistar rats. The unexpected results regarding cholesterol levels and tumor development underscore the intricate nature of these relationships, indicating a need for further investigation to better understand the effects of dietary factors on cancer outcomes. We plan to conduct a study with extended exposure to the high-fat diet to gain a deeper understanding of its long-term effects on the organism and tumor development.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

JA, TA: conceptualization and writing (original and draft preparation); JS, TA, IA: methodology. FD, MJN, FLQ, FS, RF, AIF-R, JAD, and PAO: supervision, validation, and writing (reviewing and editing). All Authors have read and agreed to the published version of the manuscript.

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