Nutrition Research 150 (2026) 90–103

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/NTR

Adult cardiovascular dysfunction induced by peri-pubertal high-fat diet exposure is mediated by the renin-angiotensin axis in male rats

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ARTICLE INFO

Article history:
Received 30 July 2025
Revised 31 March 2026
Accepted 31 March 2026

Keywords:
High-fat diet
Overweight
Hypertension
Angiotensin II
Developmental Origins of Health and Disease (DOHaD)

ABSTRACT

This study investigated whether the renin-angiotensin system (RAS) is associated with the hypertension and metabolic syndrome induced by high-fat diet exposure during the peri-pubertal phase in rats. At postnatal day (PN) 30, male Wistar rats were randomly selected to be fed a normal diet (NF, 4.5% of fat) until PN 120 or a high-fat diet (HF, 35% lard) until PN 60 and then fed with a normal diet until PN 120. At PN 120, the function of the cardiovascular and RAS was assessed (5-16 animals per group). Statistical analyses were performed with the Student's T-test or 2-way ANOVA. HF rats showed increased arterial pressure (+ 9%, $P = .004$). The HF animals showed an increased pressor response at the lower (50 ng/kg/iv; + 50%, $P = .048$) and higher (300 ng/kg/iv; + 32%, $P = .033$) dose of Angiotensin II (Ang II), compared with NF animals. A higher depressor response to enalapril (5 mg/kg/iv; -100%, $P = .014$) was observed in HF rats. Greater endogenous circulating Ang II (+ 16%, $P = .010$) and lower endogenous circulating Ang (1-7) (-28%, $P = .005$) were observed in HF animals. The Ang II

Abbreviations: ACE, angiotensin-converting enzyme; ACE1, angiotensin-converting enzyme 1; ACE2, angiotensin-converting enzyme 2; AIN, American Institute of Nutrition; Ang, II angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; AU, arbitrary units; BW, body weight; cDNA, complementary DNA; DAP, diastolic arterial pressures; DOHaD, Developmental Origins of Health and Disease; FI, food intake; HF, high-fat; HR, heart rate; IP, pulse interval; MASR, MAS receptor; MAP, mean arterial pressure; MS, Metabolic syndrome; mRNA, messenger RNA; NF, animals fed a normal fat diet; PN, postnatal day; RAS, renin-angiotensin system; SAP, systolic arterial pressures; SEM, standard error of the mean.

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https://doi.org/10.1016/j.nutres.2026.03.013
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type 1 receptor (AT1R) mRNA expression increased (+ 81%, $P = .037$) in the heart of HF animals; however, Ang II type 2 receptor (AT2R; -91%, $P = .039$) and MAS receptor (MASR; -67%, $P = .022$) mRNA expression were reduced. In the aorta, AT1R mRNA expression increased (+ 124%, $P = .046$) in HF animals, whereas MASR mRNA expression decreased (-70%, $P = .035$), compared with NF animals. RAS is disrupted in adult hypertensive rats exposed to an HF diet during peri-pubertal life.

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1. Introduction

Metabolic syndrome (MS) is a cluster of metabolic conditions characterized by the presence of 3 or more components, including abdominal obesity, hyperglycemia, dyslipidemia (triglyceride >150 mg/dL and/or HDL cholesterol < 40 mg/dL in men), and elevated blood pressure. MS is primarily the result of a combination of overnutrition and a sedentary lifestyle, both of which lead to excess adiposity and other cardiometabolic disorders [1–5]. This condition is a growing global health problem that can increase 1.5-fold all-cause mortality rates and increase cardiovascular disease mortality rates by up to 2-fold [6,7].

The Developmental Origins of Health and Disease (DOHaD) concept suggests that exposure to adverse conditions, such as a high-fat (HF) diet, during critical developmental periods increases the risk of developing chronic diseases later in life [8,9], such as hypertension, obesity, dyslipidemia, and hyperglycemia [8,10,11]. In the DOHaD field, early development phases, such as gestation and lactation, which are primarily responsible for organogenesis and early tissue differentiation, have been extensively explored [12–17]. Gestation establishes organ structures [17,18], and lactation supports early postnatal physiological maturation [14,19]. Later development periods, such as the peri-pubertal phase, are also considered crucial for programming effects in adulthood [20,21]. This phase is characterized by the simultaneous maturation of the neuroendocrine and reproductive systems, including hormonal changes, brain development, and metabolic function [22–26]. Transitional stress is heightened [27], as a variety of simultaneous transitions experienced, including physical maturation, the drive for independence, the increased importance of social interaction, and biological maturation [22,23,28], are some of the conditions that may drive metabolic programming induced in peri-puberty. This phase has also been established as a sensitive window of development [29] linked to programming for MS, diabetes, hypertension, and reproductive dysfunction, among others [30–34]. Previous studies from our group have demonstrated that adult male Wistar rats exposed to an HF diet during the peri-pubertal period develop metabolic alterations, including increased visceral fat accumulation, dyslipidemia, hyperglycemia, hyperinsulinemia [35], increased blood pressure [36], and reproductive dysfunction [37]. Furthermore, the peri-pubertal phase is particularly vulnerable as organs mature, increasing sensitivity to environmental challenges [25,29,38].

The renin-angiotensin system (RAS) regulates cardiovascular function, and its dysregulation is implicated in the development and progression of cardiometabolic diseases, as well as hypertension, diabetes, hyperinsulinemia, and dyslipidemia [39]. RAS is overexpressed in MS [39,40]. Furthermore, programming studies with an HF diet during gestation and lactation showed that hypertension was associated with RAS dysfunction in the adipose tissue (increased AT1 receptor (AT1R), AT2 receptor (AT2R), and angiotensin-converting enzyme (ACE)) [12] and in the kidneys (increased mRNA expression of angiotensinogen and ACE) [13] in adult offspring. Angiotensin II (Ang II) is the main endogenous agonist of AT1R and mediates vasoconstriction, cell proliferation, oxidative stress, and hypertension [41,42]. Counter-regulation occurs via the AT2R, also activated by Ang II, and the MAS receptor (MASR), activated by Ang (1-7) [43]. The activation of AT2R and MASR promotes vasodilation and diuresis/natriuresis, countering the effects of AT1R [43]. To the best of our knowledge, little is known about the long-term implications of the RAS on hypertension induced by a peri-pubertal HF diet. Therefore, this study hypothesized that the RAS is disrupted by peri-pubertal HF diet exposure in male rats that show hypertension associated with MS in adulthood.

2. Methods and materials

All experimental procedures were conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) and COBEA guidelines (Brazilian College of Animal Experimentation) and were approved by the Ethics Committee on Animal Use and Experimentation of the State University of Maringá (CEUA), protocol number 2588060422. Male Wistar rats were obtained from the Central Animal Facility of the State University of Maringa and housed in the Facility of the Department of Biotechnology, Genetics, and Cell Biology, in polypropylene boxes (40 × 34 × 17 cm, 4 animals per box), under controlled temperature (22±2 °C) and photoperiod (07:00-19:00, light cycle), with *ad libitum* access to water and food. The animals underwent a 5-day adaptation period (postnatal day (PN) 25-30) before the experiment began.

In this study, we considered the beginning of the peri-pubertal period (PN 30) [32,35,37,44], when animals were distributed into 2 groups: a control group fed a normal fat (NF; Nuvital, Curitiba, Paraná, Brazil; 4.5% of fat; 3.80 kcal/g; n = 35) and a group fed with an HF diet (HF; 35% of lard; 5.81 kcal/g; n = 35) for 30 days (from PN 30 to PN 60) (Table 1), as previously

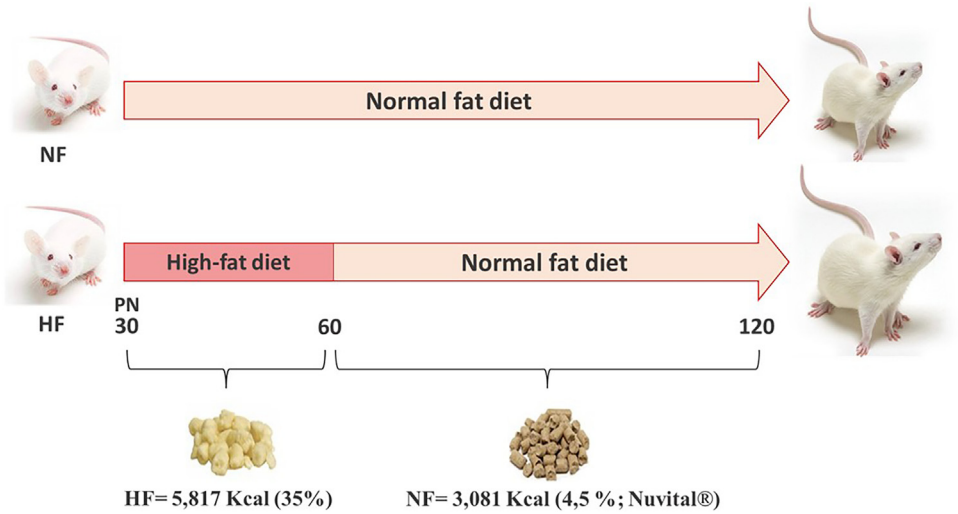

Fig. 1 – Schematic representation of experimental design. Animals were divided into two groups. One group was fed a normal diet (NF: 4.5% fat; 3.80 kcal/g) for 30 d. The other group was fed a high-fat diet (HF: 35% lard; 5.81 kcal/g) for 30 d. Subsequently, both groups were fed a normal diet until the end of the experiment. Analyses were performed after PN (postnatal day) 120.

Table 1 – Composition and caloric value of the diets for male rats Wistar throughout the experimental protocol, from PN 25 to PN 120.

Ingredients (g kg/diet)	NF (AIN-93G)	HF
Casein	200	200
Sucrose	100	100
Cornstarch	427.5	115.5
Dextrinate dstarch	132	132
Lard	-	312
Soybean oil	40	40
Cellulose	50	50
Mineral mix (AIN-93)	35	35
Vitamin mix (AIN-93)	10	10
L-cystine	3.0	3.0
Choline bitartarate	2.5	2.5
Caloric value (kcal/g)	3.80	5.81

Abbreviations: AIN, American Institute of Nutrition; HF, high-fat diet; NF, normal fat diet.

described [35,37]. Animals from both groups were fed NF from PN 60 to PN 120 (Fig. 1).

The sample size was determined using a power analysis in G Power software (Faul, Erdfelder, Lang, and Buchner, 2007), ensuring that the number of animals was sufficient to detect significant differences between groups at an appropriate confidence level [45]. Animals were randomly assigned to experimental groups to minimize bias. At PN 120, both groups underwent evaluation of biochemical and biometric parameters, food intake (FI), blood pressure regulation by RAS, and gene expression of RAS components.

2.1. *Body composition and FI*

Body weight (BW) was recorded once a week, and FI was assessed 3 times a week (n = 16 animals per group). Weekly FI was calculated using the following formula: [absolute FI of the week × 100/average weekly mean BW of the animals in each box].

At PN 120, a cohort of animals was subjected to an overnight fast (12 h), followed by euthanasia by decapitation guillotine (Insight–Ribeirão Preto) without prior anesthesia to avoid the biochemical changes induced by anesthesia [46–49]. Blood samples and tissues (fat depots, thoracic aorta, and heart) were also collected. The BW and length of the left tibia were evaluated (n = 12 animals per group). The length of the left tibia was used to normalize heart mass for quantification of cardiac hypertrophy [50].

2.2. *Biochemical parameters*

Immediately following guillotine decapitation, without prior use of anesthesia, according to ethical guidelines [51], blood was collected directly from the exposed cervical region, and the serum was separated for biochemical measurements (n = 8 animals per group). Glycemia (samples 2 µL) was quantified by the glucose oxidase method (intra-assay coefficient of variation (CV) < 2.60% and inter-assay CV < 2.50%). Total cholesterol and triglycerides (samples 2 µL) were measured by enzymatic colorimetric methods (cholesterol oxidase and glycerol-3-phosphate oxidase, respectively; intra-assay CV < 2% and inter-assay CV < 2.20% for total cholesterol and intra-assay CV < 1.70% and inter-assay CV < 2.60% for triglycerides). HDL-cholesterol (samples 2 µL) was determined after the precipitation of chylomicrons and low-density lipoproteins (intra-assay CV < 2% and inter-assay CV < 2.20%) and subsequent determination of HDL-cholesterol, using the method described above for measuring total cholesterol. The measurements were performed using spectrophotometry in a microplate reader (SpectraMax Plus 384 Microplate Reader from Molecular) with specific commercial kits (Gold Analisa, Belo Horizonte/MG, Brazil). The results were expressed in mg/dL.

The plasma concentrations of circulating Ang II and Angiotensin 1-7 (n = 12 animals per group) were measured using a rat ELISA kit according to the manufacturer's instructions (Kit Elabscience/Wuhan Fine Biotech Co., Ltd/Catalogue No.: E-EL-R1430, intra-assay CV < 6.41% and inter-assay CV < 6.94%; and Kit FineTest/Wuhan Fine Biotech Co., Ltd/Catalogue No.: ER1616, intra-assay CV < 8%, and inter-assay CV < 10%). Briefly, samples (50 µL), in duplicate, and Biotin-labeled antibodies (50 µL) were added to each well and incubated at 37°C for 45 min. The plates were washed at least 3 times with the washing buffer. HRP-Streptavidin Conjugate (SABC; 100 µL) was added to each well and incubated for 30 min at 37 °C, and then washed 5 times. After adding TMB substrate solution (90 µL) and incubating for 10-20 min at 37 °C, stop solution (50 µL) was added to terminate the reaction. Absorbance at 450 nm was measured using a microplate reader (SpectraMax Plus 384 Microplate Reader by Molecular).

2.3. *Arterial and venous catheterization*

At PN 120, a separate batch of adult offspring from both groups (n = 13 animals per group) was anesthetized (ketamine-xylazine; 75 mg + 15 mg/kg BW intraperitoneally). Two cannulae (P10-Micro-Renathane connected to 2 P50 cannulas, ClearTygon) were inserted: one into the femoral artery for monitoring arterial pressure and another in the femoral vein for drug administration. At the end of the surgeries, the animals received anti-inflammatory analgesic (Meloxicam, 0.4 mg/kg BW, IV) and antibiotic (Enrofloxacin, 5 mg/kg BW, IV, 8/8 h) coverage. Direct evaluations were performed 48 h after cannulation recovery [32,52].

2.4. *Arterial pressure recording*

After 1 hour of room adaptation, baseline arterial pressure was recorded for 30 min on each of the 3 days of the experimental protocol. Following baseline recordings, intravenous injections were administered according to the protocol described below. Experiments were carried out between 10:00 and 15:00 on conscious, free-moving animals.

The arterial cannula was connected to a pressure transducer (MLT0670, ADInstruments, Dunedin, New Zealand), which was connected to a recording system (Insight, Ribeirão Preto, Brazil) that used the DI 158 System for signal acquisition and analog-to-digital signal conversion (WinDaq lite, DataQ instruments, USA). The recordings were sampled at 1000 Hz.

Data were preanalyzed using Advanced CODAS software (Data Q Instruments, United States of America, https://www.dataq.com/products/windaq/, paid software) to objectively identify stable recording periods and exclude erratic signals. Baseline values, over 10 min of recording under stable conditions, of systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), and pulse interval (PI) were transferred to a spreadsheet for analysis (Microsoft Excel, Redmond, WA, USA). The heart rate (HR) was calculated from the PI (ms) using the formula (60,000/PI in ms), and the pulse pressure was calculated based on the difference between SAP and DAP.

2.5. *Effect of ACE inhibitor*

On the first day of the protocol (n = 10 animals per group), following baseline recording over 30 min, enalapril (5 mg/kg BW, IV; Sigma–Aldrich, San Luis, MO, USA), an ACE inhibitor, was used to estimate the implication of ACE on arterial pressure [44,53–55]. Arterial pressure and HR were recorded over 30 min after injection, and the responses were calculated from the MAP and HR baselines.

2.6. *Effect of angiotensin II*

On the second and third days of the protocol (n = 9 animals per group), following baseline recordings, 2 doses of Ang II (50 and 300 ng/kg of BW, IV; Sigma–Aldrich, San Luis, MO, EUA) were injected into different NF and HF animals according to the rat body weight [44,53]. To avoid receptor desensitization, only 2 doses of Ang II were administered per animal, with 24h interval. MAP and HR responses were calculated from the baseline.

2.7. *Relative expression of RAS component genes*

In a separate cohort of animals (n = 10 animals per group), samples of the left ventricle and thoracic aorta were collected after euthanasia using a guillotine. Tissues were frozen in nitrogen and subsequently stored at -80°C for molecular analyses. Total RNA was extracted using the TRIzol reagent and chloroform. The isolated RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Promega, Madison, WI, USA), and samples were run using AccessQuickTM Master Mix (Promega, Madison, WI, USA). RT-qPCR was performed using the Promega Access RT-PCR System (Promega, Madison, WI, USA). The first strand of cDNA synthesis was obtained by 1 cycle of 5 min at 25 °C, followed by 1 cycle of 1 min at 42 °C and 1 cycle of 15 min at 70 °C. The solution was then sequentially thermocycled at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, and 40 cycles for 1 min at 60 °C. The expression patterns of interest genes were normalized by beta-actin expression, and the primers used were described in Table 2. RNA purity was evaluated using spectrophotometric analysis (A260/A280 ratio). The $2^{-\Delta\Delta CT}$ method was used for the relative quantification analysis, and data were expressed as arbitrary units (AU) [56,57].

2.8. *Statistical analysis*

Data analysis was performed using GraphPad Prism Software version 8.00 for Windows (GraphPad Software Inc., La Jolla, California, USA, Home - GraphPad). The results are presented as mean ± standard error of the mean (SEM). Data normality and distribution were verified using the Shapiro–Wilk test. The data were analyzed using Student's T-test (unpaired) for parametric data, the Mann–Whitney test for nonparametric data, and 2-way ANOVA, followed by the Bonferroni post hoc test (with time, dose, and diet as independent factors) for repeated measures (such as FI, BW, and pressor response doses of Ang II). Statistical significance was set at $P < 0.05$.

Table 2 – List of primer sequences used in PCR.

Gene	5' → 3'	3' → 5'
β-actin	GTCGTACCACTGGCATTGTG	CTCTCAGCTGTGGTGGTGAA
ACE	GCTTGACCCTGGATTGCAGC	CTCCGTGATGTTGGTGTCGT
ACE2	GCCCAAAAGATGAACGAGGC	GACGCTTGATGGTCGCATTC
AT1R	CTCTGCCACATTCCCTGAGTTA	CACTTTCTGGGAGGGTTGTGT
AT2R	GGTCTGCTGGGATTGCCTTA	GGAAGGGAAGCCAGCAAATG
MASr	CAATCGTGACGTTATCGGTG	TCTCTCCACACTGAT GGCTG

Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MASr, MAS receptor.

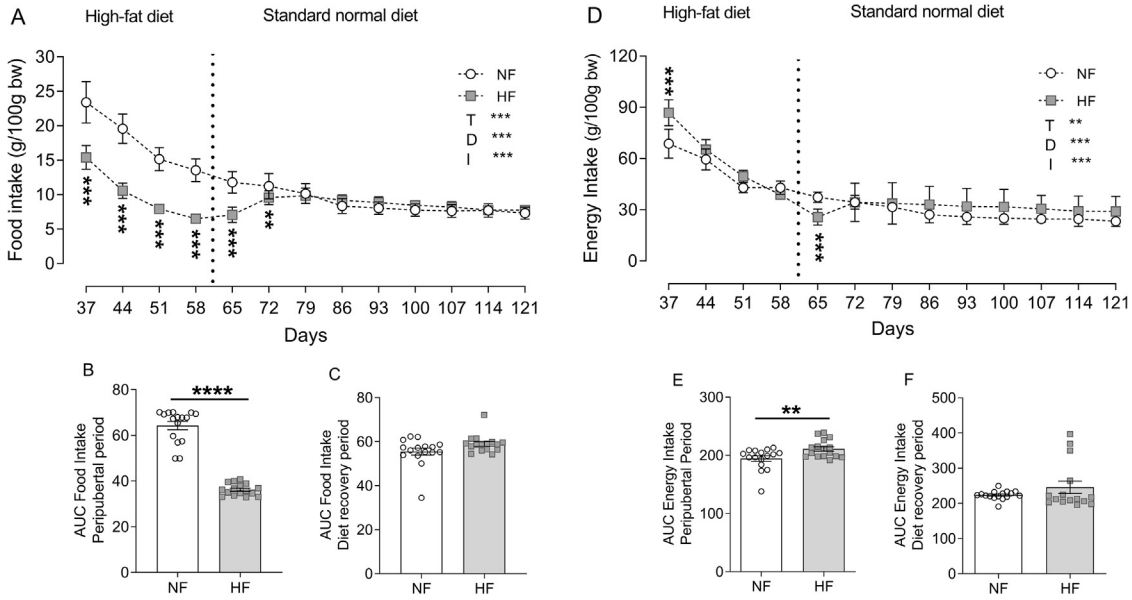

Fig. 2 – Food intake (NF n = 16; HF n = 16). During the peri-pubertal period (PN 30-PN 60), HF diet rats showed reduced food consumption but higher caloric intake. Food intake curve throughout the experiment, in grams (A); area under the curve of food intake during the peri-pubertal period (B); and area under the curve of food intake during the dietary recovery period (C). Food intake curve through the experiment, in Kcal (D); area under curve of food intake during the peri-pubertal period (E); and food intake during the dietary recovery period (F). NF, normal fat diet; HF, high-fat diet; T, time; D, diet; and I, the interaction between diet and time. Values are presented as mean ± SEM. ***P < .001 and **P < .01 indicate statistical significance (two-way ANOVA (A and D; Factors: time and diet) or Student's T-test (B, C, E, and F)). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

3. Results

3.1. *FI, BW, and biochemical parameters*

During the peri-pubertal period (PN 30-PN 60), HF diet rats showed a reduction of cumulative relative to BW FI calculated by area under curve (AUC) of FI (AUC NF: 64.3±21.5 vs. HF: 36.2±28.1 g; $P = .0001$; Fig. 2A and B); however, these animals showed higher caloric intake (AUC NF: 194.0±24.5 vs. HF: 211.0±23.1 g; $P = .009$; Fig. 2D and E), than that of the NF group. In contrast, during dietary recovery (PN 60-PN 120), both groups showed similar absolute and caloric FI.

During the peri-pubertal period, the HF diet animals started to show greater BW in the fourth week, which was sustained throughout the following experimental weeks (Fig. 3A). The AUC of BW was increased during the HF diet exposure and dietary recovery in HF diet animals (AUC NF: 642.7±15 vs. HF: 700.2±30; $P = .004$ and NF: 2778±15 vs. HF: 3013±30; $P = .003$; Fig. 3B and C, respectively).

HF diet animals had an increased BW (NF: 410±6.97 vs. HF: 440±3.28 g; $P = .001$) (Table 3). HF diet rats also showed higher retroperitoneal fat pad (NF: 1.49±0.06 vs. HF: 1.86±0.08 g/100 g BW; $P = .003$), mesenteric fat pad (NF: 0.69±0.03 vs. HF: 0.87±0.04 g/100 g BW; $P = .003$), heart mass (NF: 0.30±0.008 vs. HF: 0.33±0.008 g/100 g BW; $P = .001$), thoracic aorta mass (NF: 0.050±0.005 vs. HF: 0.090±0.003 g/100 g BW; $P = .001$) and heart/tibia index length (NF: 0.36±0.011 vs. HF: 0.42±0.006 mg/cm; $P = .004$) (Table 3).

HF diet animals showed an increase in fasting blood glucose (NF: 100±2.50 vs. HF: 110±1.24 mg/dL; $P = .016$) and triglycerides (NF: 163.6±8.49 vs. HF: 204.8±9.22 mg/dL;

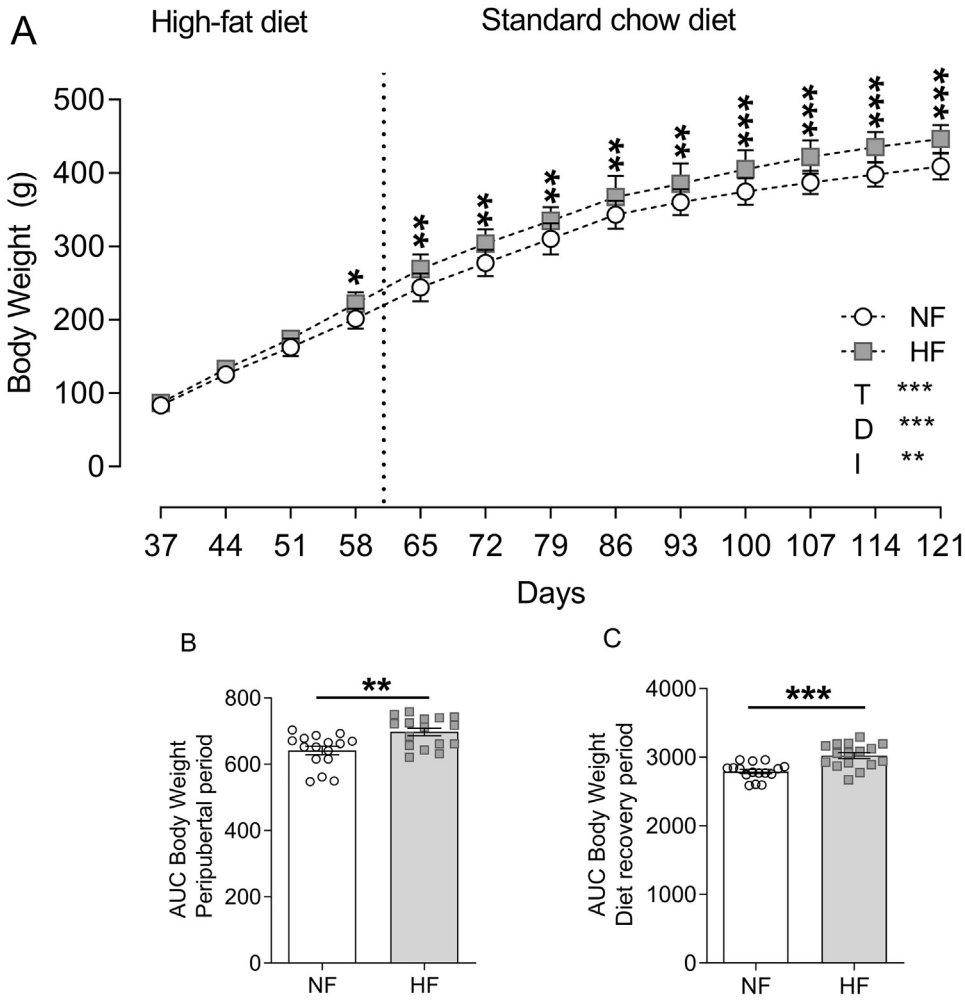

Fig. 3 – Body weight (NF n = 16; HF n = 16). During the peri-pubertal period (PN 30-PN 60), HF diet animals showed greater body weight in the fourth week, which was sustained until 120 days. Body weight curve throughout the protocol (A), area under curve of body weight during the peri-pubertal period (B), area under curve of body weight during the dietary recovery period (C). NF, normal fat diet; HF, high-fat diet; T, time; D, diet; and I, interaction between diet and time. Values are presented as mean ± SEM. ***$P < .001$, **$P < .01$, and *$P < .05$ indicate statistical significance (two-way ANOVA (A; Factors: time and diet) or Student's T-test (B and C)). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

Table 3 – Biometric parameters of male Wistar rats at PN 120.

Biometric parameters	NF	HF
Body weight (g)	410±6.97	440±3.28**
Retroperitoneal fat pad (g/100 g bw)	1.49±0.06	1.86±0.08**
Mesenteric fat pad (g/100 g bw)	0.69±0.03	0.87±0.04**
Heart (g/100 g bw)	0.30±0.008	0.34±0.008*
Thoracic aorta (g/100 g bw)	0.050±0.005	0.090±0.003***
Heart/tibia length (mg/cm)	0.36±0.011	0.42±0.006***

The HF animals increased body weight, adipose tissue, cardiac tissue, aorta, and cardiac hypertrophy index when compared with NF animals. NF: Normal fat diet; HF: high-fat diet; (NF n = 12; HF n = 12). Values are expressed as mean ± SEM.
*** $P < .001$, ** $P < .01$, and * $P < .05$ indicate statistical significance for Student's T-test (asterisk in black) or Mann–Whitney (asterisk in grey).

Table 4 – Biochemical parameters of male Wistar rats at PN 120.

Biochemical parameters	NF	HF
Blood glucose (mg/dL)	100.7±1.24	110.2±2.50**
Triglycerides (mg/dL)	150.0±9.22	203.0±8.49*
Total cholesterol (mg/dL)	109.5±3.67	115.5±3.72
HDL - cholesterol (mg/dL)	75.57±3.25	73.43±3.81
Angiotensin II (pg/mL)	37.23±1.71	42.88±1.12*
Angiotensin 1-7 (pg/mL)	375.8±4.07	269.6±8.09**

The HF animals showed increased glycemia, triglycerides, and angiotensin II, while showing decreased concentrations of angiotensin 1-7 compared to NF animals. NF: Normal fat diet; HF: high-fat diet; Blood glucose, triglycerides, total and HDL cholesterol (NF n = 8; HF n = 8); Angiotensin II and Angiotensin 1-7 (NF n = 12; HF n = 12); Values are expressed as mean ± SEM. ** $P < .01$ and * $P < .05$ indicate statistical significance for Student's T-test (asterisk in black) or Mann–Whitney (asterisk in grey).

$P = .007$) compared with NF, and HDL-cholesterol and total cholesterol were similar between the groups (Table 4). In the HF diet animals, an increase in endogenous circulating Ang II (NF: 37.23±1.71 vs. HF: 42.88±1.12 pg/mL; $P = .010$), and a decrease in Ang (1-7) (NF: 375.8±4.07 vs. HF: 269.6±8.09 pg/mL; $P = .005$) were observed compared with the control group (Table 4).

3.2. *Arterial pressure and HR*

HF group increased SAP (NF: 125±1.91 vs. HF: 136±1.83 mmHg; $P = .0017$; Fig. 4A), DAP (NF: 80±1.16 vs. HF: 89±2.11 mmHg; $P = .006$; Fig. 4B), and MAP (NF: 100±1.54 vs. HF: 108±1.92 mmHg; $P = .004$; Fig. 4C) compared with NF animals. However, similar HR were observed between the groups (Fig. 4D).

3.3. *Effects of enalapril on MAP and HR*

HF diet animals showed a greater depressor response (NF: -6.54±1.19 vs. HF: -13.79±2.31 mmHg; $P = .014$; Fig. 5A) compared with the NF group. Tachycardic responses were similar between groups (Fig. 5B).

3.4. *Effects of angiotensin II on MAP and HR*

In the lower dose of Ang II (50 ng/Kg of BW), the HF group had a greater pressor response of MAP (NF: 19.94±2.90 vs. HF: 30.33±3.90 mmHg; $P = .048$; Fig. 6A) compared with NF animals. The HF diet animals showed an exacerbated bradycardic response compared to the NF animals (NF: -13±10.55 vs. HF: -46±15.90 bpm; $P = .030$; Fig. 6B). In the higher dose of Ang II (300 ng/Kg of BW), the HF diet animals showed an increased pressor response of MAP (NF: 34.73±2.82 vs. HF: 45.70±3.05 mmHg; $P = .018$; Fig. 6A) compared with the NF group, while the bradycardia responses were similar between the groups (Fig. 6B).

3.5. *Relative expression of RAS components*

In the heart, HF diet animals showed increased expression of ACE1 mRNA (NF: 0.74±0.26 vs. HF: 1.82±0.34 AU; $P = .024$) and AT1R mRNA (NF: 1.05±0.25 vs. HF: 1.91±0.99 AU; $P = .037$), compared with the NF group (Fig. 7A). The mRNA expression of ACE2, AT2R, and MASR were lower in HF diet animals compared with NF diet animals (ACE2, NF: 1.64±0.23 vs. HF: 0.73±0.17 AU, $P = .048$; AT2R, NF: 2.22±0.78 vs. HF: 0.41±0.17 AU, $P = .039$; and MASR, NF: 2.55±0.53 vs. HF: 0.82±0.29 AU, $P = .022$; Fig. 7A).

In the aorta, ACE1 and AT1R mRNA expression were increased in animals with HF compared with NF diet animals (NF: 0.63±0.18 vs. HF: 1.16±0.13 AU; $P = .044$; and NF: 0.82±0.12 vs. HF: 1.84±0.43 AU; $P = .046$, respectively; Fig. 7B). In the counter-regulatory axis, HF group also showed lower mRNA expression of AT2R and MASR (NF: 2.06±0.68 vs. HF: 1.14±0.29 AU; $P = .040$; and NF: 1.34±0.33 vs. HF: 0.40±0.14 AU; $P = .035$, respectively; Fig. 7B).

4. Discussion

This study shows that the RAS is disrupted by exposure to a peri-pubertal HF diet in male rats, resulting in hypertension associated with MS in adulthood, confirming the initial hypothesis. Little is known about the susceptibility of the RAS to peri-pubertal nutritional insults in the context of DOHaD. To the best of our knowledge, this is the first study to demonstrate that RAS dysfunction is involved in adult hypertension programmed by peri-pubertal HF diet exposure. Previous studies have targeted precocious susceptibility windows of development, such as pregnancy and lactation, which are primarily responsible for organogenesis and early tissue differentiation [14,18]. HF diet exposure in pregnancy and lactation [12,13] or an early postnatal overfeeding [38,58] induces hypertension and dysfunction of RAS in rats. However, the main finding of the present study is that RAS dysfunction was programmed by an insult during peri-puberty, a life period in which organs and systems are already formed; however, neuroendocrine and reproductive maturation, including relevant sexual, brain, and metabolic adjustments, are observed [22,24–26,28]. The present findings reinforce the susceptibility of peri-puberty as a "later window of development," in the DOHaD context [30–35,37,44], indicating that the hypertension observed in these animals is dependent on RAS hyperactivity; evidenced by the: (1) greater pressor response to low and high doses of Ang II (50 and 300 ng/Kg); (2) greater depressor response to ACE inhibitor (enalapril, 5 mg/kg); (3) increased circulating endogenous Ang II and decreased circulating endogenous Ang (1-7); and (4) greater expression of AT1R mRNA and decrease in expression of ACE2, AT2R, and MASR mRNA, in the heart and the thoracic aorta.

HF diet animals exhibited increased blood pressure and higher endogenous circulating Ang II concentration under basal conditions. Santos et al. [59] also showed that chronic exposure to an HF diet (60% fat PN 35 to PN 120) induced an increase in blood pressure and endogenous circulating Ang II, although this occurred only after the end of HF diet exposure. Furthermore, the main effects of Ang II on blood pressure are mediated by the AT1R, including, among other mechanisms, vasoconstriction and water retention by reabsorption in the kidney, leading to hypertension [41,60]. HF diet animals also show increased AT1R mRNA expression in the thoracic aorta

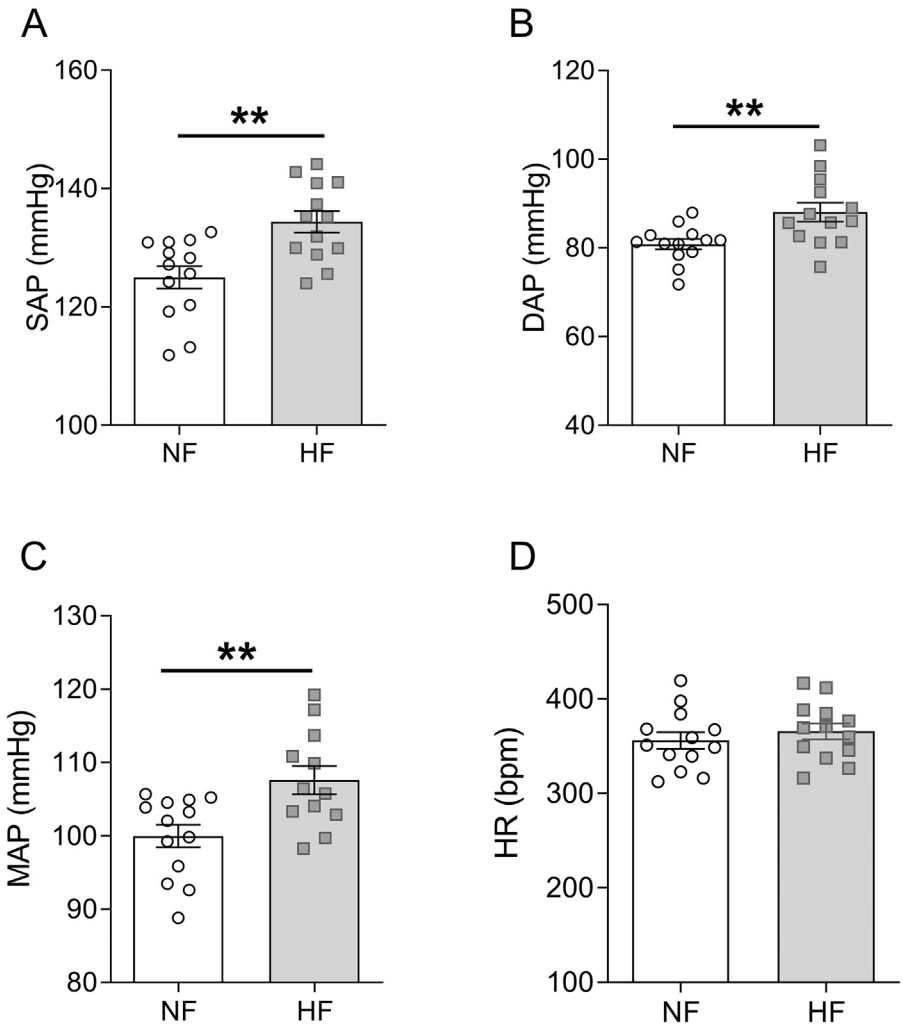

Fig. 4 – Basal arterial pressure (NF n = 13; HF n = 13). The HF diet animals showed an increase in blood pressure parameters, including systolic, diastolic, and mean, in adult rats. Systolic arterial pressure (A), diastolic arterial pressure (B), mean arterial pressure (C), and heart rate (D). NF, normal fat diet; and HF, high-fat diet. Values are presented as mean ± SEM. **P < .01 indicates statistical significance (Student's T-test). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

and heart tissues. Studies in mice have shown that hypertension induced by chronic exposure to a HF diet in adulthood (40% fat PN 84 to PN 140) also leads to increased AT1R mRNA expression in the cardiac [61,62], thoracic aorta [63], and adipose tissues [59]. Considering the mouse studies cited above, the animals were exposed to more than 50 d of HF dietary insult and the blood pressure conditions were explored just after the insult exposure; in the present study, the animals were exposed to the dietary insult for a shorter period (30 days) and evaluated after a dietary recovery period (60 days), considering the puberty phase.

When the HF diet animals were challenged with increasing doses of Ang II, a greater pressor response was observed, which may depend on the overexpression of AT1R. Schreier et al. [64] showed that exposure to the HF diet (60% fat PN 42 to PN 162) in mice induced an increased blood pressure response to a dose of 1 μg/kg of exogenous Ang II just after the end of the HF diet exposure (PN 162). Although the response patterns were similar between the 2 studies, our study evaluated blood pressure after 60 days of dietary recovery. Ghatta et al. [65] showed that animals fed an HF diet have an increased response to Ang II in vascular reactivity owing to overexpression of AT1R in arterioles, leading to increased vasoconstriction, reduced nitric oxide (endothelial dysfunction), and a consequent increase in arterial pressure. As AT1 mRNA was increased in these animals, we may suggest that the increased blood pressure response in the HF animals may be due to a programmed increase in AT1R expression; protein quantification still needs to be explored. Furthermore, the cardiac RAS is critical for regulating cardiac function in response to injury. Activated AT1R enhances NADPH oxidase activity, increasing reactive oxygen species and oxidative stress, thereby impairing nitric oxide bioavailability and leading to endothelial dysfunction [66]. Sustained AT1R signaling promotes vascular re-

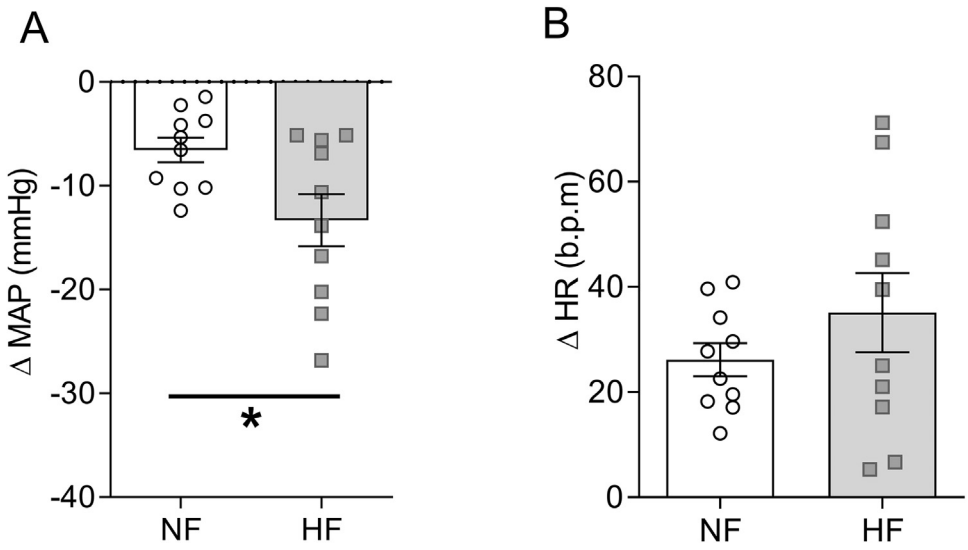

Fig. 5 – Pressure response to enalapril (NF n = 10; HF n = 10). The HF diet animals showed an increased depressor response to an angiotensin-converting enzyme (ACE) inhibitor, enalapril. Changes in mean arterial pressure (A) and changes in heart rate (B) in response to enalapril. NF, normal fat diet; and HF, high-fat diet. Values are presented as mean ± SEM. *P < .05 indicates statistical significance (Student's T-test). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

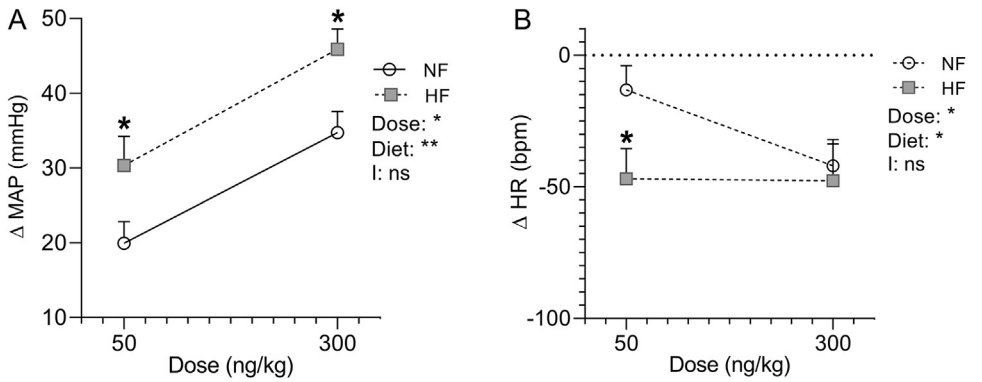

Fig. 6 – Blood pressure response to angiotensin II (NF n = 9; HF n = 9). The HF diet animals showed an increased pressure response to angiotensin II. Changes in mean arterial pressure (A) and changes in heart rate (B) in response to angiotensin II (50 ng/kg and 300 ng/kg). NF, normal fat diet; HF, high-fat diet; and I, interaction between dose and diet. Values are presented as mean ± SEM. *P < .05 indicates statistical significance (two-way ANOVA; factors: dose and diet). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

modeling and fibrosis via activation of the TGF-β pathway and increased extracellular matrix deposition, leading to cardiac hypertrophy and fibrosis in hypertension [61,62,67]. Additionally, RAS dysregulation triggers proinflammatory responses by increasing Ang II concentrations, thereby stimulating cytokine release and adhesion molecule expression, thereby exacerbating vascular inflammation [59,68]. In this context, further studies are needed to explore the potential mechanisms underlying RAS dysfunction in the present model of HF diet exposure during puberty (e.g., inflammation).

Animals with an HF diet showed a greater depressor response to enalapril, an ACE1 blocker, suggesting that this enzyme was overactive. Studies that targeted ACE1 function by chronic inhibition of this enzyme via 12 weeks or 7 days of treatment with enalapril also showed a greater depressor response in animals exposed to an HF diet [59,69]. Pulmonary ACE1 drives the synthesis of circulating Ang II [70,71]; however, local dysregulation of RAS components can affect the systemic RAS pathway [72,73]. We did not explore the pulmonary expression of ACE1, but we may suggest that the increased concentration of circulating Ang II may be dependent on ACE1 overexpression induced by peri-pubertal HF diet exposure, as increased expression of ACE1 mRNA was observed in the heart and thoracic aorta of the HF diet animals, indicating overac-

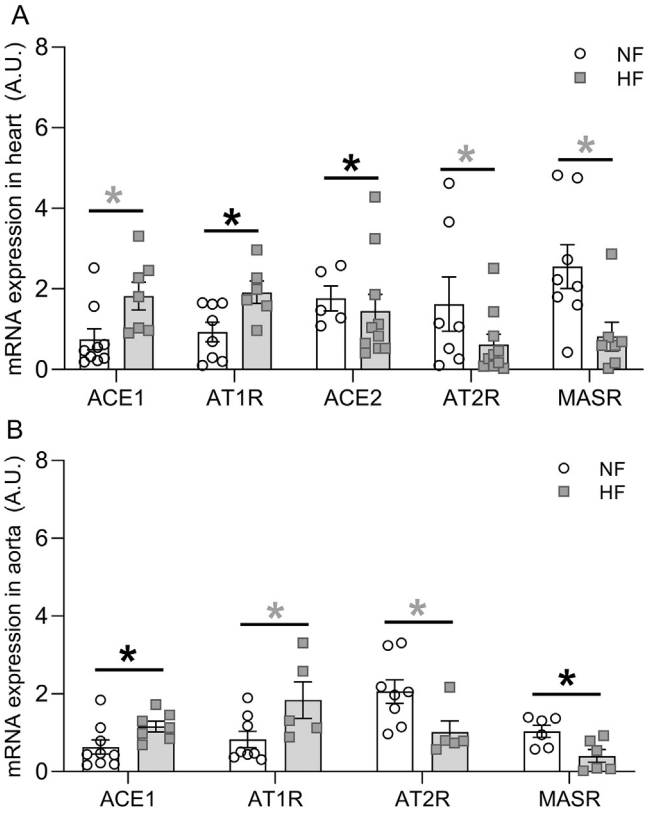

Fig. 7 – RAS mRNA expression in the left ventricle and thoracic aorta. Peri-pubertal high-fat diet induced long-term increased expression of the regulatory ACE1–AT1R axis and reduced expression in the counter-regulatory ACE2–AT2R–MAS axis of the renin-angiotensin system. mRNA expression of RAS components in the heart (A), ACE1 (NF n = 9; HF n = 7); AT1R (NF n = 8; HF n = 6); ACE2 (NF n = 5; HF n = 10); AT2R (NF n = 7; HF n = 10); MASR (NF n = 8; HF n = 7); mRNA expression of RAS components in the thoracic aorta (B), ACE1 (NF n = 9; HF n = 7); AT1R (NF n = 8; HF n = 5); AT2R (NF n = 8; HF n = 5); MASR (NF n = 6; HF n = 6); Beta-actin was used as an internal control to normalize selected gene expression. NF, normal fat diet; HF, high-fat diet; ACE1, angiotensin-converting enzyme 1; ACE2, angiotensin-converting enzyme 2; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; and MASR, MAS receptor. Values are presented as mean ± SEM. *P < .05 indicates statistical significance for Student's T-test (asterisk in black) or Mann–Whitney (asterisk in grey). Animals were divided into two groups: a control group exposed to a normal fat diet (4.5%) and a group exposed to a high-fat diet (35%) for 30 days (PN 30 to PN 60 - (PN - postnatal day)). Both groups then received a normal fat diet from PN 60 to PN 120.

tivity of ACE1 in this tissue. This study suggests, by inhibiting ACE1, that the HF diet induced higher RAS "activity" due to the overactivity of ACE1 observed in these animals. The mechanism driving changes in gene expression is unknown; however, histone acetylation and DNA methylation are major epigenetic mechanisms that regulate ACE1 expression in hypertension [74–76]. Epigenetic alterations are relevant to DOHaD studies across developmental phases and in response to diverse environmental stimuli [29]. Epigenetic alterations were not examined in the present study, and future experiments (e.g., chromatin immunoprecipitation, methylation profiling, and pharmacological receptor studies) are needed to elucidate the mechanisms underlying RAS changes in this animal model.

Considering the potentially increased expression/activity of ACE1 and AT1R (as assessed by mRNA expression) associated with elevated basal endogenous Ang II concentrations in the present animal model, the role of AT1R in this model warrants further exploration. In this context, future studies should employ pharmacological approaches, specifically AT1R blockade, accurate evaluation of the pressure response to exogenous Ang II following prior ACE1 blockade, and assessment of AT1R protein expression and intracellular signaling [53,76,77]. Another limitation of the present study was that only male rats were evaluated, and the extrapolation of the present data to female rats was restricted. Previous studies have demonstrated sex differences in RAS regulation [78,79], MS [80], and hypertension [81], with females generally exhibiting protection, likely mediated by ovarian hormones and an increased RAS activity counter-regulatory axis (Ang-(1–7)/ACE2/Mas) [80,82]. In addition, future studies that include females are warranted to investigate sex-specific effects of HF diet exposure during the pubertal phase in this programming model.

In addition to the ACE1–AngII–AT1R axis, studies have identified a parallel axis that drives counter regulation via the RAS, characterized by depressor, vasodilatory, antiproliferative, antioxidant, and anti-inflammatory actions [83]. On this axis, Ang (1–7) is produced from Ang I or Ang II by the catalytic activity of ACE2 [43] and activates MASR [84]. AT2R also mediates this pathway via Ang II binding. The activation of MASR and AT2R induces vasodilation, natriuresis, and antiproliferative action [85]. Present hypertension induced by a peri-pubertal HF diet is characterized by deregulation of the RAS counter-regulatory pathway, as evidenced by lower basal concentrations of endogenous circulating Ang (1-7), reduced MAS and AT2 mRNA expression in the heart and thoracic aorta, and reduced ACE2 mRNA expression in cardiac tissue. Studies in rodents have shown that HF diet exposure induces a reduction in the circulating Ang 1-7 concentrations, and expression of MASR and AT2 mRNA, just after the exposure to the HF diet, suggesting that the "protective" RAS pathway is dysregulated in these animals [62,69]. Another model of programmed hypertension, induced by perinatal exposure to an HF diet, also showed reduced AT2R expression in the adipose tissue [12]. Recent studies have indicated that ACE2 is the primary enzyme involved in Ang (1–7) generation in vital organs, including the heart [73]. In this sense, a reduction in the RAS counter-regulatory pathway (Ang-(1-7)/Mas receptor) may lead to adverse effects, including increased oxidative stress, inflammation, cardiac fibrosis, and cardiac remodeling [59,61].

Despite the fundamental role of the RAS in blood pressure regulation, it is not only present in the circulatory system, but also present in local tissues, such as the brain, pancreas, adipose tissue, heart, blood vessels, and kidneys [83,86,87].

Our study highlights that exposure to an HF diet during the peri-pubertal period, followed by 60 d of nutritional recovery, leads to the development of adult hyperglycemia, hypertension, hypertriglyceridemia, and obesity, key risk factors of MS diagnoses. Furthermore, a previous study using the same programming model with an HF diet during peri-puberty [35] reported metabolic alterations, including insulin resistance, hyperglycemia, and dyslipidemia, although it focused on hepatic dysfunction in the present model. Studies have suggested that metabolic changes may be related to RAS dysfunction, as the RAS is implicated in dyslipidemia, alterations in glycemic homeostasis [39], and insulin resistance [40,88]. In addition, increases in adipose tissue and changes in its structure and distribution may lead to overactivation of the RAS via increased local production of angiotensinogen [12,89], thereby contributing to increased plasma angiotensinogen [90] and affecting the systemic RAS [72]. We suspect that the increase in circulating endogenous Ang II present in these animals may also be related to the increase in adipose tissue and angiotensinogen production in animals programmed with the HF diet mentioned above. However, the relationship between RAS, MS, and hypertension is complex and was not examined in the present study. Future studies are needed to explore this question in the present animal model. Furthermore, considering that the HF diet animals showed higher caloric intake, which may induce metabolic changes, the absence of a pair-fed control group is a limitation of this study and should be addressed in future studies.

5. Conclusion

In conclusion, the current results point to the RAS dysregulation in hypertension associated with MS induced by peri-pubertal HF diet exposure. The imbalance between the hypertensive/deleterious and hypotensive/protective axes of the RAS, characterized by hyperactivity of Ang II via and attenuated counterregulatory via, is associated with hypertension in the present animals. These findings indicate a potential physiopathological mechanism involved in human hypertension associated with MS, which may depend on the peri-pubertal dietary lifestyle when young people are exposed to a fatty diet, such as the consumption of fast food and ultra-processed foods. Therefore, exploring the role of RAS in MS is important, and further studies are needed. Additionally, these findings point to the potential of pharmacological interventions targeting RAS function to control hypertension associated with developmental insults, such as a peri-pubertal HF diet. Furthermore, public health policies targeting adequate consumption diets during the peri-pubertal period, implementing school-based nutrition education programs, and promoting regular physical activity among adolescents to prevent the long-term development of chronic cardiometabolic dysfunction are important.

Author declarations

None.

CRediT authorship contribution statement

Maiara Vanusa Guedes Ribeiro: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Rebeka Oliveira Ferreira:** Writing – review & editing, Methodology, Investigation. **Letícia Ferreira Barbosa:** Writing – review & editing, Investigation. **Gabriel Kian Guimarães Lopes:** Writing – review & editing, Investigation. **Silvano Piovan:** Writing – review & editing, Investigation. **Lucas Paulo Jacinto Saavedra:** Writing – review & editing, Investigation. **Scarlett Rodrigues Raposo:** Writing – review & editing, Investigation. **Rodrigo Mello Gomes:** Writing – review & editing. **Ananda Malta:** Writing – review & editing, Visualization, Investigation. **Douglas Lopes Almeida:** Writing – review & editing. **Gessilda de Alcantara Nogueira de Melo:** Writing – review & editing. **Paulo Cezar de Freitas Mathias:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Kesia Palma-Rigo:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Acknowledgment

The authors have no acknowledgments to declare.

Sources of support

This research was funded by the Coordination for the Improvement of Higher Education Personnel – CAPES (grant number 888876851072022-00) and the Brazilian Research National Foundation: Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (grant number 4674819003837990), JBS (José Batista Sobrinho), and Brazilian National Institute of Science and Technology of DOHaD-INCT.

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