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Angiotensin System Blockers in Experimental NAFLD/MASLD: Is there a Preferential Pathway or Drug?

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Authors' contributions

This work was carried out in collaboration among all authors. Author MCSS conceptualized and designed the study and provided and interpreted data. Author FCC wrote and prepared the original draft. Authors JMP, IHJ, CCC, GS, MPM, KMW and TLS collaborated in revising the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Nonalcoholic fatty liver disease (NAFLD), currently known as metabolic dysfunction-associated steatotic liver disease (MASLD), is a persistent challenge in medical practice. Its global prevalence has progressively increased, with an estimated 30.1%. NAFLD has been associated with an increased risk of developing systemic hypertension, with an estimated prevalence of

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hypertension among patients with this liver condition at 39.34%. However, there are no established criteria for selecting antihypertensive medications for the treatment of hypertensive patients with NAFLD.

Aims: To evaluate and compare the effects of the preventive use of olmesartan and ramipril in an animal model of NAFLD. Assess the potential differences and determine if there are specific aspects that may account for these differences. In addition to histological analysis and metabolic variables, evaluate oxidative stress through immunohistochemistry.

Methods: A total of 41 rabbits were distributed into four groups: normal control group (NG) with 9 animals; placebo group (PG) with 10 animals; olmesartan group (OG) with 12 animals; and ramipril group (RG) with 10 animals. The NG received a standard diet without additives, while the OG, PG, and RG were given the standard diet with added cholesterol. The OG and RG were treated with their respective medications from baseline until euthanasia over an 8-week period. Hematoxylin-eosin stained slides were assessed using a scoring system for histological evaluation of NAFLD, and hepatic oxidative stress was evaluated through immunohistochemistry.

Results: There was a significant attenuation of steatosis, lobular inflammation, ballooning, fibrosis, and steatohepatitis, as indicated by the activity score, in both the ramipril group (RG) and olmesartan group (OG) compared to the placebo group (PG). Additionally, hepatic oxidative stress was notably decreased in these treatment groups.

Conclusion: The preventive use of ramipril and olmesartan significantly attenuated the development of fundamental histological changes and hepatic oxidative stress in an animal model of NAFLD. Therefore, it can be inferred that olmesartan and ramipril may add benefit in the treatment of hypertensive patients with NAFLD. However, such findings require substantiation based on clinical studies.

Keywords: Nonalcoholic fatty liver disease; metabolic dysfunction-associated steatotic liver disease; experimental model of NAFLD; hypertension; renin-angiotensin-aldosterone system.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) remains a persistent challenge in medical practice, even four decades after its first description and naming in 1980, when Ludwig and colleagues reported biopsy findings similar to alcoholic steatohepatitis in mostly moderately obese patients without a significant history of alcohol consumption [1]. Since then, a growing body of research has been conducted to elucidate its complex and multifaceted nature in terms of epidemiology, pathophysiology, potential therapeutic targets, and association with other comorbidities, such as obesity, metabolic syndrome (MS), type 2 diabetes mellitus (T2DM), and cardiovascular diseases (CVD). However, this important chronic liver disease remains inadequately understood in many aspects.

The global prevalence of NAFLD is rising at an alarming rate. In a meta-analysis published in 2016, the global prevalence of NAFLD was estimated at 25.23% [2]. However, in a systematic review published in 2023, this prevalence was even higher, estimated at 30.1% [3]. Furthermore, it is projected that by 2040, about half of the adult population will have NAFLD [4]. Additionally, NAFLD has significantly contributed to the increasing burden of chronic liver disease worldwide [5] and has been described as one of the leading causes of advanced fibrosis, cirrhosis, hepatocellular carcinoma, liver failure, and liver transplantation.

NAFLD is often associated with MS and has been characterized as a hepatic manifestation and an emerging component of this syndrome [6]. Obesity, T2DM, and CVD share common cardiometabolic risk factors with NAFLD, suggesting overlapping pathophysiological mechanisms [7]. It is estimated that 51.34% of NAFLD patients have obesity, 22.51% have diabetes, 69.16% have dyslipidemia, 39.34% have hypertension, and 42.54% meet the diagnostic criteria for MS. These percentages tend to increase as the disease progresses to non-alcoholic steatohepatitis (NASH) [3].

The diagnosis of NAFLD is established by the presence of steatosis detected through imaging or histology, while excluding significant alcohol consumption, prolonged use of steatogenic medications, viral hepatitis, hereditary disorders, and other causes of secondary steatosis [8]. Symptoms such as fatigue, nausea, and discomfort in the right upper quadrant of the

abdomen may be associated with NAFLD; however, most patients are asymptomatic and exhibit no physical abnormalities at the time of diagnosis. Mild to moderate elevations in serum aminotransferase levels are the most common – and often the only – laboratory abnormalities observed. Notably, only 2.8% to 5.4% of individuals with steatosis without an identifiable cause exhibit elevated aminotransferase levels [9,10].

Cardiovascular disease (CVD) is a leading cause of mortality among NAFLD patients. The frequent presence of well-established cardiovascular risk factors in these individuals indicates an association between this chronic liver disease and an increased risk of CVD. However, the exact role of NAFLD in this context remains under investigation. On the other hand, shared pathophysiological characteristics of both conditions suggest significant potential links between them [11, 12]. Cross-sectional studies have shown a significant association between NAFLD and subclinical carotid atherosclerosis, independent of traditional cardiovascular risk factors. [13, 14]. Additionally, NAFLD has been linked to coronary artery disease (CAD) [15, 16] and increased arterial stiffness [17].

The prevalence of hypertension in NAFLD patients, estimated at 39.34% [18], is higher than the estimated prevalence for the general population aged 30 to 79 years – 34% in men and 32% in women [19]. The continuous relationship between elevated blood pressure and the risk of stroke, CAD, heart failure, and chronic kidney disease development and progression has been well-documented [20]. In a systematic review and meta-analysis, NAFLD was significantly associated with an increased risk of developing hypertension [21]. Therefore, antihypertensive pharmacotherapy will often be necessary in NAFLD patients.

The pathophysiology of NAFLD is complex, and in many aspects, the mechanisms involved still need to be adequately elucidated [22]. Insulin resistance (IR) is believed to play a key role in the accumulation of triglycerides in hepatocytes, triggering oxidative stress and an inflammatory response that culminates in cell damage, death, and fibrotic replacement [6]. The activation of the renin-angiotensin system (RAS) has been associated with IR [23], increased lipid influx into hepatocytes [24], oxidative stress [24, 25], inflammatory response, and hepatic fibrogenesis [25, 26]. On the other hand, RAS blockade has been associated with improvements in these parameters [27-30]. Therefore, IR and RAS blockade may represent potential pharmacological therapeutic targets for NAFLD.

Despite the high global prevalence and the morbidity and mortality associated with NAFLD, due to its complexity, only resmetirom has been approved by the U.S. Food and Drug Administration for the treatment of adults with non-cirrhotic NASH who have moderate to advanced fibrosis, and this is to be used alongside lifestyle changes such as diet and physical activity. As of March this year, no other medications had been approved by regulatory agencies specifically for the treatment of NAFLD [31], and it is essential to note that resmetirom is intended for a specific profile of NAFLD. Therefore, established recommendations should continue to emphasize lifestyle modifications aimed at weight loss through dietary changes and increased physical activity, as well as pharmacological treatment for associated conditions.

Olmesartan, an angiotensin II receptor blocker (ARB), and ramipril, an angiotensin-converting enzyme inhibitor (ACEI), have shown potential benefits in animal models of NAFLD. In these studies, the preventive administration of both drugs significantly attenuated the development of the entire basic histological spectrum of NAFLD [30,32-36]. However, there is currently no comparative data between them in the context of NAFLD. Furthermore, clear criteria for selecting antihypertensive medications in the treatment of hypertensive patients with NAFLD have yet to be established. In this context, the present study aims to analyze and compare the preventive effects of olmesartan and ramipril in an animal model of NAFLD.

2. MATERIALS AND METHODS

For the analysis and comparison of results, well-preserved slides were utilized, along with blocks of liver tissue samples embedded in paraffin, to create new slides that could replace those unsuitable for histological and immunohistochemical analysis. Additionally, data on metabolic and serum variables from two previously conducted studies using the same NAFLD animal model at the Pontifical Catholic University of Paraná (PUC-PR) were incorporated into the analysis. These studies were approved by the university's animal ethics committee. The materials used for histological

analysis are appropriately stored in the experimental pathology laboratory at PUC-PR, and the researchers provided the metabolic variables results.

2.1 Sample

Slides and data from 41 male albino rabbits (Oryctolagus cuniculus) of the New Zealand strain from the Central Animal Facility at PUC-PR were used. The rabbits, initially healthy, weighing between 2,996 and 3,255 grams and with an average age of 111 days, were divided into four groups: Normal Control Group (NG), with 9 animals; Placebo Group (PG), with 10 animals; Olmesartan Group (OG), with 12 animals; and Ramipril Group (RG), with 10 animals. The NG group was fed a standard laboratory rabbit diet without additives (Nuvilab Rabbits®-Nuvital). The OG, PG, and RG groups received the same diet but with added cholesterol – 1% for the OG and 0.925% for the PG and RG. The drugs were administered via oral solution once daily to their respective groups (olmesartan 1 mg/kg and ramipril 0.35 mg/kg) from baseline until euthanasia. These doses, calculated through allometric extrapolation, correspond to approximately 20 mg for olmesartan and 5 mg for ramipril in humans. The study lasted for 8 weeks, during which all groups had ad libitum access to water and their respective diets, and were sacrificed at the end of this period.

2.2 Histological Analysis

The slides, containing two histological sections – one from the right lateral lobe and another from the left medial lobe – were stained with hematoxylin-eosin (HE) and Gomori trichrome. The analysis was conducted in a blinded manner using an Olympus® microscope, based on a validated histological scoring system for assessing NAFLD in both clinical and experimental studies [37]. The presence of NASH was estimated using the NAFLD Activity Score (NAS), a component of this scoring system. NAS is the unweighted sum of scores for steatosis, hepatocyte ballooning, and lobular inflammation. In the validation study, NAS ≤ 2 was significantly correlated with the absence of NASH; NAS between 3 and 4 showed no discriminative value, being similarly distributed among three diagnoses (absence of NASH, borderline, and NASH); and NAS ≥ 5 was correlated with the presence of NASH (Table 1). After analyzing and consolidating the results, the groups were compared.

2.3 Immunohistochemistry

Immunohistochemistry was conducted to assess hepatic expression of inducible nitric oxide synthase (iNOS) as an indirect indicator of nitro-oxidative stress. Monoclonal anti-iNOS antibodies from rats (dilution 1/200, ab129372, Abcam) were used for this purpose. The stained slides were evaluated in a blinded manner, following the Allred scoring system [38], using an Olympus BX 50 microscope. The Allred score is calculated as the sum of the proportion and intensity scores: the proportion score reflects the total number of stained cells, while the intensity score indicates the staining intensity of the positive cells (Table 2).

Table 1. Scoring System for Histological Analysis of NAFLD

Alteration	Definition	Score
Degree of Steatosis	<5%	0
	5 to 33%	1
	33 to 66%	2
	>66%	3
Lobular Inflammation	None	0
	<2 foci/field (200x)	1
	2-4 foci/field (200x)	2
	>4 foci/field (200x)	3
Ballooning	Absent	0
	Few cells	1
	Many cells	2
NAFLD Activity Score (NAS)	NAFLD absent	≤ 2
	Indeterminate	3 and 4
	NAFLD present	≥5

NAFLD (Non-Alcoholic Fatty Liver Disease).
Source: authors' composition

Table 2. Scores for Proportion of Stained Cells and Intensity of Staining

Parameter	Definition	Score
Total Proportion of Stained Cells	None	0
	<1/100	1
	1/100 to <1/10	2
	1/10 to <1/3	3
	1/3 to 2/3	4
	>2/3	5
Intensity of Positive Cell Staining	None	0
	Weak	1
	Intermediate	2
	Strong	3

Source: authors' composition

2.4 Comparison of Results Related to Serum Variables

Comparisons were conducted between the weights and serum levels of AST, ALT, creatinine, glucose, total cholesterol, and triglycerides, both at baseline and at the time of euthanasia.

2.5 Statistical Analysis

Data were analyzed using IBM SPSS Statistics v.20 software (Armonk, NY: IBM Corp). The results were described using means, medians, minimum and maximum values, standard deviations, or frequencies and percentages. The interaction between the groups (placebo, ramipril, olmesartan, and normal control) and phases (baseline and euthanasia) for the quantitative variables was evaluated using the non-parametric Kruskal-Wallis test. To compare baseline and euthanasia measurements within each group, the non-parametric Wilcoxon test was employed. The normality of the data was assessed using the Shapiro-Wilk test. Statistical significance was defined as *P*-values < 0.05.

3. RESULTS

3.1 Weight and Serum Variables

At baseline, the weights were similar across the groups, with the exception of the normal control group, which had a higher average weight than the others. Over time, both the olmesartan and normal groups exhibited more significant weight gain, with higher averages at the time of euthanasia (Fig. 1).

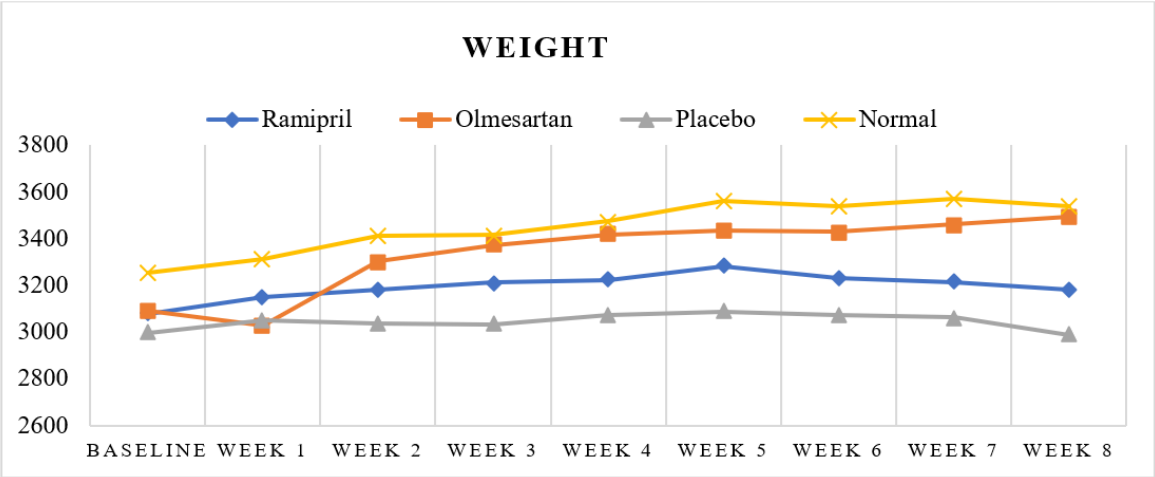

Fig. 1. Distribution of Average Animal Weight
Values are described in grams.
Source: authors' composition

Table 3. Analysis of Glucose, Cholesterol, Triglycerides, AST, and ALT Variables

Phase	Group	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	AST (U/L)	ALT (U/L)
Baseline	PG	141,0 ± 32,6	31,9 ± 16,2	49,5 ± 16,3	52,8 ± 14,8	85,9 ± 29,7
	RG	123,7 ± 48,0	38,4 ± 28,0	45,7 ± 19,4	51,2 ± 18,1	68,7 ± 29,2
	OG	219,3 ± 31,1	50,7 ± 18,2	61,8 ± 43,5	35,8 ± 16,1	40,7 ± 14,6
	NG	163,7 ± 37,6	18,4 ± 10,7	70,7 ± 34,6	70,0 ± 43,7	92,2 ± 47,8
	P	< 0,001	0,003	0,278	< 0,001	< 0,001
Euthanasia	PG	150,8 ± 22,3	971 ± 157	384,9 ± 307,7	86,1 ± 32,8	98,5 ± 47,9
	RG	125,3 ± 43,1	989 ± 103	282,7 ± 175,6	86,5 ± 39,6	104,3 ± 54,1
	OG	168,0 ± 33,0	2080 ± 800	214,8 ± 126,6	43,8 ± 21,5	41,4 ± 14,7
	NG	142,0 ± 25,9	18 ± 6	76,2 ± 48,6	50,6 ± 23,0	92,7 ± 37,7
Difference (euthanasia – baseline)	PG	9,8 ± 24,3	939 ± 149	335,4 ± 307,8	33,3 ± 38,4	12,6 ± 43,4
	RG	1,6 ± 63,2	951 ± 92	237,0 ± 176,9	35,3 ± 42,2	35,6 ± 65,6
	OG	- 51,3 ± 32,2	2029 ± 786	153,0 ± 118,3	8,0 ± 23,3	0,8 ± 10,2
	NG	-21,7 ± 51,2	-0,3 ± 9,6	5,6 ± 54,9	-19,4 ± 43,7	0,4 ± 42,1
	P	0,012	< 0,001	0,001	0,001	0,420
Baseline vs Euthanasia (*P*-value)	PG	0,046	0,001	0,001	0,001	0,944
	RG	0,799	0,005	0,005	0,022	0,139
	OG	0,003	0,002	0,003	0,814	0,859
	NG	0,314	0,999	0,722	0,236	0,767

Values are expressed as mean ± standard deviation. PG = placebo group; RG = ramipril group; OG = olmesartan group; NG = normal group; P = P-value for comparisons.
Source: authors' composition

Table 4. Difference between euthanasia and baseline for serum variables: pairwise group comparisons (*P*-value)

Comparisons	Glucose	Creatinin	Cholesterol	AST	ALT
RG vs OG	<0,001	<0,001	0,035	0,013	0,003
RG vs PG	<0,001	<0,001	<0,378	<0,001	0,003
OG vs PG	0,093	0,615	0,171	0,075	0,925
RG vs NG	0,023	0,457	0,030	0,466	0,377
OG vs NG	0,001	<0,001	<0,001	0,002	<0,001
PG vs NG	0,043	< 0,001	0,002	< 0,001	<0,001

PG = placebo group; RG = ramipril group; OG = olmesartan group; NG = normal group;
Source: authors' composition

Regarding glycemia, differences were noted among the groups at baseline, with the highest averages found in the olmesartan and normal groups. When comparing the baseline and euthanasia phases within each group, significant differences were observed in both the placebo and olmesartan groups. In the placebo group, the average glycemia at euthanasia was higher than at baseline, whereas the opposite trend was observed in the olmesartan group (Table 3).

There was a marked increase in serum total cholesterol levels at euthanasia compared to baseline, except in the normal control group (Table 3). Pairwise comparisons between groups revealed significant differences, with the exception of the comparison between the olmesartan and placebo groups (Table 4).

Serum triglyceride levels were similar at baseline and, similar to total cholesterol, increased significantly at euthanasia in all groups except the normal control group (Table 3). However, pairwise comparisons did not show significant differences between the olmesartan, placebo, and ramipril groups (Table 4).

AST and ALT levels differed significantly among the groups at baseline, with higher averages in the normal control and placebo groups. When comparing the baseline and euthanasia phases within each group, significant differences in AST levels were observed in both the placebo and ramipril groups. In contrast, ALT levels remained similar across all groups (Table 3).

Regarding creatinine levels, there was a significant difference among groups at baseline, with the highest average recorded in the normal control group. The difference between the baseline and euthanasia phases was significant, except in the normal control group (Table 3). However, at euthanasia, pairwise comparisons between the olmesartan, placebo, and ramipril groups did not show significant differences.

3.2 Histological Analysis

Steatosis was observed in all animals of the placebo group, with more than 33% to 66% of hepatocytes affected (score 2) in half of the group, and more than 66% affected (score 3) in the remaining half. In the ramipril and olmesartan groups, 40% and 50% of animals, respectively, had a steatosis score of 1, with 10% of the ramipril group not developing steatosis. Pairwise comparisons showed no significant difference between the ramipril and olmesartan groups (*P*=1). However, both the ramipril and olmesartan groups had significantly lower steatosis scores compared to the placebo group, with P-values of 0.032 and 0.015, respectively (Fig. 2, Table 5).

Regarding lobular inflammation, 80% of the placebo group developed significant inflammation (scores 2 and 3), 90% of the ramipril group developed mild inflammation (score 1), and 100% of the olmesartan group showed no signs of inflammation. As with steatosis, significant differences were observed between the placebo group and both the olmesartan and ramipril groups, but no significant difference was found between the olmesartan and ramipril groups (*P*=0.454), with both groups showing significantly lower lobular inflammation scores (Fig. 2, Table 5).

Ballooning degeneration, at its highest score, was observed in 80% of the placebo group. In contrast, the lowest score for this histological change was noted in 70% of the ramipril group and in 100% of the olmesartan group. Significant differences were found between the placebo group and both the ramipril and olmesartan groups (Fig. 2, Table 5), but no significant difference was observed between the olmesartan and ramipril groups (*P*=0.195).

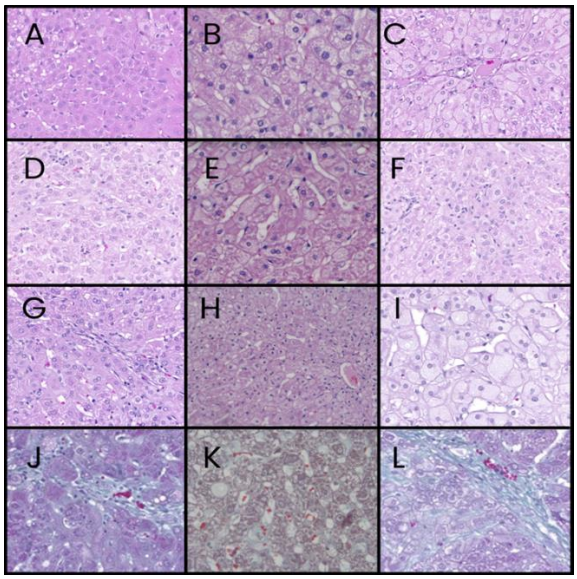

Fig. 2. Steatosis, lobular inflammation, ballooning, and fibrosis

(A) Rabbit from the ramipril group with the lowest steatosis score (HE, 200x). (B) Rabbit from the olmesartan group with steatosis grade 1 (HE, 200x). (C) Rabbit from the placebo group with the highest degree of steatosis (HE, 200x). (D) Rabbit from the ramipril group with a score of 1 for lobular inflammation (HE, 200x). (E) Rabbit from the olmesartan group with no lobular inflammation (HE, 400x). (F) Rabbit from the placebo group with the highest score for lobular inflammation (HE, 200x). (G) Rabbit from the ramipril group with preserved hepatic lobular architecture, without ballooning (HE, 200x). (H) Rabbit from the olmesartan group with ballooning grade 1 (HE, 200x). (I) Rabbit from the placebo group with the highest score for ballooning (HE, 200x). (J) Fibrosis score 1A was observed in the ramipril group (Gomori Trichrome, 200x). (K) Absence of fibrosis in a rabbit from the olmesartan group (Gomori Trichrome, 200x). (L) Rabbit from the placebo group with a fibrosis score 2 (Gomori Trichrome, 200x).

Source: authors' composition

Table 5. Histological Analysis Results According to the Scoring System for NAFLD Evaluation

Histological Alteration	Score	Percentage/Group			Two by two comparison with PG	
		PG	RG	OG	RG	OG
Steatosis	0	0%	10%	0%	*P*=0,032	*P*=0,015
	1	0%	40%	50%		
	2	50%	40%	33,3%		
	3	50%	10%	16,7%		
Lobular Inflammation	0	0%	0%	100%	*P*=0,006	*P*< 0,001
	1	20%	90%	0%		
	2	40%	10%	0%		
	3	40%	0%	0%		
Ballooning	0	0%	10%	0%	*P*=0,023	*P*< 0,001
	1	20%	70%	100%		
	2	80%	20%	0%		
Fibrosis	0	0%	0%	100%	*P*=0,020	*P*=0,001
	1	0%	0%	0%		
	1A	20%	60%	0%		
	1B	10%	30%	0%		
	1C	50%	10%	0%		
	2	20%	0%	0%		
NAFLD Activity Score	0-2	0%	20%	50%	*P*=0,005	*P*< 0,001
	3-4	0%	50%	50%		
	≥5	100%	30%	0%		

PG = placebo group; RG = ramipril group; OG = olmesartan group; NG = normal group; P = P-value for comparisons

Source: authors' composition

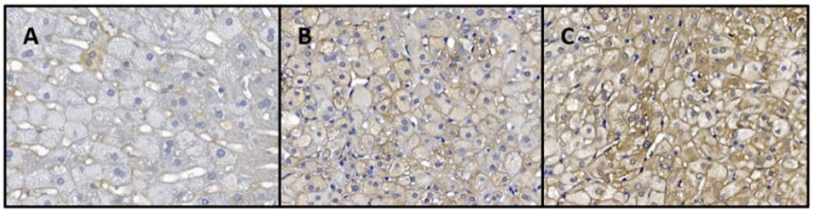

Fig. 3. Immunohistochemistry: iNOS Immunostaining, Photomicrographs, 200x Magnification
(A) Rabbit from the olmesartan group with an Allred score of 4. (B) Rabbit from the ramipril group with an Allred score of 5. (C) Rabbit from the placebo group with an Allred score of 8.
Source: authors' composition

Table 6. Comparison between groups two by two regarding Allred score components

Score	Compared Groups	*P*	Adjusted *P*
Intensity Score	Olmesartan vs ramipril	0,932	1
	Olmesartan vs placebo	0,034	0,101
	Ramipril vs placebo	0,034	0,103
Proportion Score	Olmesartan vs ramipril	0,047	0,141
	Olmesartan vs placebo	<0,001	<0,001
	Ramipril vs placebo	<0,009	0,028
Allred Score	Olmesartan vs ramipril	0,062	0,187
	Olmesartan vs placebo	<0,001	<0,001
	Ramipril vs placebo	0,004	0,012

P = P-value for comparisons.
Source: authors' composition

Fibrosis was not identified in the olmesartan group, occurred at a mild intensity in 60% of the ramipril group, and was classified as greater than moderate (scores 1C and 2) in 70% of the placebo group. There was a significant difference between the placebo group and the olmesartan and ramipril groups (Fig. 2, Table 5), but this difference was not observed between the olmesartan and ramipril groups (*P*=0.45).

Regarding the NAFLD activity score, 70% of the ramipril group and 100% of the olmesartan group did not have scores indicative of NASH, whereas 100% of the placebo group had scores consistent with NASH. Consistent with other histological parameters, there was a significant difference between the placebo group and both the olmesartan and ramipril groups (Table 5); however, no difference was observed between the olmesartan and ramipril groups (*P*=0.084).

3.3 Immunohistochemistry

The expression of iNOS, assessed through immunohistochemistry, was significantly different among the three groups exposed to the hypercholesterolemic diet (placebo, ramipril, and olmesartan) when comparing both the proportion scores and the sum of intensity and proportion scores. However, when comparing intensity scores alone, no significant difference was observed among the three groups, although the *P*-value was borderline (*P*=0.052). Pairwise comparisons between the placebo group and the ramipril and olmesartan groups showed significant differences, with the same *P*-value for both comparisons (*P*=0.034). However, after applying the Bonferroni correction, the statistical significance was lost for both comparisons (Table 6).

Regarding the proportion score of stained cells, pairwise comparisons revealed significant differences between the placebo group and both the ramipril (*P*<0.009) and olmesartan (*P*<0.001) groups. This difference remained significant for both comparisons (*P*=0.028 and *P*<0.001, respectively) even after applying the Bonferroni correction (Table 6). Similarly, the Allred score, which is the sum of the proportion and intensity scores, was significantly higher in the placebo group compared to the ramipril (*P*=0.004) and olmesartan (*P*<0.001) groups. This statistical significance was also maintained (*P*=0.012 and *P*<0.001) after the Bonferroni correction (Table 6). In pairwise comparisons between the ramipril and olmesartan groups, a significant difference was observed only in the proportion scores of stained cells (*P*=0.047). However, statistical

significance was lost after applying the Bonferroni correction (Fig. 3, Table 6).

4. DISCUSSION

NAFLD has recently been renamed metabolic dysfunction-associated steatotic liver disease (MASLD), while NASH is now referred to as metabolic dysfunction-associated steatohepatitis (MASH). This new definition requires the presence of at least one cardiometabolic risk factor, in addition to the absence of alcohol consumption exceeding 20 grams per day for women and 30 grams per day for men, as well as other causes of hepatic steatosis [38]. The lack of a standardized animal model for MASLD, as was the case for NAFLD, reinforces the importance of developing animal models with greater elucidative potential. The animal model employed in this study utilizes a natural induction method (hyperlipidemia) and exhibits the fundamental histological characteristics of NAFLD/MASLD found in humans (steatosis, lobular inflammation, ballooning, and fibrosis). Specifically related to MASLD, this model also presents hypertriglyceridemia as an additional diagnostic criterion. Furthermore, a study that analyzed the NAFLD database revealed that 99.8% of patients met the MASLD criteria [38]. Therefore, it can be inferred that it is a viable animal model of MASLD.

Obesity has been associated with increased prevalence and worsening prognosis of NAFLD [39]. However, in the animal model used in the present study, animal weight had no apparent influence on histological outcomes. At both baseline and euthanasia, the highest mean weight was recorded in the normal control group, while the placebo group had the lowest mean weight. Furthermore, at euthanasia, the olmesartan group exhibited the second-highest mean weight, lower only than the mean of the normal group (Fig. 1). A similar finding was observed in experiments with Fisher rats subjected to a choline-deficient diet and treated with losartan [40]. This outcome is attributed to the drug's role in attenuating the progression of liver disease.

Similar to obesity, the presence of T2DM has also been associated with increased prevalence and progression of NAFLD [41]. Up to 75% of patients with T2DM are estimated to have NAFLD, while approximately 10 to 18% of adults with NAFLD have T2DM [42]. In our study, the highest mean blood glucose levels at baseline were observed in the normal and olmesartan groups, but both evolved with a reduction in these levels at euthanasia. In contrast, the placebo group evolved with increased blood glucose levels at euthanasia, which suggests a potential influence of the induction method used, as well as an attenuation related to the preventive use of olmesartan. Nevertheless, the lack of well-established normal blood glucose values for laboratory animals and the limitations inherent to the animal models make this result limited.

The significantly elevated levels of triglycerides and particularly total cholesterol in the groups subjected to the hypercholesterolemic diet at euthanasia, combined with the lack of significant differences between the olmesartan and placebo groups, indicate that the drugs did not influence these metabolic variables. The similarity between ALT levels at baseline and euthanasia in each group, along with comparable creatinine levels at euthanasia among the three groups exposed to the hypercholesterolemic diet, further suggest that the drugs had no impact on these parameters. While there was a difference in AST levels at baseline, significant changes were noted only in the placebo and ramipril groups when comparing the baseline and euthanasia phases within each group.

Regarding the histological analysis, the steatosis observed in the placebo group was moderate, affecting more than 33 to 66% of the hepatocytes (score 2) in 50% of the rabbits, and marked or severe, affecting more than 66% of the hepatocytes (score 3) in the other half of the group. In contrast, 50% of the olmesartan group and 40% of the ramipril group developed mild steatosis (score 1), and 10% of the ramipril group did not develop steatosis. Pairwise comparisons with the placebo group demonstrated significant differences, characterizing attenuation of steatosis in the olmesartan group (P=0.015) and in the ramipril group (P=0.032). However, the comparison between the olmesartan and ramipril groups demonstrated no significant difference (P=1). Therefore, it can be inferred that olmesartan and ramipril attenuated the development of steatosis in this experimental model (Table 4).

There are few studies published in high-impact journals that utilize animal models with methods and findings similar to those of the present study. In an experiment using Wistar rats fed a methionine- and choline-deficient diet (MCD),

there was a significant attenuation of the development of steatosis in the group receiving olmesartan, which aligns with the results observed in our study [30]. Similarly, in an animal model of obesity and diabetes using Otsuka Long–Evans Tokushima Fatty (OLETF) also fed an MCD diet, olmesartan significantly attenuated the development of steatosis [33]. However, differences related to the specific animal model and the methods used to induce liver disease complicate direct comparisons with the present study.

In our study, lobular inflammation was classified as mild, moderate, and severe (scores 1, 2, and 3), with 20%, 40%, and 40% of the placebo group showing these scores, respectively. In contrast, the ramipril group exhibited 90% with mild inflammation (score 1) and 10% with moderate inflammation (score 2), while no lobular inflammation was observed in the olmesartan group. Compared to the placebo group, olmesartan effectively prevented lobular inflammation, and ramipril significantly attenuated its severity (Table 4). However, the comparison between the olmesartan and ramipril groups showed no significant difference (P=0.454). In previously cited experiments, results were similar to those observed in the present study [33,34].

Ballooning degeneration, which characterizes hepatocellular injury, was observed in the placebo, olmesartan, and ramipril groups. In the placebo group, 80% of the rabbits exhibited the highest score (score 2), whereas this score was present in only 20% of the ramipril group and was absent in the olmesartan group. Furthermore, 100% of the olmesartan group and 70% of the ramipril group developed the lowest grade (score 1), with 10% of the ramipril group not developing ballooning at all. Compared to the placebo group, both olmesartan and ramipril significantly attenuated hepatocellular injury, with no significant difference observed between the olmesartan and ramipril groups (Table 4).

In the present experiment, the highest fibrosis scores were recorded in the placebo group, with 60% of the ramipril group exhibiting mild fibrosis, while the olmesartan group showed no signs of fibrosis. Compared to the placebo group, olmesartan effectively prevented fibrosis, and ramipril significantly attenuated its development (Table 5), with no significant difference observed between the olmesartan and ramipril groups. Similar results were reported in a previously cited study, which used LETO rats fed an MCD diet and treated with olmesartan 5 mg/kg/day [33], and in a study that used Fisher rats fed a choline-deficient diet and treated with losartan 30 mg/kg/day [40].

The NAFLD activity score has been used in important clinical studies such as PIVENS [43] and MAESTRO-NASH [44] to estimate the presence of NASH, now referred to as metabolic dysfunction-associated steatohepatitis (MASH). In our study, 70% of the ramipril group and 100% of the olmesartan group showed no scores indicative of NASH, whereas all animals in the placebo group exhibited scores consistent with the condition (Table 4). Compared to the placebo group, Olmesartan and ramipril significantly attenuated the development of NASH, and similarly to the other criteria of this scoring system, the comparison between the olmesartan and ramipril groups showed no significant difference (P=0.084).

Oxidative stress related to lipid peroxidation is one of the factors that culminate in hepatocellular injury and death in NASH. Hepatic expression of iNOS has been used to estimate nitro-oxidative stress, which has been associated with the severity of NAFLD in animal models [45,46]. In our experiment, the hepatic expression of iNOS, assessed using the Allred score, was significantly lower in the olmesartan and ramipril groups compared to the placebo group, and this significance remained after applying the Bonferroni correction. When analyzing the individual parameters of the Allred score, we observed a significant reduction in the proportion of stained cells in both the ramipril and olmesartan groups relative to the placebo group, with this significance maintained after the Bonferroni correction. However, regarding the staining intensity score, while significant differences were found between the placebo group and both the olmesartan and ramipril groups, this significance did not hold after Bonferroni correction. Additionally, comparisons between the olmesartan and ramipril groups revealed no significant differences across all Allred score parameters. Therefore, we can infer that olmesartan and ramipril, when used preventively, similarly attenuated hepatic oxidative stress (Table 6). Similar findings were reported in a previously cited study involving OLEFT rats subjected to an MCD diet and treated with olmesartan [33].

In addition to the inherent limitations of animal models, such as the lack of clearly defined normal values for serum variables and the potential influence of animal handling on these values, it is important to note that the olmesartan group was fed a diet with 1% cholesterol, while the placebo and ramipril groups received a diet with 0.925% cholesterol. However, this slight difference in concentration alone would not fully explain the higher serum cholesterol levels observed in the olmesartan group. Aside from this discrepancy, all groups were subjected to identical conditions throughout the study period.

The significant and often independent relationship between NAFLD and CVD has been documented in various studies. Although the mechanisms behind this connection remain unclear, it has increasingly gained recognition over time. Around a decade ago, the notion emerged that NAFLD might serve as a marker of cardiovascular risk. In 2022, the American Heart Association declared that NAFLD could be considered a risk enhancer when evaluating atherosclerotic cardiovascular disease risk in patients [47,48]. In 2024, research highlighted the high prevalence of NAFLD in individuals with very high-risk coronary atherosclerotic disease in specific regions [49], as well as its association with the severity of acute coronary syndrome [50].

Considering that hypertension is important both as cardiovascular disease and as a risk factor for complications related to atherosclerotic CVD, and recognizing the importance of renin-angiotensin system blockade as a therapeutic target for hypertension and potentially NAFLD, this study aims to clarify the therapeutic landscape. Given that some effects of specific drugs may not be class-dependent, and that established criteria for selecting antihypertensive medications in patients with NAFLD are lacking, our research seeks to contribute valuable insights. Despite the inherent limitations of basic research, this study may contribute to paving the way for the definition of one of the therapeutic targets for NAFLD or MASLD, as well as the appropriate way to achieve it.

5. CONCLUSION

In the present study, all histological parameters that characterize NAFLD or MASLD were significantly attenuated with the preventive use of olmesartan and ramipril. However, no significant differences were observed in any comparisons between the two groups treated with their respective drugs. Therefore, in this experimental model, olmesartan and ramipril significantly and similarly attenuated the development of NAFLD or MASLD. Consequently, we can infer that both drugs may be potentially beneficial in the treatment of hypertensive patients with NAFLD or MASLD. However, adequately designed clinical studies are necessary to substantiate these findings.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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