Rifaximin prophylaxis in MASLD-hepatocellular carcinoma: Lessons from a negative animal model

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Abstract. The incidence of hepatocellular carcinoma (HCC) has been rising, particularly among individuals diagnosed with metabolic dysfunction-associated steatotic liver disease. In the present study, the prophylactic effects of rifaximin (RIF) on HCC, inflammatory markers and cardiovascular risk (CVR) were investigated in an animal model. Adult Sprague-Dawley rats were randomly allocated into three groups (n=10, each): Control [standard diet/water plus gavage with vehicle (Veh)], HCC [high-fat choline deficient diet (HFCD)/diethylnitrosamine (DEN) in drinking water/Veh gavage] and RIF [HFCD/DEN/RIF (50 mg/kg/day) gavage] groups. After euthanasia at week 16, biochemical/inflammatory markers and the liver histology were assessed. The results demonstrated that the HCC and RIF animals had a significant increase in fresh liver weight, liver weight/body weight ratio, serum total cholesterol (TC), high-density lipoprotein-cholesterol, triglycerides, hepatic lipid accumulation and hepatic concentration of triglycerides and TC, relative to the controls (P<0.001, for all). Additionally, the HCC and RIF animals had higher plasminogen activator inhibitor, intercellular adhesion molecule-1, E-selectin and CVR scores than the controls (P<0.001, for all). The HCC animals had higher interleukin (IL)-1β (P=0.011), IL-10 (P<0.001), toll-like receptor-2 (P=0.012), lipopolysaccharide-binding protein (P=0.018) and metalloproteinase-2 (P=0.003) levels than the RIF animals. Furthermore, liver steatosis, inflammation and fibrosis, along with increased collagen fiber deposition occurred in the HCC and RIF groups. However, HCC occurred only in 2 RIF rats. In conclusion, although most animals did not develop HCC in the present study, RIF positively affected liver inflammation markers involved in steatohepatitis pathogenesis.

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Abbreviations: AC, atherogenic coefficient; CRI, Castelli's Risk Index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; H&E, hematoxylin and eosin; HFCD, high-fat and choline-deficient; ICAM, intercellular adhesion molecule; ICIs, immune checkpoint inhibitors; IL, interleukin; LDL, low-density lipoprotein; LBP, lipopolysaccharide-binding protein; MASH, non-alcoholic steatohepatitis associated with metabolic dysfunction; MASLD, metabolic dysfunction-associated liver disease; MCP, monocyte chemoattractant protein; MMP, metalloproteinase; PAI, plasminogen activator inhibitor; TC, total cholesterol; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor; RIF, rifaximin; VAP-1, vascular adhesion protein-1; Veh, vehicle

Key words: animal model, cardiovascular risk, hepatocellular carcinoma, metabolic-dysfunction associated steatotic liver disease, rifaximin

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is the latest term used to define non-alcoholic fatty liver disease associated with metabolic syndrome (1). MASLD is characterized by excessive lipid accumulation associated with obesity, type 2 diabetes mellitus, hypertension, dyslipidemia and metabolic syndrome, with insulin resistance being the common denominator. Alternative diagnoses such as viral hepatitis and significant alcohol intake should also be ruled

out before diagnosing MASLD (1). The initial stage of development of this multisystem disorder shows simple hepatic steatosis that can progress to non-alcoholic steatohepatitis associated with metabolic dysfunction (MASH), fibrosis, cirrhosis and eventually to hepatocellular carcinoma (HCC), depending on the clinical, genetic and epigenetic predispositions of the patient (2-4). MASLD is the most common cause of liver-related morbidity and mortality, affecting >30% of individuals worldwide due to the global prevalence of obesity and diseases associated with this clinical condition (3,5). Additionally, cardiovascular diseases feature among the leading causes of death in patients with MASLD, causing ~40% of all deaths (6,7). Overall, HCC is the sixth most common cancer and the third leading cause of cancer-related mortality worldwide (2). However, MASLD/MASH-derived HCC shows distinct characteristics (including lower survival rates than other pathologies), but its underlying pathogenic mechanisms remain only partially understood (2).

Several oncogenic mechanisms are associated with the progression of MASLD, one of which refers to the accumulation of lipids in hepatocytes and its associated lipotoxicity creating a dynamic pro-inflammatory environment (3), in which multiple oncogenic pathways are associated with HCC development, markedly changing regulatory and signaling pathways and fostering a conducive hepatic microenvironment for disease progression (3,8). This process concurrently increases the risk of cardiovascular diseases by the activation and generation of metabolic and inflammatory components (6,9). Due to the difficulty treating and improving HCC outcomes (10), immune checkpoint inhibitors (ICIs) represent an effective treatment strategy for HCC, the mechanisms of action of which are based on activating the immune system by modulating T lymphocyte responses and targeting immune checkpoints (11,12). As a previous meta-analysis has shown, despite the acceptable safety profile of ICI monotherapy and its immunological combinations, ICIs have a specific set of treatment-related adverse events (11,12), including a higher risk of hypertransaminasemia, warranting liver function monitoring and the evaluation of potential prognostic biomarkers, such as albumin, which is related to inflammatory pressure (11,13). Lower levels of albumin occur in cases of chronic inflammatory disorders and cancer, acting as a negative acute-phase reactant (13). In recent years, new systemic therapies for advanced HCC have been developed, and it has been suggested that the combination of new and old treatments with locoregional approaches be implemented (14). MASLD/MASH treatment is necessary to prevent irreversible chronic liver diseases, such as cirrhosis and HCC. At present, regulatory agencies have not approved specific pharmacological therapies to treat MASH, and several clinical studies currently target its different symptoms (1,15). Rifaximin (RIF), a non-absorbable broad-spectrum oral antibiotic, can positively modulate the components of the intestinal microbiota, attenuating the inflammatory process and energetic metabolism (16-18). These mechanisms contribute to the progression from MASLD to HCC, making it an important target of study.

Based on the above, MASLD-related HCC configures a global health issue as its forecast impact on HCC morbidity and mortality is expected to rise in the future. Thus, developing pre-clinical studies is of fundamental importance for understanding the mechanisms linked to the development and progression of MASLD and for evaluating prognostic markers and potential therapeutic targets (15,19). The complex and multifaceted pathophysiology of MASLD challenges the search for animal models that can replicate the disease in its advanced stages, which more urgently require treatment (15,19). Hence, the present study aimed to evaluate the impact of RIF treatment on MASLD-associated hepatocarcinogenesis and to assess the hepatic and systemic inflammatory processes associated with the risk of developing cardiovascular diseases.

Materials and methods

Animals. In total, 30 adult male Sprague Dawley rats aged 60 days and weighing 290-330 g were included in the present study. The rats were housed in pairs in polypropylene cages with sawdust-covered floors and allowed to acclimatize to the maintenance room for 2 weeks prior to this experiment. The rats were kept under a standard 12-h light/dark cycle in a temperature-controlled environment (22±2˚C). Before starting the study, measures for anticipating the euthanasia of animals as a refinement procedure and to protect/preserve their well-being whenever the animals showed altered behavior or signs of suffering that could not be controlled with handling or analgesics were adopted. Additionally, performing chronic gavage (16 weeks) can result in adverse events such as irritations in the upper gastric tract (mouth, pharynx, esophagus and stomach), physical stress, passive reflux if the stomach is overloaded and aspiration pneumonia. In such situations, comfort measures should also be adopted. However, these measures were not necessary for any animal during the present study. All experiments and procedures involving the use of animals were approved by the Institutional Ethics Committee of Hospital de Clinicas de Porto Alegre (Porto Alegre, Brazil; approval no. 2019-0311). The procedures for the use of scientific animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and Law number 11,794 (Brazil, 2008).

Study design. Diethylnitrosamine (DEN; to stimulate the development of HCC) doses and experimental duration for this protocol were based on a prior study (20). Briefly, after acclimatizing to the environment, the rats were randomly assigned to three groups based on their weight: A control group (n=10), which received a standard diet and water without DEN along with a daily gavage of vehicle (Veh) solution throughout the 16-week experiment period; an HCC group (n=10), which received a high-fat and choline-deficient diet (HFCD), 135 mg/l DEN (MilliporeSigma) in drinking water, and a daily gavage of Veh solution for the experimental period; and the RIF group, which received the HFCD diet plus DEN and prophylactic RIF (MilliporeSigma) administered daily by gavage for 16 weeks. The experimental design is depicted in Fig. S1. The weight of the rats was logged twice a week throughout the experiment. Additionally, naso-anal length (cm) was measured in the initial and final week of the study for determination of the change in Lee index. This index was calculated as the ratio between the cube root of the body weight and the naso-anal length of animals multiplied by 10 (g/cm) (21). At the end of the 16-week period, all rats were anesthetized via inhalation

with isoflurane (BioChimico) in 100% oxygen at a dose of 5% for induction and 3-4% for maintenance, at 0.5 l/min, and then euthanized via cardiac exsanguination. The rat livers were completely excised and weighed. Serum samples, abdominal adipose tissue and liver fragments were also collected under sterile conditions, flash-frozen in liquid nitrogen and stored at -80˚C until experimental procedures were conducted. A portion of each liver sample was fixed in 10% formalin at room temperature for 24 h for histological analysis.

Nutritional intervention. The diet administered to the intervention groups was selected to replicate a number of the phenotypes in humans with MASLD, as previously shown by our research team (22). Rats in the control group were provided with a standard rodent diet (Nuvilab CR-1; Quimtia), with an energy content of 2.93 kcal/g (carbohydrates, 55.0%; protein, 22.0%; fat, 4.5%; other nutrients, 18.5%). Rats in the intervention groups were fed an HFCD diet (RH19576; Rhoster) with an energy content of 4.3 kcal/g (carbohydrates, 54.5%; protein, 14.0%; fat, 31.5, and 54.0% from trans fatty acids). The diet for all groups was replaced every 2 days. Throughout the experimental period, groups had *ad libitum* access to water and food.

RIF administration. The RIF dose administered followed a previous study in the literature (8,23). The RIF group received a daily dose of 50 mg/kg/day of RIF (Biolab Sanus Farmaceutica Ltd.) by daily gavage until the 16th week of the experiment. The animals in the control and HCC groups received a daily gavage with a Veh solution (0.5 ml/kg distilled water). The therapeutic intervention by administering gavage daily followed the same previously standardized procedures that had been performed by our research group (8). In summary, the administration of RIF or the provision of Veh solution via gavage to the respective experimental groups occurred from the first day of the experiment until the date of euthanasia.

Biochemical analysis and atherogenic ratios. The rats were fasted for 8 h before euthanasia via cardiac exsanguination under isoflurane anesthesia. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride (TG) serum levels were determined using Labmax 560, in the Laboratory Diagnostic Service, Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil).

The atherogenic ratios, calculated based on the lipid profile results, were used as a tool to predict cardiovascular risk (CVR). The atherogenic ratios were calculated as follows: Castelli's risk index (CRI)-I=TC/HDLc; CRI-II=LDLc/HDLc; and atherogenic coefficient (AC)=(TC-HDLc)/HDLc (24), where 'c' indicates cholesterol.

Quantitative analysis of liver fat deposition. Previously frozen liver tissue samples were thawed on ice and homogenized in phosphate-buffered saline at a concentration of 20 mg of tissue/ml, to analyze the hepatic lipid content. From this homogenate, TG, TC and overall lipid accumulation levels were assessed. The hepatic TG and TC levels were enzymatically determined by colorimetric assays (Labtest Diagnóstica S.A), at wavelengths of 505 and 500 nm, respectively. The total lipid concentration was determined following the modified protocol outlined by Gómez-Lechón *et al* (25). Briefly, the liver tissue was homogenized in phosphate-buffered saline and incubated with 1 μl Nile Red solution (1 mg/ml in acetone) at 37˚C for 15 min. The fluorescence was measured at excitation and emission wavelengths of 488 and 550 nm, respectively, using a SpectraMax M3 spectrophotometer. The obtained values were normalized to the total protein content of the homogenate (26). The results are presented as fluorescence/μg protein. All analyses were performed in duplicate.

Assessment of the gene expression of hepatic inflammation. Total RNA was extracted from liver tissue fragments using a RNeasy Mini Kit (Qiagen, Inc.). A high-capacity cDNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to convert cDNA from 2 μg of RNA according to the manufacturer's instructions. To assess the gene expression of interleukin (IL)-1β, IL-6, IL-10, tumoral necrosis factor-α (TNF-α), lipopolysaccharide-binding protein (LBP), myeloid differentiation primary response 88, toll-like receptor (TLR) 4, TLR2, transforming growth factor-β1 (TGF-β1), metalloproteinase (MMP)2 and MMP9 in the liver, a quantitative polymerase chain reaction with the TaqMan assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) was performed according to the manufacturer's instructions. The probes used are listed in Table SI. The β-actin gene was used to normalize gene expression in the liver tissues. The changes in gene expression levels were calculated using the formula $2^{-\Delta\Delta Cq}$ (27).

Inflammatory status and endothelial injury. By a multiplex assay using the Luminex platform (Merck KGaA), the serum levels of inflammatory and endothelial dysfunction markers, such as IL-1β, IL-6, TNF-α, monocyte chemoattractant protein (MCP-1), E-selectin, intercellular adhesion molecule (ICAM-1), plasminogen activator inhibitor (PAI-1), insulin, leptin and adiponectin, were evaluated using the following kits: Rat Adipokine (cat. no. RADPKMAG-80K) for the assessment of IL-1β, IL-6, insulin, leptin, MCP-1, PAI-1 and TNFα; Rat Vascular Injury Panel 2 (cat. no. RV2MAG-26K) for the assessment of adiponectin, E-selectin and ICAM-1. The serum evaluation of soluble vascular adhesion protein-1 (VAP-1) was performed by an enzyme-linked immunosorbent assay (cat. no. MBS2515661; MyBioSource, Inc.). Absorbance was measured in a spectrophotometer (Zenyth 200 rt) at a wavelength of 450 nm. The results are presented in ng/ml or pg/ml. All procedures followed the manufacturers' instructions, and all analyses were performed in duplicate.

Histopathological analysis. Formalin-fixed liver tissue samples (4-μm sections) were embedded in paraffin and stained with hematoxylin and eosin (H&E) and picrosirius red. The histopathological lesions of the several evolutionary stages of MASLD were assessed according to the score by Liang *et al* (28), which is a highly reproducible scoring system applicable to experimental rodent models. The degree of fibrosis was evaluated using the slides stained with picrosirius red and cancerous lesions were graded according to the Edmondson and Steiner classification (29). The analysis was performed by an experienced pathologist, who was blinded to the experimental groups. The evaluation was conducted in

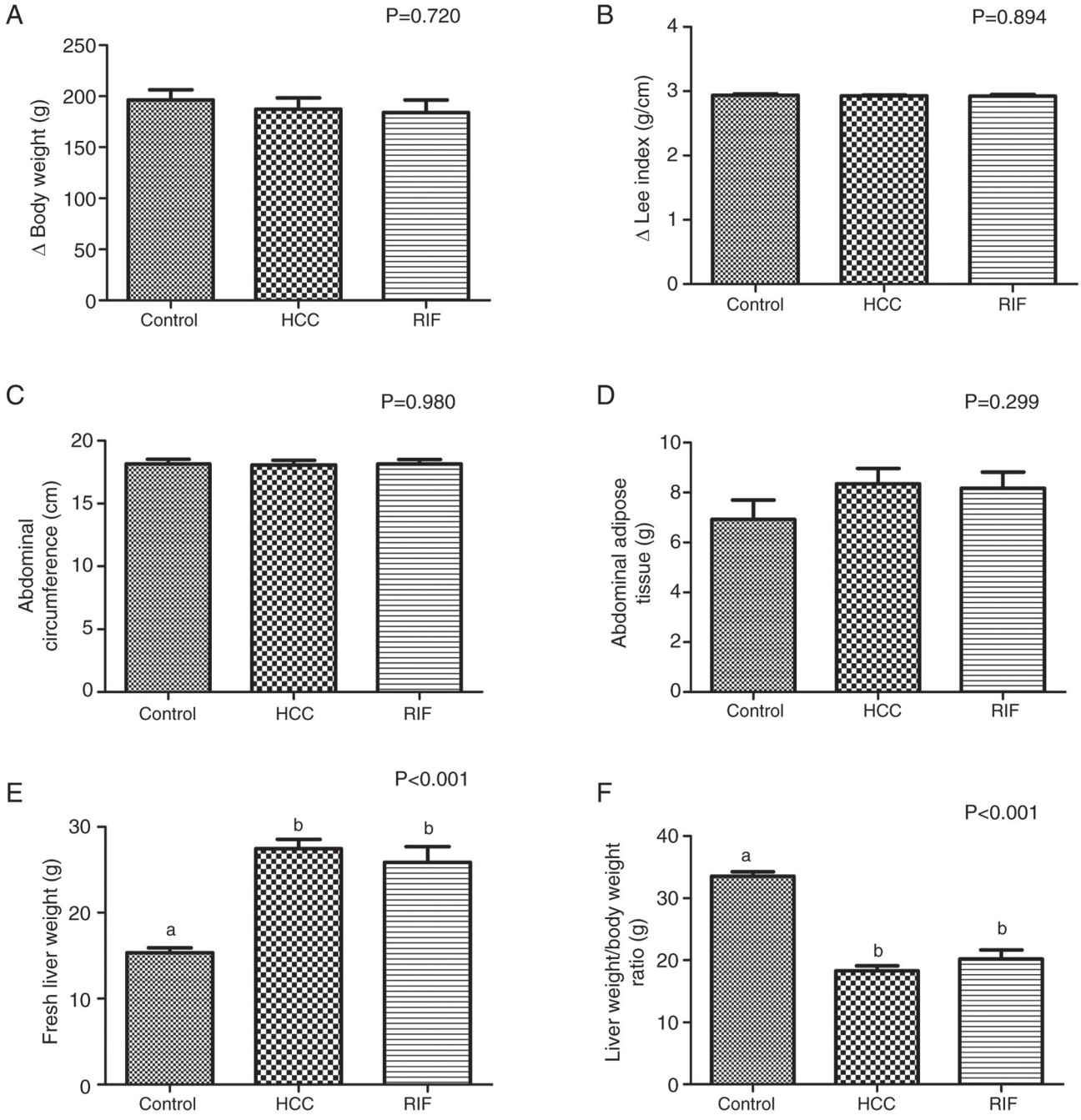

Figure 1. (A) Change in body weight, (B) change in Lee index, (C) abdominal circumference, (D) abdominal adipose tissue, (E) fresh liver weight, (F) liver weight/body weight ratio of the rats. Data are presented as the mean ± standard deviation. Statistical significance was determined by Tukey's test. Different letters indicate a significant difference between groups (P<0.05). If groups share a letter, they are not significantly different. HCC, hepatocellular carcinoma; RIF, rifaximin.

the Surgical Pathology Service at the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil).

Sample size calculation and statistical analysis. The sample size was estimated using WINPEPI 11.20 software (Brixton Health) following a prior study by our group (20,30). With a power of 80% and a significance level of 5%, it was determined that a minimum of 10 animals per experimental group would be necessary. Data were analyzed using SPSS version 28.0 (IBM Corp.). The normality of all variables was assessed using the Shapiro-Wilk test and histograms. Parametric data were analyzed using one-way analysis of variance, followed by the Tukey's post-hoc test. Quantitative variables are shown as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

General characteristics of the experimental model. The animals in all experimental groups showed similar baseline body weights (P=0.797), evincing homogeneity. After the first week of the experiment, HFCD was introduced to the HCC and RIF groups, and there was no significant difference (P=0.720) between these experimental groups and the control group in

Table I. Biochemical parameters and atherogenic ratios.

Variable	Control, n=10	HCC, n=9	RIF, n=10	P-value (ANOVA)
ALT, U/l	69.2±32.9	61.3±19.0	61.5±22.6	0.757
AST, U/l	100.9±26.7[a]	124.9±25.0[a,b]	132.0±28.7[b]	0.045
Glucose, mg/dl	148.0±26.4[a]	114.3±9.5[b]	128.2±28.6[a,b]	0.018
Total cholesterol, mg/dl	60.7±10.8[a]	130.2±29.7[b]	127.9±22.8[b]	<0.001
HDL cholesterol, mg/dl	24.9±3.11[a]	40.7±8.6[b]	40.6±9.8[b]	<0.001
LDL cholesterol, mg/dl	20.4±6.9[a]	78.4±27.2[b]	78.2±14.3[b]	<0.001
Triglycerides, mg/dl	76.9±18.0[a]	56.0±13.7[b]	45.7±10.5[b]	<0.001
AC	1.43±0.3[a]	2.28±0.7[b]	2.22±0.4[b]	<0.001
CRI-I	0.77±0.3[a]	1.77±0.7[b]	2.49±1.1[b]	<0.001
CRI-II	0.8±0.3[a]	2.0±0.7[b]	2.0±0.4[b]	<0.001

Variables are presented as the mean ± standard deviation. The superscript letters [a, b] refer to the results of the post hoc statistical test (Tukey's test). Identical letters indicate no statistical difference, while different letters between experimental groups show a significant difference for the variable under analysis. AC, atherogenic coefficient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRI, Castelli's Risk Index; HCC, hepatocellular carcinoma; HDL, high density lipoprotein; LDL, low density lipoprotein; RIF, rifaximin.

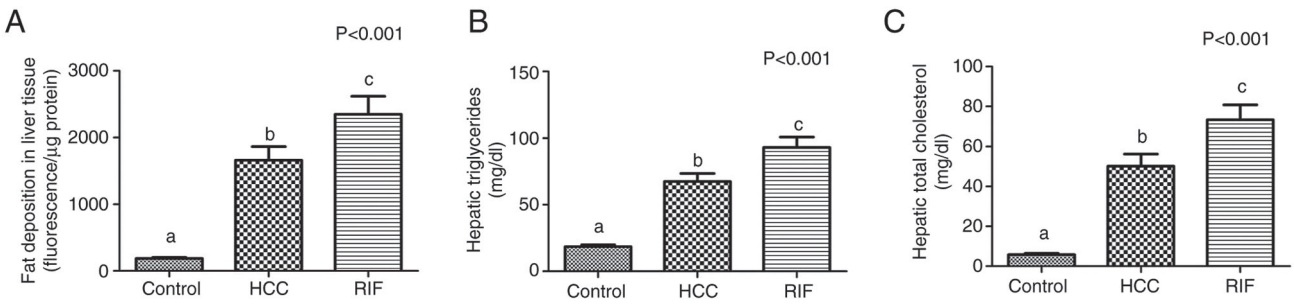

Figure 2. Quantitative analysis of the (A) accumulation of liver lipids, (B) hepatic triglycerides, and (C) hepatic total cholesterol. Data are presented as the mean ± standard deviation. Statistical significance was determined by Tukey's test. Different letters indicate a significant difference between groups (P<0.05). If groups share a letter, they are not significantly different. HCC, hepatocellular carcinoma; RIF, rifaximin.

terms of the change in body weight (Fig. 1A). Additionally, no significant differences in the change in Lee index (P=0.894; Fig. 1B), abdominal circumference (P=0.980; Fig. 1C) and abdominal adipose tissue accumulation (P=0.299; Fig. 1D) was found between the groups. However, the fresh liver weight significantly increased and the liver weight/body weight ratio significantly decreased in the HCC and RIF groups relative to the control group (both P<0.001; Fig. 1E and F).

Biochemical parameters and atherogenic ratios to assess the CVR. Table I shows the biochemical parameter and atherogenic ratio data. The serum AST levels in the RIF group significantly increased compared with the control group (P=0.045). The glucose levels significantly increased in the HCC group compared with the control group (P=0.018). There were no significant differences in serum ALT levels between the experimental groups (P=0.757). Regarding the lipid profiles, the HCC and RIF groups showed significantly increases in TC, HDLc and triglyceride serum levels relative to the control (P<0.001, for all). However, the inverse occurred for serum LDLc levels (P<0.001). Regarding the atherogenic ratios, rats in the HCC and RIF groups showed a significant increase in AC, CRI-I and CR-II (P<0.001, for all) compared with the control group.

Analysis of fat deposition in liver tissue. In the quantitative analysis of lipid deposits in liver tissue, the rats in the HCC and RIF groups showed a significant increase in the accumulation of lipids, TGs and TC concentration relative to the control group (P<0.001, for all; Fig. 2A-C).

Expression of genes involved in steatohepatitis pathogenesis. Table II shows the data obtained of the hepatic gene expression of inflammatory parameters related to steatohepatitis pathogenesis. The HCC group showed a significant increase in IL-1β (P=0.011) and IL-10 (P<0.001) gene expression relative to the RIF group. The RIF group showed significantly lower expression levels of TLR2 (P=0.012), LPB (P=0.018) and MMP2 (P=0.003) than the HCC group, obtaining values that resembled the control group. No significant differences between the experimental groups in the gene expression of TNF-α (P=0.174), IL-6 (P=0.187), TLR4 (P=0.140), TGF-β1 (P=0.687) and MMP9 (P=0.479) were found.

Systemic inflammation and endothelial dysfunction. Table III shows the data obtained on the protein concentrations of inflammatory and endothelial dysfunction parameters. The HCC and RIF groups showed a significant increase in the serum

Table II. Gene expression of liver inflammation markers involved in steatohepatitis pathogenesis.

Variables	Control, n=10	HCC, n=9	RIF, n=10	P-value (ANOVA)
TNF-α	13.9±23.8	7.6±9.7	0.1±0.01	0.174
IL-1β	1.3±0.7[a,b]	2.1±0.8[b]	0.7±0.9[a]	0.011
IL-6	1.2±0.9	3.1±4.4	0.8±1.1	0.187
IL-10	1.3±0.7[a]	1.6±0.5[a]	0.4±0.5[b]	<0.001
TLR4	1.6±1.1	1.2±0.9	0.7±0.9	0.140
TLR2	1.2±0.7[a]	4.4±4.2[b]	0.8±1.3[a]	0.012
LPB	1.3±0.7[a]	2.4±1.2[b]	1.2±0.8[a]	0.018
Myd88	1.3±0.8[a]	1.0±0.9[a,b]	0.3±0.4[b]	0.009
TGF-β1	1.2±0.8	1.6±1.9	1.1±0.1	0.687
MMP2	1.3±0.8[a]	3.9±2.3[b]	1.0±1.5[a]	0.003
MMP9	1.5±1.4	2.7±1.5	3.2±4.6	0.479

Variables are presented as the mean ± standard deviation. The superscript letters [a, b] refer to the results of the post hoc statistical test (Tukey's test). Identical letters indicate no statistical difference, while different letters between experimental groups show a significant difference for the variable under analysis. IL, interleukin; HCC, hepatocellular carcinoma; LBP, lipopolysaccharide-binding protein; MMP, metalloproteinase; MyD88, myeloid differentiation primary response 88; RIF, rifaximin; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor.

Table III. Inflammation and endothelial dysfunction.

Variables	Control, n=10	HCC, n=9	RIF, n=10	P-value (ANOVA)
IL-1β, pg/ml	1.5±2.1	6.4±6.6	7.5±11.1	0.194
IL-6, pg/ml	39.0±31.6	17.5±18.7	29.7±44.3	0.393
TNF-α, pg/ml	1.6±0.9	1.7±0.4	1.7±0.5	0.918
PAI-1, pg/ml	25.2±14.5[a]	91.3±60.2[b]	135.6±82.8[b]	0.001
MCP-1, pg/ml	304.3±109.3[a]	336.6±119.9[a]	534.6±84.4[b]	<0.001
ICAM-1, ng/ml	0.1±0.001[a]	1.6±0.8[b]	1.8±0.7[b]	0.001
E-selectin, ng/ml	1.2±0.4[a]	2.6±0.2[b]	2.6±0.4[b]	0.001
VAP-1, ng/ml	5.7±1.5[a]	7.1±1.4[a,b]	7.8±1.6[b]	0.008

Variables are presented as the mean ± standard deviation. The superscript letters [a, b] refer to the results of the post hoc statistical test (Tukey's test). Identical letters indicate no statistical difference, while different letters between experimental groups show a significant difference for the variable under analysis. HCC, hepatocellular carcinoma; IL, interleukin; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; PAI, plasminogen activator inhibitor; RIF, rifaximin; TNF, tumor necrosis factor; VAP-1, vascular adhesion protein-1.

concentrations of PAI-1 (P=0.013 and P<0.001, respectively), ICAM-1 (P<0.001, for both) and E-selectin (P<0.001, for both) relative to the control group. The RIF group showed a significant increase in MCP-1 protein concentration compared with the HCC and control groups (P<0.001, for both). The RIF group had a significantly higher concentration of VAP-1 than the control group (P=0.041). No significant differences in the protein concentration of IL-1β (P=0.194), IL-6 (P=0.393) and TNF-α (P=0.918) between the groups were found.

Liver histopathological analysis. No abnormalities in the macroscopic appearance of the liver of the control rats (Fig. 3A) were found, whereas those in the HCC (Fig. 3B) and RIF (Fig. 3C-E) groups had the yellowish and greasy livers that characterize steatosis. Additionally, no abnormalities in the liver histopathological evaluation of the control group (Fig. 4A and B) were observed, whereas the rats in the HCC group had predominantly microvesicular steatosis, mild or moderate macrovesicular steatosis, inflammatory activity, and local fibrosis (Fig. 4C and D) and rats in the RIF group had predominantly microvesicular steatosis, moderate or severe macrovesicular steatosis, inflammatory activity, and local fibrosis (Fig. 4E and F).

Rodent-standardized MASLD activity scores showed that 7 (77.8%) rats in the HCC group developed steatosis and 2 (22.2%) steatohepatitis; 1 animal from this experimental group died, the biological samples of which were ignored in the proposed analyses. In the RIF group, 7 (70.0%) rats developed steatosis and 3 (30.0%) steatohepatitis. No animals in the HCC group developed liver cancer and only 2 (20.0%) in the RIF group developed grade IV (Fig. 3D) and grade II (Fig. 3E) HCC. The control group showed no hepatic histopathological

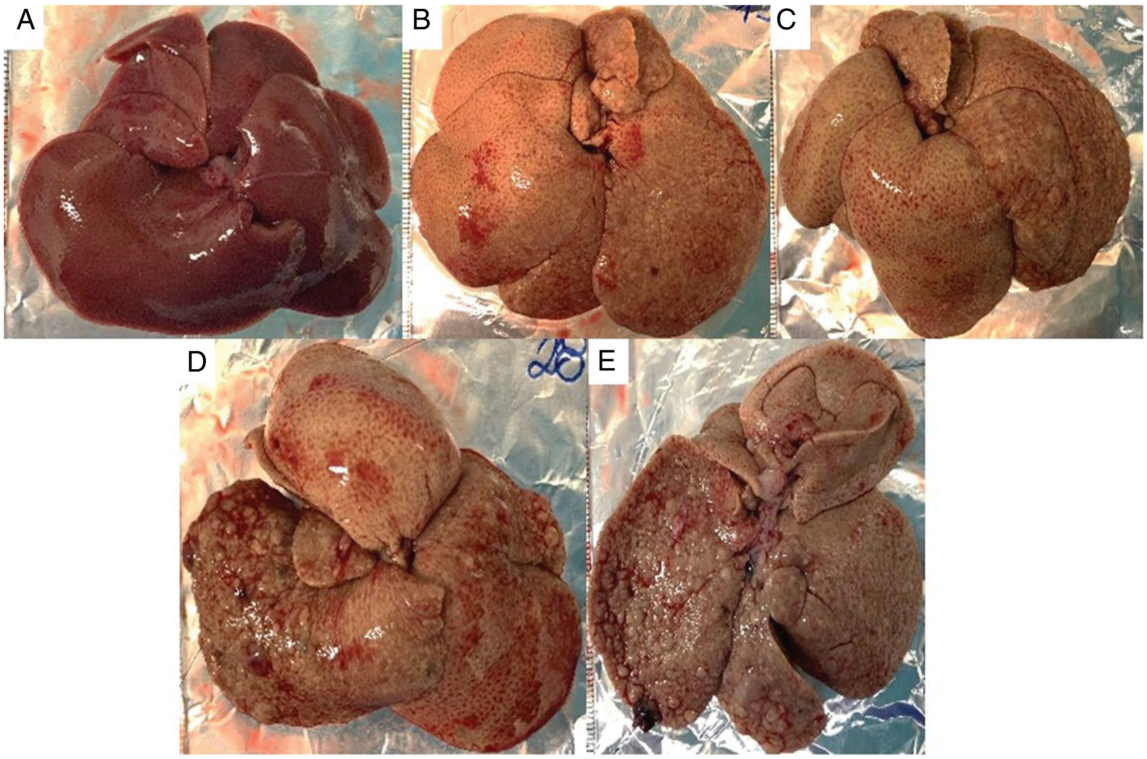

Figure 3. Macroscopic appearance of the liver in the (A) control group, (B) HCC group, (C) RIF group, (D) RIF group with cancer grade IV and (E) RIF group with cancer grade II. HCC, hepatocellular carcinoma; RIF, rifaximin.

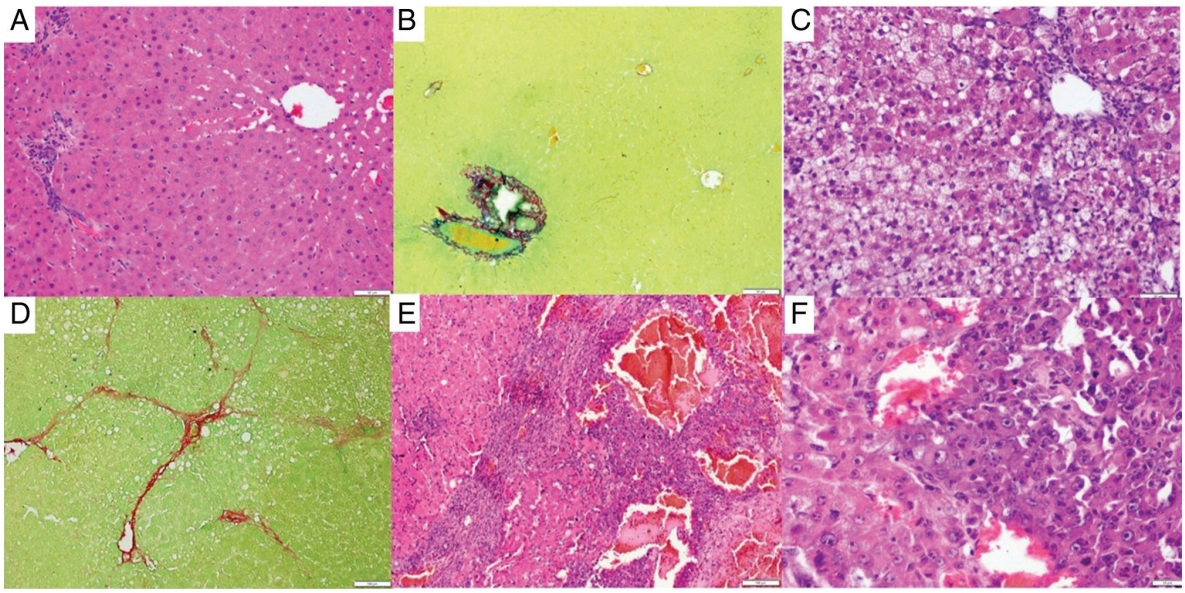

Figure 4. Hepatic histological evaluation. (A) H&E stain in the control group; magnification, x10. (B) Picrosirius stain in the control group; magnification, x10. (C) H&E stain in the HCC group, magnification, x20; (D) Picrosirius stain in the HCC group, magnification, x10. (E) H&E stain in the RIF group, magnification, x10. (F) H&E stain in the RIF group (magnification, x40) demonstrating metabolic dysfunction-associated liver disease-related liver injury secondary to the development of HCC. Scale bar, 10 μm. HCC, hepatocellular carcinoma; RIF, rifaximin.

changes. Table IV summarizes the data obtained in the evaluation of the hepatic histopathological scores.

Discussion

Recent decades have seen a significant increase in the prevalence of MASLD, which is associated with cardiometabolic risk factors. The progression of the disease is not only linked to the development of cardiovascular diseases, the main cause of mortality in this clinical condition, but also to the development of HCC (6,31). This context evinces the utmost importance of experimental studies to evaluate the pathophysiological mechanisms and the potential therapeutic targets of HCC. Indeed, there is a growing body of evidence that shows that

Table IV. Distribution of liver histopathological findings.

	General NAFLD scoring system for the rat models			
Variables	No NAFLD, n (%)	Steatosis, n (%)	Steatohepatitis, n (%)	HCC development, n (%)
Control, n=10	10 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
HCC, n=9	0 (0.0)	7 (77.8)	2 (22.2)	0 (0.0)
RIF, n=10	0 (0.0)	7 (70.0)	3 (30.0)	2 (20.0)

HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; RIF, rifaximin.

the use of RIF can contribute to reducing the complications of cirrhosis by relieving portal pressure (8,32-34). However, this is not the same as protection against HCC, whose pathogenesis does not involve portal hypertension. A search on PubMed (https://pubmed.ncbi.nlm.nih.gov/) using the keywords 'RIF' and 'HCC' yields 20 articles. Careful reading shows that only one article, published by our group, investigated this issue (8). On the contrary, a study has shown that the use of antibiotics, including RIF, can worsen the outcome of patients with HCC treated with sorafenib (34). Based on this evidence, the present study introduces a brand-new aspect to the discussion. In the present study, an experimental model of HCC secondary to MASLD was developed with the overall objective of evaluating the effect of prophylactic RIF treatment on the inflammatory markers and CVR of the disease. It was shown that the HCC and RIF experimental groups generally evinced alterations in the serum lipid profile, increased lipid and cholesterol deposition in hepatic tissue and significant changes in the atherogenic indices and the concentration of systemic inflammatory markers and endothelial adhesion molecules when compared with healthy rats. This indicated the triggering of metabolic and CVR-associated changes in the development of MASLD. Additionally, when comparing the two intervention groups, a significant increase in the gene expression of inflammatory mediators and hepatic fibrogenesis in the HCC group were observed when compared with the RIF-treated rats. These differences suggest a certain attenuation of the inflammatory and metabolic stimulus due to RIF. The hepatic histological evaluation showed that all animals in the HCC and RIF groups developed the local steatosis, inflammation and fibrosis that characterize MASLD. However, the development of HCC, the main objective of the present study, occurred in only 2 animals in the RIF group, showing the difficulty in reproducing experimental models previously described in the literature.

The need to fully understand the pathogenesis and progression of MASLD and to conduct preclinical tests for potential therapeutic agents has led to the development of experimental models that can reproduce hepatic phenotypes that resemble that in humans with MASLD and that can progress to inflammation, MASH cirrhosis, and HCC (35,36). The results of the present study showed that the use of HFCD for 16 weeks was able to induce changes in the serum and hepatic lipid profile, serum concentration of systemic inflammatory markers and adhesion molecules and in the atherogenic indices. These results corroborate other studies from our research group, which have reported a significant increase in the risk of cardiovascular disease associated with MASLD, which configures a pathophysiological mechanism that needs to be better evaluated with possible therapeutic targets (22,37). Regarding the serum levels of hepatic transaminases in the present study, significant differences in ALT levels between the experimental groups were not observed; however, rats in the RIF group showed a significant increase in AST levels compared with the control group. In the context of MASLD, the evaluation of both markers, but especially ALT due to its localization in the hepatocellular cytosol, serves as an indicator of liver damage. However, these transaminase levels fluctuate, and imaging or histological studies are necessary for diagnosis. Additionally, normal levels of liver enzymes are observed in individuals across the spectrum of MASLD, which may underestimate the presence of the disease (38,39). The present study was primarily developed to evaluate these hepatic inflammatory and CVR parameters in an experimental model of HCC secondary to MASLD. Analysis was hindered by the absence of HCC in most studied animals. The literature includes several experimental models that promote the development of HCC by diet, chemicals, xenografts and genetic induction (19,40). The previously described and standardized mixed experimental model of HCC secondary to MASLD induced by DEN and HFCD mimics the disease phenotype in humans, including excessive caloric intake, the development of obesity and dyslipidemia and a similar evolutionary profile to that in humans living with the several evolutionary stages of disease progression up to HCC (20). Recently, our research group published studies that used this same experimental model to evaluate the therapeutic effect of RIF on modulating the composition of the intestinal microbiota, epigenetic markers and autophagy. These studies observed the development of HCC secondary to MASLD, and treatment with RIF showed a beneficial effect on modulating the intestinal microbiota and epigenetic markers, preventing/retarding hepatic carcinogenesis (8,30). Considerable efforts have been made to generate experimental models that share numerous physiological, anatomical and metabolic characteristics with humans (35,41). However, in addition to these factors, the limitation in reproducing standardized experimental models in the literature configures a significant factor to be considered.

RIF is an oral, safe and poorly absorbed antibiotic that is widely used in clinical practice, especially to treat irritable bowel syndrome, traveler's diarrhea and hepatic encephalopathy (17,42,43). RIF plays a notable role in modulating the

intestinal microbiota due to its selective antimicrobial activity in the intestine, affecting both gram-positive and gram-negative bacteria (17,42). Due to these characteristics, the use of RIF has been the subject of preclinical and clinical studies to treat MASLD at its different evolutionary stages (8,43,44). However, the effect of RIF on MASLD/MASH must be better understood due to controversial results in the literature. Cheng *et al* (45) showed an adverse effect of prolonged (6-month long) administration of RIF in mice, resulting in the activation of genes involved in lipid uptake, leading to hepatic steatosis. Fujinaga *et al* (44) reported that the use of RIF combined with an angiotensin II receptor blocker was able to reduce intestinal permeability, portal endotoxemia and hepatic fibrogenesis by suppressing the TLR4/NF-κB signaling pathway in an experimental model of non-alcoholic steatohepatitis. Clinically, studies have reported that short-term treatment with RIF is beneficial in reducing endotoxemia, inflammatory cytokine levels and insulin resistance (18,46). In the present study, although MASLD failed to progress to HCC, both intervention groups showed mild to moderate steatosis, inflammation and local fibrosis. In this context, an interesting result in the present study refers to the significant reduction in the gene expression of inflammatory mediators and markers of hepatic fibrogenesis in rats treated with RIF compared with the HCC group (which received no treatment). A critical factor for the development and progression of MASLD refers to intestinal dysbiosis and, as shown in a previous study by our research group, treatment with RIF managed to promote modulation of the intestinal microbiota (8). This previous study found a significant decrease in the gene expression of LBP and TLR2, and consequently a reduction in the expression of IL-1β and MMP2 in animals treated with RIF compared with animals with HCC. LBP is a soluble acute phase protein that binds to bacterial lipopolysaccharide, which in turn can activate TLRs (including TLR-2) thereby triggering an inflammatory and hepatic fibrogenesis response (44,47,48). In this scenario, the differences in the present study resulting from RIF treatment suggest the partial attenuation in inflammatory and metabolic stimulation. In this context, a prior experimental model study recently published shows the potential beneficial effect of RIF in preventing/delaying the development of carcinogenesis (8). Additionally, our research group is developing *in vitro* studies with hexachlorobenzene, which can stimulate hepatic proliferation. Unpublished results show that RIF reduces cell proliferation in Huh-7 cells through antiproliferative, antimigratory and pro-apoptotic effects. The lack of *in vitro* experiments is a limitation of the present study. However, as aforementioned, the *in vitro* data should be published soon.

Although the potential beneficial effect of RIF administration was shown, the main limitation of the present study refers to the absence of HCC development. A previously described mixed HCC experimental model that has been reproduced by our research group in the past was utilized in the present study. However, the same success was not obtained. Animal models are essential for studying the initiation and progression of MASLD. In MASLD, an ideal preclinical model is triggered by the same causes of the disease in humans (such as caloric excess) and is associated with the same risk factors (49,50). In this context, the ideal assessment of HCC secondary to MASLD would trigger the lesion by the progression of the disease, rather than administering a chemical carcinogen (49). However, the use of chemical additives is very common, as spontaneous development of HCC only via diet occurs from 50 weeks of experimentation, increasing the costs of studies (49,50). The present study likely showed no development of HCC due to an issue regarding the DEN used, dosage and/or administration since its histopathological evaluation showed that the studied animals had steatosis and inflammation, probably due to the use of HFCD. In the present study, the histopathological evaluation of liver tissue was conducted only through staining with H&E and picrosirius red. The lack of evaluation by α-smooth muscle actin, fibronectin and Masson staining is therefore a limitation. The issue widely stems from the notion that experimental models must be reproducible, reliable, simple, easy and accessible for the development and preclinical validation of new therapeutic targets (49-51). However, the literature shows few reports on experimental models that have failed to reproduce the expected phenotype in a disease (as occurred in the present study), complicating the discussion of the topic.

This line of research of our group has developed unpublished *in vitro*, experimental and clinical studies and shows significant potential in the field of hepatology and metabolic disorders. Overall, it has been observed that RIF can reduce the expression of inflammatory mediators and modulate the expression of epigenetic markers, autophagy and the composition of the gut microbiota (8,30). This suggests the beneficial effect of RIF beyond its current clinical uses, particularly in the modulation of inflammatory and metabolic pathways, including the cardiometabolic pathways involved in MASLD. However, some knowledge gaps still require further exploration in future studies, including the reproducibility of experimental models. The development and reproduction of reliable and consistent experimental models of HCC secondary to MASLD are of utmost importance to evaluate the pathophysiological mechanisms associated with disease progression and to identify new biomarkers and therapeutic targets. In this context, the primary objective in developing the present study was to evaluate markers of autophagy and epigenetics and to assess their relationship with the microbiota composition in these different study groups. However, due to the non-development of HCC, the objectives had to be modified. It is difficult to explain the reason for this limitation as the experimental parameters in our previous study (the species of rats, the diet and the medication doses) in which the tumor developed were repeated exactly in the present study (8). Considering all the variables, perhaps DEN itself could be responsible for the negative results. Thus, the long-term effects of RIF on MASLD/MASH and its progression to HCC remain unclear. Therefore, further studies should evaluate inflammatory and metabolic pathways to assess the potential beneficial effects associated with this process and ensure their clinical applicability.

Despite the aforementioned gaps, the present study showed that, although most rats studied did not develop HCC, RIF treatment reduced metabolic stimulus and inflammatory markers compared with rats that received no MASLD treatment. As the reproducibility of experimental models is key to allowing the evaluation of pathophysiological mechanisms associated

with disease progression and to identify new biomarkers and therapeutic targets, it is important to show negative results to the academic community.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LL and MRA performed the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft and the review and editing of the manuscript; GTSG, LB, MHMP, CEP, CTSC and CUC performed the methodology, formal analysis and the review and editing of the manuscript. LL, CUC and MRA confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments and procedures involving the use of animals were approved by the Institutional Ethics Committee of Hospital de Clinicas de Porto Alegre (Porto Alegre, Brazil; approval no. 2019-0311). The procedures for the use of scientific animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and Law number 11,794 (Brazil, 2008).

Patient consent for publication

Not applicable.

Competing interests

Biolab Sanus Farmaceutica Ltd. donated the rifaximin used in our study, although this company had no influence on either the design or conduct of the study, the analysis or interpretation of the data or the writing of the manuscript. Therefore, we do not believe this constitutes a competing interest.

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