


# Homoarginine treatment of rats improves cardiac function and remodeling in response to pressure overload

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## Abstract

Low serum concentrations of the amino acid homoarginine (HA) are associated with increased cardiovascular mortality by incompletely understood mechanisms. This study sought to assess the influence of HA on cardiac remodeling in rats undergoing either transaortic banding or inhibition of nitric oxide synthesis by N<sup>ω</sup>-Nitro-L-arginine methyl ester hydrochloride (L-NAME). Male Wistar rats ( $n = 136$ ) underwent sham operation (SH) or aortic banding (AB). Both groups were equally divided into 14 subgroups, receiving different doses of HA alone or in combination with lisinopril, spironolactone, or L-NAME for 4 weeks. HA treatment in AB animals resulted in a dose-dependent improvement of cardiac function up to a concentration of 800 mg·kg<sup>-1</sup>·day<sup>-1</sup>. Combining 800 mg·kg<sup>-1</sup>·day<sup>-1</sup> HA with spironolactone or lisinopril yielded additional effects, showing a positive correlation with LV ejection fraction (+33%,  $p = 0.0002$ ) and fractional shortening (+41%,  $p = 0.0014$ ). An inverse association was observed with collagen area fraction (-41%,  $p < 0.0001$ ), myocyte cross-sectional area (-22%,  $p < 0.0001$ ) and the molecular markers atrial natriuretic factor (-74%,  $p = 0.0091$ ), brain natriuretic peptide (-42%,  $p = 0.0298$ ), beta-myosin heavy chain (-46%,  $p = 0.0411$ ), and collagen type V alpha 1 chain (-73%,  $p = 0.0257$ ) compared to placebo-treated AB animals. Co-administration of HA and L-NAME was found to attenuate cardiac remodeling and prevent NO-deficient hypertension following AB. HA treatment has led to a dose-dependent improvement of myocardial function and marked histological and molecular changes in cardiac remodeling following AB. Combining HA with standard heart failure medication resulted in additional beneficial effects boosting its direct impact on heart failure pathophysiology.

## KEYWORDS

amino acids, aortic stenosis, cardiac remodeling, heart failure, rats

**Abbreviations:** AB, aortic banding; AGAT, arginine-glycine amidinotransferase; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; BW, body weight; Col5a1, collagen type V alpha 1 chain; DMEM, Dulbecco's modified Eagle's medium; EF, ejection fraction; FS, fractional shortening; H&E, hematoxylin/eosin; HA, homoarginine; HFpEF, heart failure with preserved ejection fraction; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HW, heart weight; IL-17A, interleukin-17A; LiW, liver weight; L-NAME, N<sup>ω</sup>-Nitro-L-arginine methyl ester hydrochloride; LuW, lung weight; LV, left ventricle; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; NO, nitric oxide; RV, right ventricle; SEM, standard error of the mean; SH, sham; SR, sirius red; TL, tibia length; TNFSF14, tumor necrosis factor superfamily member 14; β-MHC, beta-myosin heavy chain.

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## 1 | INTRODUCTION

Cardiovascular diseases remain the leading cause of death worldwide with approximately 17.8 million deaths annually [1]. Despite the widespread use of therapeutics interfering with different targets of the neurohumoral axis and strenuous efforts aiming at improving survival, cardiovascular mortality remains unacceptably high.

Recently, homoarginine (HA) attracted scientific attention due to its role in the pathogenesis of cardiovascular diseases [2–5]. HA is a naturally occurring amino acid that is structurally related to L-arginine [6]. Several previous epidemiological studies identified low concentrations of the endogenous and non-proteinogenic amino acid HA as an independent risk marker for adverse cardiovascular events, cerebrovascular outcomes, and all-cause mortality [2, 5–9].

While the exact underlying mechanisms remain to be elucidated, accumulating evidence suggests that HA interferes with the L-arginine/nitric oxide (NO) pathway by serving as an alternative substrate for NO synthase [2]. Prolonged inhibition of NO synthesis by administration of the NO synthase inhibitor N $\omega$ -Nitro-L-arginine methyl ester hydrochloride (L-NAME) was found to induce cardiac remodeling resulting in cardiovascular damage with ventricular fibrosis, left ventricular hypertrophy, and decreased cardiac output [10, 11]. Since cellular hypertrophy and fibrosis are processes retaining a certain degree of reversibility, identification of metabolic pathways responsible for the underlying changes is crucial for detecting novel therapeutic targets.

Recent experimental studies demonstrated that HA may exert direct protective cardiovascular and metabolic effects rather than being merely a risk marker for adverse outcomes [12–17]. Dietary supplementation of HA was found to directly influence heart failure pathophysiology in a murine model of post-myocardial infarction heart failure [12]. However, current literature provides insufficient experimental data regarding the application of HA for therapeutic purposes, especially if combined with standard medical care. The present animal study was designed to investigate whether HA treatment alone or in combination with standard heart failure medication is capable of preventing cardiac remodeling upon pressure overload or NO-deficient hypertension.

## 2 | METHODS

We used male Wistar rats (Charles River Laboratories, Sulzfeld, Bavaria, Germany) at the age of 5–6 weeks for our animal model. All experiments were performed blinded on male animals to avoid hormonal fluctuations, to ensure comparability between study groups, and to reduce group sizes by avoiding mixed-gender populations. L-homoarginine hydrochloride, spironolactone,

lisinopril, and L-NAME were provided by Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Hamburg, Germany).

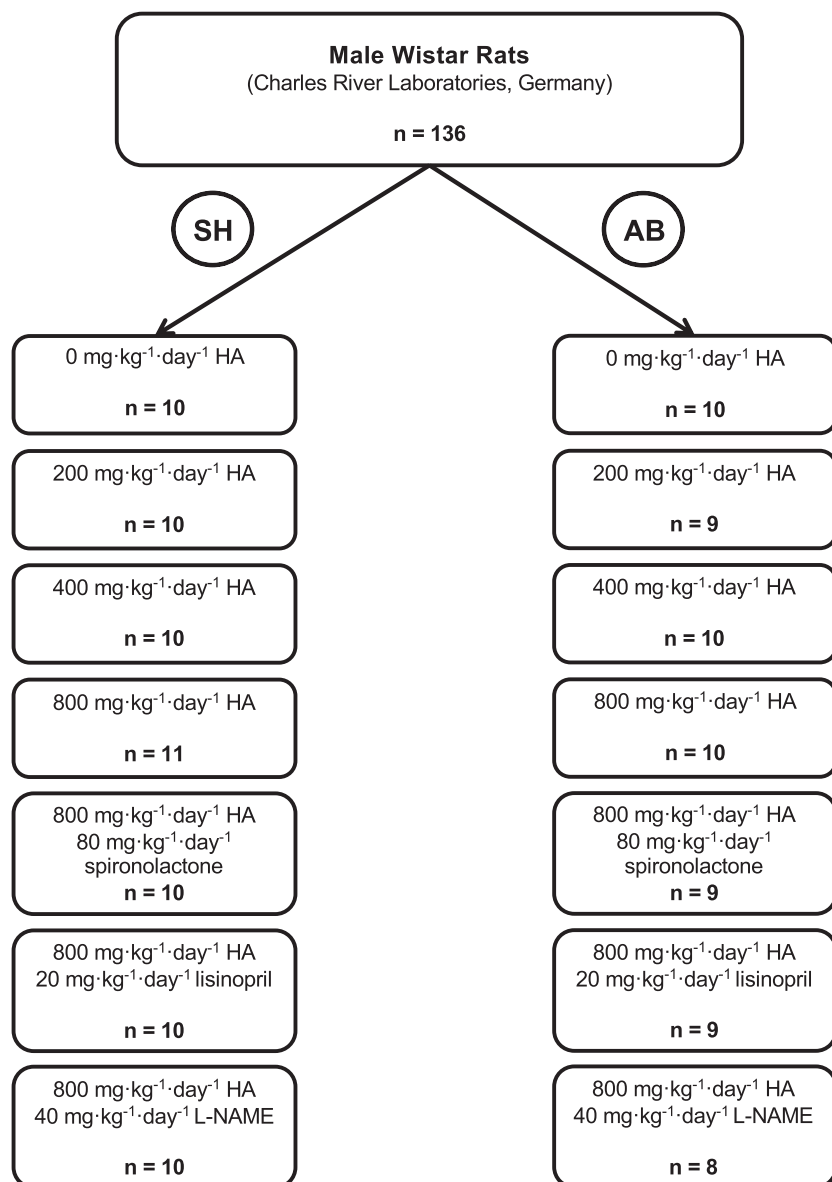
The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the institutional review board and the local authorities of the Regierungspräsidium Karlsruhe (Karlsruhe, Baden-Wuerttemberg, Germany). Animals were housed in cages under controlled conditions of temperature, light/dark cycle, and free access to water and standard diet. The animal protocol complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

### 2.1 | Aortic banding and heart failure model

Pressure overload was induced in 65 male Wistar rats by clipping the ascending aorta, as previously described [18]. Briefly, anesthesia was performed with ketamine (70 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. After orotracheal intubation, animals were ventilated (Harvard Apparatus Inc., Holliston, Massachusetts, USA) with 2.5% isoflurane in 100% oxygen to maintain anesthesia. The thorax was opened in the third left intercostal space and the ascending aorta was carefully dissected free of the surrounding tissue. Aortic banding (AB) was induced by clipping the ascending aorta using tantalum hemostatic clips with a diameter of 0.75 mm (Weck Hemo-clip Plus, Research Triangle Park, North Carolina, USA). Finally, the muscle layers and the skin were closed with 5-0 sutures (Polysorb, Synture, Mansfield, Massachusetts, USA). Animals were extubated and placed in an incubator at approximately 30°C. For pain relief, subcutaneously administered buprenorphine at 0.05 mg/kg (Temgesic, Indivior Europe Ltd., Dublin, Ireland) was used in the critical postoperative phase twice a day, combined or followed by subcutaneous injections of carprofen at 5 mg/kg (Rimadyl, Zoetis, Berlin, Brandenburg, Germany) once a day. Sham (SH) operated rats ( $n = 71$ ), undergoing the same surgical procedure without clip insertion, served as controls.

### 2.2 | Study design

SH- and AB animals were treated with different doses of HA (200 mg·kg<sup>-1</sup>·day<sup>-1</sup>, 400 mg·kg<sup>-1</sup>·day<sup>-1</sup>, and 800 mg·kg<sup>-1</sup>·day<sup>-1</sup>), spironolactone (80 mg·kg<sup>-1</sup>·day<sup>-1</sup>), lisinopril (20 mg·kg<sup>-1</sup>·day<sup>-1</sup>), L-NAME (40 mg·kg<sup>-1</sup>·day<sup>-1</sup>), or placebo by oral gavage. In detail, rats were randomly assigned to one of 14 subgroups (Figure 1).



**FIGURE 1** Illustration of the study design

Drug treatment was initiated on the third postsurgical day and continued for 4 weeks, respectively. HA was administered by oral gavage, which guaranteed a constant daily ingestion. Carrier solution was 0.9% sodium chloride (B. Braun Melsungen AG, Melsungen, Hesse, Germany). After 4-week treatment, the animals were sacrificed for further characterization. Peripheral blood samples were taken from the tail vein and centrifuged at 13000 rpm for 5 minutes. The separated plasma was stored at  $-80^{\circ}\text{C}$ . HA was measured in plasma samples by using tandem-mass spectrometry [19]. Echocardiographic and hemodynamic measurements were performed before hearts were excised, weighed, and subjected to further analysis. The heart was arrested in diastole by injection of saturated potassium chloride solution and heart weight (HW), left ventricular (LV) weight, right ventricular (RV) weight, liver weight (LiW), lung weight (LuW), and tibia length

(TL) were determined. Myocardial samples from the LV were snap-frozen for biochemical measurements or fixed in formalin for further histological evaluation.

### 2.3 | Transthoracic echocardiography

Echocardiography was performed in a modified setting, as previously described [18, 20]. Briefly, after anesthesia, the chest was shaved, and the heart was imaged in the left lateral decubitus position using the two-dimensional mode in the parasternal short-axis view [21]. M-mode measurements of LV dimensions were averaged from at least three consecutive cycles with good signal quality. Fractional shortening (FS, %) was calculated according to the following equation:  $\text{FS} = \text{LVEDD} - \text{LVESD} / \text{LVEDD} \times 100\%$ , where LVEDD is the LV end-diastolic diameter and LVESD is the LV

end-systolic diameter [22]. Experiments were recorded using dynamic focused 10-MHz probes with an ATL 5000 echocardiography machine (ATL Ultrasound, Philips, Bothell, Washington, USA). Analysis was performed with Scion Image (Scion Image, Scion Corporation, Frederick, Maryland, USA).

## 2.4 | Pressure measurements

For invasive measurements, the aorta was catheterized via the right carotid artery using a 2.0-F impedance micromanometer catheter (SPR-838, Millar Instruments Inc., Houston, Texas, USA). To simultaneously measure LV hemodynamics, a second 1.4-F micromanometer catheter (Millar Instruments Inc., Houston, Texas, USA) was inserted into the apex of the LV. The raw conductance volumes were corrected for parallel conductance by the hypertonic saline dilution method. Data were recorded with a sampling rate of 1000 Hz using a standard data-acquisition system (ADInstruments, Colorado Springs, Colorado, USA), and analyzed using PVAN Software (Millar Instruments Inc., Houston, Texas, USA) [23]. The peak-to-peak pressure gradient was defined as the difference between the peak left ventricular pressure and the peak aortic pressure.

## 2.5 | Primary cultures of ventricular cardiac fibroblasts

For isolation and culture of cardiac fibroblasts, LV tissue was homogenized and digested with Liberase 3 (Roche, Rotkreuz, Zug, Switzerland). Fibroblasts were purified by selective attachment to plastic culture ware and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% neonatal bovine serum and 5% fetal calf serum, with medium replacement every other day. All cells were used on the second passage to minimize changes in phenotype associated with culture. Before rinsing with Mosconas salt solution and serum starvation for 24 h in DMEM-F12, one million fibroblasts were seeded on culture plates and allowed to adhere to the plate for 24 h in DMEM solution. Finally, fibroblasts were treated with HA at 0.001, 0.01, 0.1 mM, and 1-M concentrations dissolved in DMEM with 1.5% FBS for 24 h.

## 2.6 | Quantitative real-time PCR

For quantitative real-time polymerase chain reaction (RT-PCR), total RNA was isolated from primary cells and fibroblasts of the LV using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesized from 500 ng of

RNA with a Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Real-time PCR was performed in duplicates with a 1:100 dilution of cDNA on a MyIQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). The reactions were carried out using 96-well plates (Thermo-Fast 96 PCR Plate, Thermo Fisher Scientific, Waltham, Massachusetts, USA), each allowing for a total volume of 20  $\mu$ l. At the end of each run, a melting curve analysis was done to confirm the amplification specificity. Copy numbers of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), beta-myosin heavy chain ( $\beta$ -MHC), collagen type V alpha 1 chain (col5a1), interleukin-17A (IL17A), and tumor necrosis factor superfamily member 14 (TNFSF14) were determined (Table S1). The amplicon length ranged from 67 to 119 base pairs (bp).

A dedicated Bio-Rad software allowed for automated analysis of each run. Relative quantification was conducted for each of the PCR samples, based on the threshold cycle. Samples were assessed with the  $2^{-\Delta\Delta C(t)}$  method being used to quantify relative expression [24]. All real-time PCRs were normalized to HPRT. A standard curve was run with the dilution series of the amplified fragment allowing for the calculation of mRNA copy numbers.

## 2.7 | Pathology

Cross-sections of the LV obtained midway between base and apex were formalin-fixed, paraffin-embedded, and stained with hematoxylin/eosin (H&E) and sirius red (SR) according to standard protocols [20, 21]. Myocyte size ( $\mu\text{m}^2$ ) was measured in LV cross-sections with ImageJ software (ImageJ, NIH, Bethesda, Maryland, USA), analyzing at least 400 cardiomyocytes and 20 paraffin slices per heart. Using light microscopy, evidence of fibrosis was evaluated in a blinded manner by two independent investigators. Collagen area fraction was calculated as collagen area to tissue area ratio in percent.

## 2.8 | Statistical analysis

Results are reported as mean  $\pm$  standard error of the mean (SEM). The normality of datasets was assessed by Shapiro-Wilk test. Comparisons between groups were performed with one-way analysis of variance (ANOVA), followed by posthoc comparisons between groups with Dunnett's test. Unpaired student's *t*-test (two-tailed) was used where appropriate. For statistical analysis, GraphPad Prism (GraphPad Inc., San Diego,

California, USA) and MedCalc software packages (MedCalc Software Ltd., Ostend, West Flanders, Belgium) were used. A  $p$  value less than 0.05 was considered to be statistically significant.

### 3 | RESULTS

#### 3.1 | HA levels after supplementation by oral gavage

HA concentration in animals with placebo treatment was  $0.9 \pm 0.6$  mg/L (0.04 mM). After 2 weeks, plasma levels of animals treated with  $200 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ,  $400 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ , and  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA increased to  $125 \pm 5$  mg/L (0.56 mM),  $274 \pm 9$  mg/L (1.22 mM), and  $561 \pm 11$  mg/L (2.50 mM), respectively.

#### 3.2 | Signs of cardiac decompensation and hypertrophy in AB animals

The average weight of Wistar rats was  $118 \pm 2$  g. AB was associated with signs of myocardial hypertrophy owing to pressure overload. HW to BW ratio (mg/g), HW to TL ratio (mg/mm), LV to BW ratio (mg/g), LV to TL ratio (mg/mm), and RV to TL ratio (mg/mm) were significantly higher (all  $p \leq 0.0017$ ) in placebo-treated AB rats compared to SH rats. Combined treatment of HA with heart failure medication led to a suppressed hypertrophic response as evidenced by a decline in heart weight ( $p = 0.0353$ ). AB animals showed higher lung weights compared to animals without intervention ( $p < 0.0001$ ), indicating fluid retention after aortic constriction. Interestingly, the lung weight of AB animals under supplementation with  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA or concomitantly applied heart failure medication was lower compared to placebo-treated AB controls ( $p = 0.0063$  and  $p = 0.0042$ , respectively).

Groups of SH-operated animals demonstrated a decrease in heart weight at simultaneously smaller tibia length following co-treatment of HA and heart failure medication (Tables S2 and S3).

#### 3.3 | Improved myocardial function together with lowered blood pressure in AB rats

Pressure overload led to an enlargement of the LV chamber as evidenced by increased EDD, aggravated systolic function, reduced ejection fraction (EF), and pronounced LV hypertrophy (Table S4 and S5). We observed a dose-dependent increase of LV systolic function compared to placebo-treated AB animals, with additionally improved EF in combination with

spironolactone (e.g.,  $48 \pm 3$  [AB  $0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA] vs.  $63 \pm 2\%$  [AB  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA w/ spironolactone],  $p = 0.0002$ ) (Figure 2).

Invasive pressure measurements confirmed the effectiveness of AB, showing high peak-to-peak pressure gradients compared to SH animals ( $6 \pm 5$  [SH  $0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA] vs.  $94 \pm 15$  mmHg [AB  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA],  $p < 0.0001$ ) (Tables S6 and S7; Figure 3). A reduction of arterial pressure was detected in the SH- and AB groups upon  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA treatment alone or combined with heart failure medication (e.g.,  $108 \pm 3$  [AB  $0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA] vs.  $91 \pm 3$  mmHg [AB  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA w/ lisinopril],  $p = 0.0014$ ).

#### 3.4 | Attenuated myocardial hypertrophy and fibrosis in AB animals

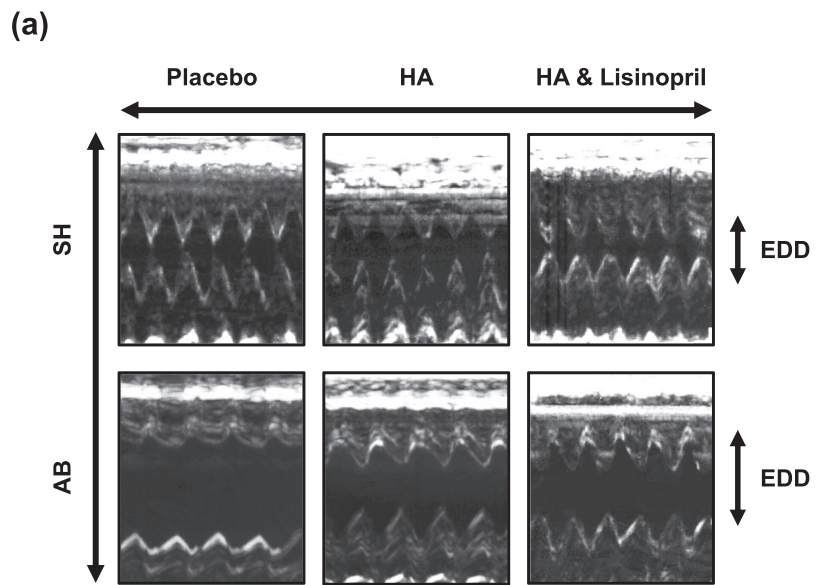
Four weeks following AB surgery, cardiomyocyte cross-sectional area was increased across all AB subgroups compared to SH animals (Tables S8 and S9; Figure 4). Myocytes from AB animals, treated with either HA alone or in combination with spironolactone or lisinopril, were significantly smaller in comparison to AB rats with placebo treatment. In this context, AB animals with lisinopril co-treatment presented the most significant decrease with approximately 22% smaller myocyte sizes than placebo-treated AB animals ( $311 \pm 3.2$  [AB  $0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA] vs.  $242 \pm 3.2 \mu\text{m}^2$  [AB  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA w/ lisinopril],  $p < 0.0001$ ), and 13% smaller myocyte sizes than solely HA treated animals with  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  ( $p < 0.0001$ ).

Quantitative evaluation of volume percent collagen (Tables S10 and S11; Figure 5) revealed large areas of collagen deposition in the interstitium and around vascular tissue after AB surgery. AB animals experienced a clearly dose-dependent reduction in myocardial fibrosis with the most significant decrease of approximately 41% among animals with spironolactone co-treatment (collagen area fraction:  $8.81 \pm 0.51$  [AB  $0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA] vs.  $5.24 \pm 0.43\%$  [AB  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA w/ spironolactone],  $p < 0.0001$ ). Neither spironolactone nor lisinopril had added value to HA treatment alone regarding the prevention of the remodeling process ( $p \geq 0.7322$ ). Interestingly, the addition of L-NAME was unable to reverse the attenuation of the remodeling process.

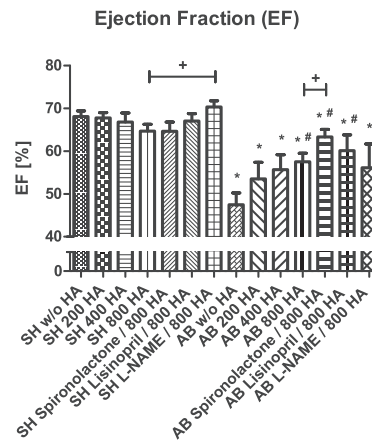
#### 3.5 | Inverse correlation of molecular hypertrophic and fibrotic markers following increasing doses of HA

In AB animals, doses of HA were inversely associated with the molecular hypertrophic markers ANF and BNP. Rats within the  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  AB group showed a

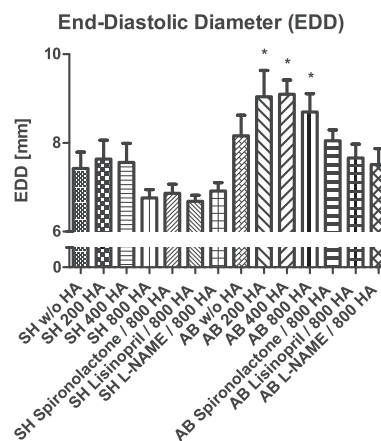
**FIGURE 2** Echocardiography following 4-week homoarginine (HA) treatment. Transthoracic echocardiographic M-mode images in SH- and AB animals treated with placebo and 800 mg.kg<sup>-1</sup>.day<sup>-1</sup> HA alone or combined with lisinopril (a). Ejection fraction (b), end-diastolic (c), and end-systolic LV diameter (d). \**p* < 0.05 versus SH 0 mg.kg<sup>-1</sup>.day<sup>-1</sup>. #*P* < 0.05 versus AB 0 mg.kg<sup>-1</sup>.day<sup>-1</sup>. +*p* < 0.05 versus SH or AB 800 mg.kg<sup>-1</sup>.day<sup>-1</sup>



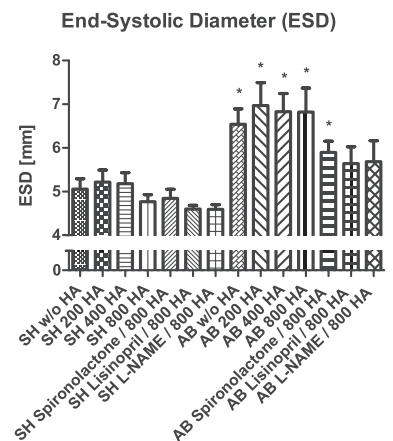
(b)



(c)

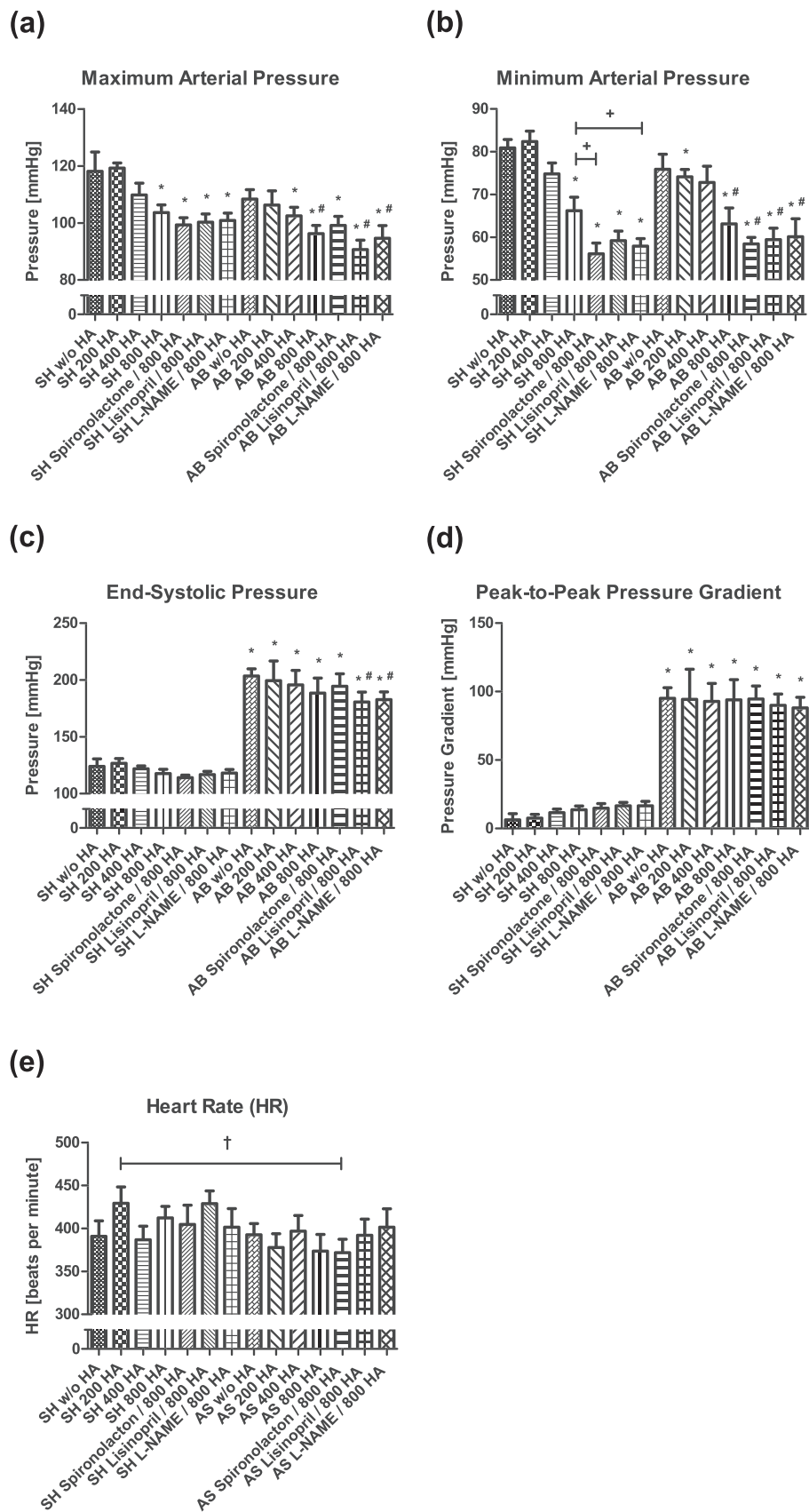


(d)



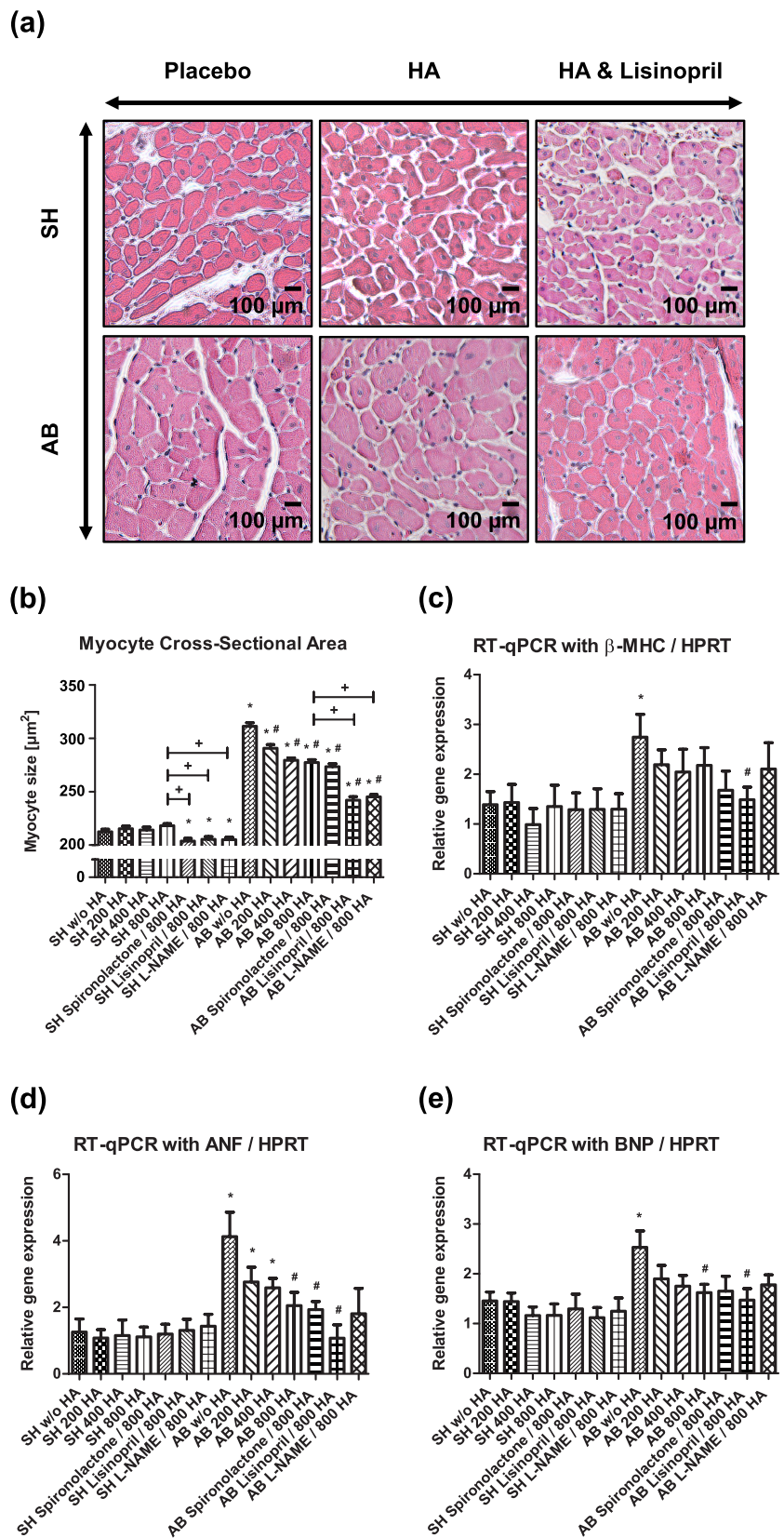
decrease of ANF by 50% (*p* = 0.0280) and BNP by 36% (*p* = 0.0295) in comparison to placebo-treated AB animals, whereas β-MHC was not significantly affected

(*p* = 0.3663). Combining HA with spironolactone or lisinopril resulted in more pronounced reductions, showing the greatest decline in AB animals following lisinopril



**FIGURE 3** Invasive hemodynamic measurements. Maximum and minimum arterial pressure (a,b). End-systolic pressure (c) and peak-to-peak pressure gradient (d). Heart rate (e). \* $p < 0.05$  versus SH 0  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ . # $p < 0.05$  versus AB 0  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ . † $p < 0.05$  versus SH 800  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ . ‡ $p < 0.05$

**FIGURE 4** Impact of homoarginine (HA) treatment on myocyte size and transcription levels. Exemplary hematoxylin eosin-stained sections of LV tissue (a) in SH- and AB animals treated with placebo and 800 mg·kg<sup>-1</sup>·day<sup>-1</sup> HA alone or combined with lisinopril. Cardiomyocyte area (b). Transcription levels of the beta-myosin heavy chain (β-MHC), atrial natriuretic factor (ANF), and brain natriuretic peptide (BNP) (c,d,e). \**p* < 0.05 versus SH 0 mg·kg<sup>-1</sup>·day<sup>-1</sup>. #*p* < 0.05 versus AB 0 mg·kg<sup>-1</sup>·day<sup>-1</sup>. +*p* < 0.05 versus SH or AB 800 mg·kg<sup>-1</sup>·day<sup>-1</sup>

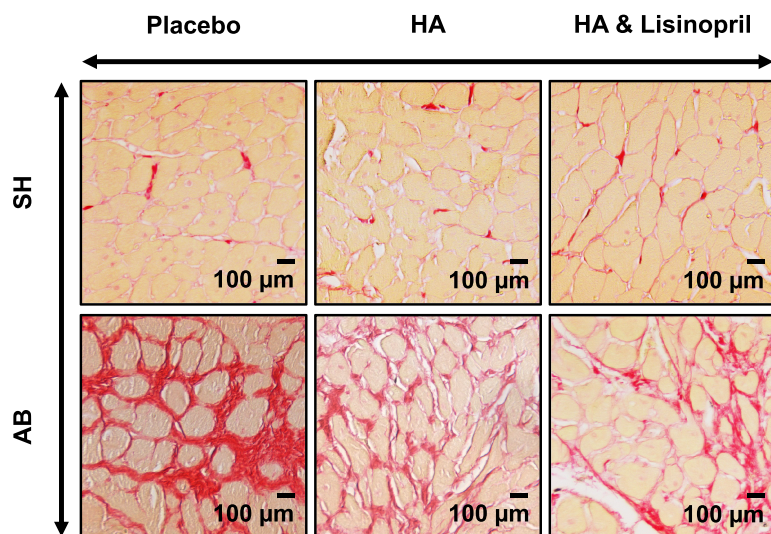


co-treatment with reductions of ANF by 74% (*p* = 0.0091), BNP by 42% (*p* = 0.0298), and β-MHC by 46% (*p* = 0.0411) (Figure 4).

Col5a1 showed an inverse relationship with rising HA concentrations (downregulation by 68% in the 800 mg·kg<sup>-1</sup>·day<sup>-1</sup> AB group compared to placebo-

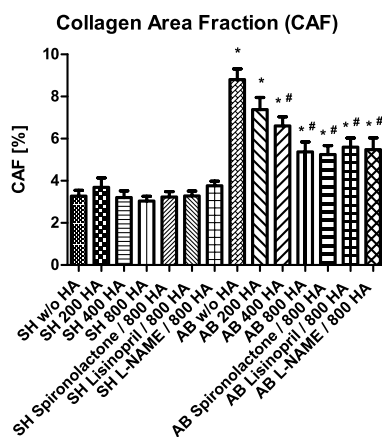


(a)

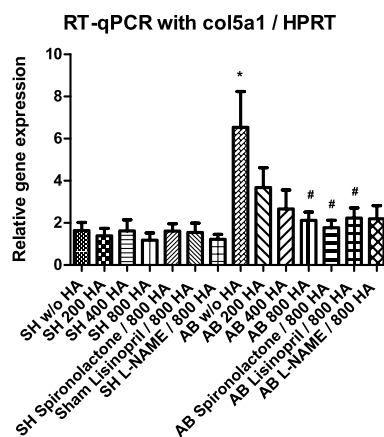


**FIGURE 5** Response of collagen deposition to homoarginine (HA) treatment. Exemplary sirius red-stained sections of SH- and AB animals treated with placebo and 800 mg·kg<sup>-1</sup>·day<sup>-1</sup> HA alone or combined with lisinopril (a). Collagen area fraction and transcription levels of collagen type V alpha 1 chain (col5a1) (b,c). Expression levels of col5a1 in fibroblasts treated with different concentrations of HA (3 animals per group, respectively) (D). \**p* < 0.05 versus SH 0 mg·kg<sup>-1</sup>·day<sup>-1</sup>. #*p* < 0.05 versus AB 0 mg·kg<sup>-1</sup>·day<sup>-1</sup>. †*p* < 0.05 versus stock 1 M

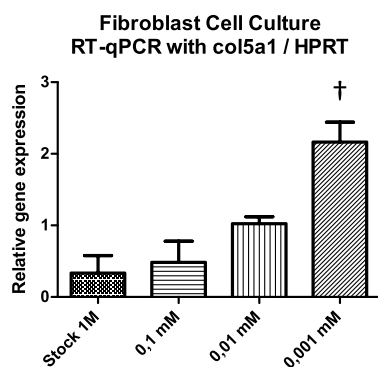
(b)



(c)



(d)



treated AB animals, *p* = 0.0210). The addition of spiro-lactone led to a downregulation of 73% (*p* = 0.0257) in comparison to AB animals with placebo treatment (Figure 5).

In a small subanalysis of in total 18 SH- and AB animals, the inflammatory markers IL-17A and TNFSF14 tended to be lower after HA treatment without reaching statistical significance

(max.  $p = 0.6319$  and  $p = 0.0615$ , respectively) (Figure S1).

### 3.6 | Response of isolated cardiac fibroblast mRNA to treatment with HA

Col5a1 mRNA expression levels showed significant concentration-dependent downregulations in cardiac rat fibroblasts upon HA treatment ( $p = 0.0382$ ) (Figure 5).

### 3.7 | Side effects and mortality

HA treatment did neither result in a deterioration of heart function nor any other serious side effect. Perioperative and postoperative mortality were approximately 30% in AB animals and <10% in SH-operated animals.

## 4 | DISCUSSION

To our knowledge, this is the first study investigating the impact of HA treatment on cardiac remodeling in an in vivo model of myocardial hypertrophy and NO-deficient hypertension. Cardiac remodeling represents a complex adaptation process in response to pressure overload to overcome the increased hemodynamic pressure required to maintain cardiac output [25]. Since changes are partially reversible, as can be seen after valve replacement, HA attracted lots of attention as being considered directly protective in cardiovascular diseases [2, 16]. However, there is still a lack of information providing new insights into the potential role of HA as a therapeutic agent and much less if combined with standard medical care for heart failure patients.

Here, we show that the remodeling process caused by aortic constriction can be rescued by dietary HA supplementation. Our findings are in line with previously published clinical and experimental data highlighting the positive correlation between HA and myocardial function [12, 26]. In our study, animals that underwent AB surgery showed an improvement in EF after 4-week treatment in a dose-dependent manner up to  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (+21%,  $p = 0.0092$ ) which further increased when adding spironolactone (+10%,  $p = 0.0476$ ). Moreover, drug treatment was accompanied by significant reductions in lung weight (max. -41%,  $p = 0.0042$ ) and lung weight to body weight ratio (max. -43%,  $p = 0.0074$ ). Considering that symptoms of heart failure result from an abnormal elevation of venous pressure with significant elevations of lung weight as a reliable marker for LV dysfunction, this observation points towards myocardial recovery [27]. In contrast, SH groups showed no significant changes after HA application, suggesting that the beneficial

effects of HA on cardiac remodeling take effect primarily under pathological conditions.

Over the past decade, several hypotheses have been generated by which HA might attenuate cardiac remodeling [2, 26, 28]. Due to its structural resemblance to L-arginine, HA has been proposed to act as an alternative substrate for NO synthase participating in the generation of NO [2]. In this context, reduced NO bioavailability and the overabundant presence of reactive oxygen species are known as major contributors to myocardial dysfunction [29]. Despite its lower catalytic activity compared to L-arginine, NO concentrations were found to be elevated even 8 h after HA application in comparison to L-arginine treated animals going to baseline levels after 4 h [26]. Interestingly, the addition of L-NAME did not revert the cardioprotective effects of HA, indicating a more complex mechanism of action.

Apart from NO signaling, HA may also take part in cellular energy metabolism [6, 13, 30]. Arginine-glycine amidinotransferase (AGAT), the rate-limiting key enzyme in the synthesis of HA, plays an important role in the generation of creatine [31]. Increased AGAT enzyme activity was found in myocardial samples of patients with end-stage heart failure, turning back to normal levels after implantation of a left ventricular assist device and myocardial recovery [31]. The AGAT upregulation was regarded as an adaptive response to depleted intracellular energy stores in the failing heart [31]. Data from AGAT knockout mice revealed that cardiac dysfunction is principally driven by HA deficiency rather than a creatine mismatch [13].

Given its proposed interactions with NO, one can infer that HA might influence blood pressure as suggested by previous investigations [6]. Chen et al have shown that intravenously injected HA increases the urinary excretion of nitrate, a degradation product of NO, lowering blood pressure in salt-sensitive hypertensive rats [32]. Our catheter-assisted pressure analysis confirmed the antihypertensive effect of HA showing lowered blood pressure values in SH- and AB animals, particularly following a dose of  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (-12%,  $p = 0.0407$ , and -11%,  $p = 0.0132$ , respectively).

In addition to improvements in LV function and reduction in blood pressure, histopathological and molecular changes were observed following HA supplementation in AB groups. In general, LV hypertrophy is followed by myocyte damage with interstitial enlargement and replacement fibrosis as the principal morphological alterations in the remodeling process [33]. Accordingly, placebo-treated animals with aortic constriction presented enlarged myocytes (+47%,  $p < 0.0001$ ) with evidence of myocyte damage and extensive collagen deposition compared to SH controls. Pharmacological treatment ameliorated cardiac hypertrophy not only by the single HA administration up to the highest dose regimen of  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  but



also in combination with lisinopril (significant added value in comparison to the single application of  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA,  $p < 0.0001$ ). Consistently, macroscopic findings showed a lowered HW/TL ratio following lisinopril co-treatment in comparison to placebo ( $p = 0.0452$ ) and solely  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA treated animals ( $p = 0.0024$ ).

It has previously been demonstrated that spontaneously hypertensive rats, given L-NAME for 3 weeks, developed severe hypertensive heart disease with similar histopathological changes as observed in our micrographs after AB surgery [10, 34]. Whether the structural myocardial changes due to chronic NO inhibition were caused by the lack of NO per se or the accompanying hypertension was addressed by several studies. L-NAME treatment with co-administration of hydralazine in rats prevented arterial hypertension but did not affect cardiac hypertrophy and microvascular remodeling caused by long-term NO blockade over 8 weeks [35]. Suppression of distinct remodeling alterations seems to be dependent on the ability of the chosen drug to influence hemodynamic processes and protein metabolism [36]. In this context, the availability of HA might be significantly affected by absorption, distribution, metabolism, excretion, or even endogenous AGAT activity [37]. In our study, particularly interstitial fibrosis was markedly reduced in HA-treated rats upon pressure overload. According to increasing HA doses in the AB group, we were able to show reduced transcription levels of col5a1 (max.  $-68\%$ ,  $p = 0.0210$ ), ANF (max.  $-50\%$ ,  $p = 0.0280$ ), BNP (max.  $-36\%$ ,  $p = 0.0295$ ), and  $\beta$ -MHC (max.  $-21\%$ ,  $p = 0.3663$ ). Co-medication with spironolactone or lisinopril resulted in a more pronounced downregulation of col5a1 (max.  $-73\%$ ,  $p = 0.0257$ ), ANF (max.  $-74\%$ ,  $p = 0.0091$ ), BNP (max.  $-42\%$ ,  $p = 0.0298$ ), and  $\beta$ -MHC (max.  $-46\%$ ,  $p = 0.0411$ ). Our in vivo results of decreased col5a1 mRNA expression levels were confirmed ex vivo showing a concentration-dependent downregulation of col5a1 in HA-treated fibroblasts (max.  $-85\%$ ,  $p = 0.0382$ ).

Our data are consistent with a recently published murine animal study reporting HA-mediated LV protection in the setting of calcified coronary artery disease [15]. HA was dissolved at a concentration of  $14 \text{ mg/L}$  in drinking tap water resulting in a threefold increase in plasma HA levels after 4 weeks (mean HA concentration of approximately  $1.1 \mu\text{mol/L}$ ) with prevented left ventricular dilatation ( $p < 0.01$ ), preserved ejection fraction ( $p < 0.05$ ), and reduced myocardial fibrosis ( $p < 0.001$ ). Despite the higher dose in our study as compared to other publications [12, 13, 15, 19], all animals showed an excellent tolerance of HA without significant side effects highlighting HA treatment as efficacious and safe.

In a clinical context, HA might represent a viable treatment option not only for patients with reduced

but also with preserved ejection function (Heart Failure with preserved Ejection Fraction, HFpEF), particularly considering the still very limited pharmacological treatment options in this patient group [38, 39].

Several study limitations have to be noticed. First, we used only a limited number of animals for our experiments requiring additional investigations for the validation of our findings. Second, direct comparisons of HA with other pharmacological interventions would be desirable, especially with L-arginine. Third, the 4 weeks of HA administration should be extended to investigate long-term outcomes. Fourth, the high dose of max.  $800 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  HA has to be noted which has possibly resulted in an artificially induced amino acid overload. Fifth, heart rate varied between groups, potentially leading to underestimation of parameters of LV function. Finally, the interpretation of our study findings is limited to male Wistar rats without including female animals. Furthermore, it makes it difficult to translate our findings to the general human population of patients affected by pressure overload.

## 5 | CONCLUSIONS

In summary, our study findings show that dietary supplementation with HA, either alone or in combination with spironolactone or lisinopril, improves cardiac function and attenuates cardiac remodeling upon pressure overload and NO-deficient hypertension. Future research is needed to evaluate the long-term effects of HA treatment on the cardiovascular outcome, elucidate underlying molecular pathomechanisms, and investigate a possible translation of our experimental findings into replications in human trials.

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### CONFLICT OF INTEREST

NF has received speaker honoraria from Daiichi Sankyo, Astra Zeneca, Boehringer Ingelheim, and Bayer Vital. All other authors have no potential conflict of interest to disclose.

### ETHICS STATEMENT

Approval from the local Institutional Review Board was obtained.

### AUTHOR CONTRIBUTIONS

All authors have contributed significantly to this work, participating in conception, design, analysis, interpretation of data, and writing/editing.

## RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

Experimental study on animals (rats).

## DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and its supplementary material.

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## SUPPORTING INFORMATION

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