

Incomplete structural reverse remodeling from late-stage left ventricular hypertrophy impedes the recovery of diastolic but not systolic dysfunction in rats

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Objective: Pressure overload-induced left ventricular myocardial hypertrophy (LVH) regresses after pressure unloading. However, distinct structural alterations become less reversible during the progression of LVH, which might influence the restoration of cardiac function. Here, we investigated how a reverse remodeling process from early versus late-stage LVH affects different aspects of left ventricular function.

Methods: Pressure overload was induced in rats for 6, 12 and 18 weeks. Sham-operated animals were used as controls. Pressure unloading was evoked by removing the aortic constriction at week 6 (early-debanded) and week 12 (late-debanded). Echocardiography and histological analyses were carried out to detect structural alterations. Pressure–volume analysis was performed to assess left ventricular function. Molecular alterations were analyzed by quantitative real-time-PCR, and western blot.

Results: Myocardial hypertrophy regressed to a similar degree in early and late-debanded groups. Accordingly, no differences were detected in the extent of regression regarding left ventricular mass, cardiomyocyte diameter, heart weight-to-tibial length ratio and beta-to-alpha myosin heavy chain expression. In contrast, resorption of interstitial and perivascular myocardial fibrosis was only detected in the early-debanded group, whereas it persisted in the late-debanded group. Removing the aortic constriction normalized ventriculo-arterial coupling and increased systolic performance in both debanded groups. However, the residual dysfunction in active relaxation and passive stiffness was more severe in the late-debanded compared to the early-debanded group.

Conclusion: Early debanding led to complete structural reverse remodeling (reduced hypertrophy and fibrosis) and full restoration of left ventricular function. In contrast, myocardial fibrosis persisted after late debanding, which impeded the normalization of diastolic but not systolic function.

Keywords: contractility, diastolic function, interstitial fibrosis, myocardial hypertrophy, perivascular fibrosis, pressure overload, pressure–volume analysis, reverse remodeling, systolic function

Abbreviations: AB, aortic banding; ANP, atrial natriuretic peptide; ANOVA, analysis of variance; AWTd, anterior wall thickness in diastole; β/α -MHC, Beta/Alpha myosin heavy chain ratio; CD, cardiomyocyte diameter; CO, cardiac output; CTGF, connective tissue growth factor; Ea, arterial elastance; EDPVR, end-diastolic pressure–volume relationship; EF, ejection fraction; ESPVR, end-systolic pressure–volume relationship; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HR, heart rate; HW/TL, heart weight to tibial length ratio; LV, left ventricle; LVH, left ventricular myocardial hypertrophy; MAP, mean arterial pressure; MF, myocardial fibrosis; PO, pressure overload; PRSW, preload recruitable stroke work; P–V, pressure–volume; PWTd, posterior wall thickness in diastole; σ , left ventricular meridional wall stress; SV, stroke volume; Tau, time constant of left ventricular pressure decay; VAC, ventriculo-arterial coupling

INTRODUCTION

In recent years, a growing body of literature has provided evidence that the termination of the pathological insult of sustained pressure overload [e.g. by aortic valve replacement (AVR) in case of aortic stenosis or by antihypertensive medications in case of arterial hypertension] induces the regression of left ventricular myocardial hypertrophy (LVH) [1–3]. This process is termed myocardial reverse remodeling and involves the restoration of the hypertrophic left ventricular phenotype on the molecular, ultrastructural, geometrical and also functional level [4]. Nevertheless, recent experiments have indicated that the

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reverse remodeling ability of the left ventricle (LV) decreases during the progression of LVH [5,6].

The latter phenomenon might be explained by the fact that the ultrastructural composition of early and late-stage LVH is characteristically different. At early stages, cardiomyocyte hypertrophy and low level of myocardial fibrosis are the dominant histopathological findings [7]. In contrast, at later stages, cardiomyocyte hypertrophy is accompanied by progressive perivascular and interstitial myocardial fibrosis [7]. Importantly, it has been shown that the reversibility of myocardial fibrosis is substantially less than that of cardiomyocyte hypertrophy [8–11]. Therefore, when pressure unloading is performed at advanced stages of LVH, cardiomyocyte hypertrophy regresses, whereas myocardial fibrosis often persists [8].

The effects of incomplete normalization of LVH-associated structural abnormalities on left ventricular function have been the focus of interest in previous clinical studies. These investigations raised the possibility that the poor resorption of collagen fibers during myocardial reverse remodeling predominantly could impede and lengthen the recovery of left ventricular diastolic dysfunction [2,3,12,13]. Of particular note is the observation that the normalization of increased myocardial stiffness is considered to be a chronic process due to the slow regression of interstitial myocardial fibrosis [2,3]. In contrast, according to prior clinical observations, removal of the sustained pressure overload often induces a rapid improvement in systolic function even before normalization of left ventricular structural abnormalities is completed [14].

Nevertheless, the question how a reverse remodeling process from early versus late-stage LVH affects distinct aspects of left ventricular function has not been addressed under experimental conditions. It is important to note that the clinical investigations share common limitations (inhomogeneity of study populations due to differences in age, sex and comorbidities) that warrants the need of experimental validation. Recently, rodent models of aortic banding and debanding were introduced to study the regression of LVH under standard laboratory conditions [5,6,8,11,15,16]. These approaches proved to be useful tools for reproducing the functional, structural and molecular alterations that occur during myocardial reverse remodeling. Furthermore, in recent years, the technique of left ventricular pressure–volume analysis became available for use in small animals, thereby allowing assessment of numerous aspects of in-vivo cardiac function in rodent models [17]. Taken together, in the present study, we aimed to evaluate the effect of pressure unloading on left ventricular systolic and diastolic function at early and late-stage LVH by pressure–volume analysis.

METHODS

Animals

The investigation conformed to the EU Directive 2010/63/EU and the Guide for the Care and Use of Laboratory Animals used by the US National Institutes of Health (NIH Publication No.85–23, revised 1996). The experiment was approved by the ethics committee of the Land Baden-Württemberg for Animal Experimentation. The study is interpreted in accordance with the ARRIVE (Animals in

Research: Reporting in Vivo Experiments) guidelines [18]. Male Sprague–Dawley rats ($n = 92$; 5–6 weeks old; 160–180 g; Janvier, France) were kept under standard conditions ($22 \pm 2^\circ\text{C}$ with 12-h light/dark cycles; maximum four rats per cage) and were allowed access to laboratory rat diet and water *ad libitum*. A 1-week long acclimatization period was utilized before performing the surgeries.

Aortic banding and debanding

Under isoflurane anesthesia, abdominal aortic banding or sham operation was performed, according to a protocol that we described elsewhere [19]. At week 6 and at week 12, part of the aortic banding animals underwent a second operation, when the aortic constriction was cut off and the reactive fibrotic tissue surrounding the aorta was removed (debanding).

Experimental groups

Randomization of the animals into the following eight experimental groups was performed immediately after aortic banding/sham operation in a blinded fashion: groups 1–3 – Sham-wk6 ($n = 10$), Sham-wk12 ($n = 10$) and Sham-wk18 ($n = 9$) groups: these rats underwent sham operation and a 6, 12, or 18-week-long follow-up, respectively; groups 4–6 – AB-wk6 ($n = 10$), AB-wk12 ($n = 11$) and AB-wk18 ($n = 13$) groups: these rats underwent aortic banding operation and a 6, 12 or 18-week-long follow-up, respectively; group 7 – early-debanded group ($n = 14$): these rats underwent aortic banding operation, after week 6 the banding suture was removed and were followed up until week 12; group 8 – late-debanded group ($n = 15$): these rats underwent aortic banding operation, after week 12 the banding suture was removed and were followed up until week 18.

Echocardiography

Echocardiographic measurements were carried out with the Vevo 2100 imaging system (FujiFilm VisualSonics Inc., Toronto, Ontario, Canada). Left ventricular internal diameters (LVDs) and anterior and posterior wall thicknesses (AWT, PWT) in diastole (d) and systole (s) were measured, and left ventricular mass was calculated. Analysis of the echocardiographic recordings was carried out by an examiner blinded to the experimental set-up.

Pressure–volume analysis

Pressure–volume analysis was performed using a 2-F pressure–conductance microcatheter (SPR-838; Millar Instruments, Houston, Texas, USA), as described previously [20]. Systolic blood pressure (SBP), diastolic BP (DBP), mean arterial BP (MAP), heart rate (HR), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) and time constant of left ventricular pressure decay (τ ; according to the Glanz method [21]) were measured. Furthermore, ejection fraction, arterial elastance [E_a ; calculated by the following equation: $E_a = \text{LVESP} / \text{stroke volume (SV)}$] [22], slope of end-systolic pressure–volume relationship (ESPVR, according to the parabolic curvilinear model [23]), preload recruitable stroke work (PRSW), ventriculo-arterial coupling (VAC; as the ratio of E_a and ESPVR) and slope of end-diastolic pressure–volume relationship (EDPVR) were calculated. Parallel conductance was calculated, and volume

calibration of the conductance system was performed as previously described [17].

From the hemodynamic and echocardiographic measurements, left ventricular meridional wall stress (σ) was estimated by the following equation: $\sigma = 0.334 \times \text{LVESP} [\text{LVDs}/(1 + \text{PWTs}/\text{LVDs})]$ [24].

After the hemodynamic measurements, the animals were euthanized by exsanguination. Heart weight and its index to the tibial length were measured.

Histology

Hematoxylin and eosin staining was utilized to measure cardiomyocyte diameter as a marker of cardiomyocyte hypertrophy [19]. Myocardial fibrosis was assessed on picrosirius-stained sections. Collagen volume fraction (ratio of collagen area/total area) was calculated to evaluate interstitial myocardial fibrosis, as also described earlier [25]. In brief, three transmural images (magnification: 50 $\times$) were randomly taken from the free left ventricular wall on each section. Image J software (NIH, Bethesda, Maryland, USA) was used to identify the picrosirius red-positive area. After background subtraction, eye-controlled auto-threshold has been determined to detect positive areas. The collagen volume fraction (picrosirius red-positive area-to-total area ratio) was determined on each images, and the mean value of three images represents each animal. Furthermore, perivascular myocardial fibrosis was assessed by the percentage of perivascular collagen area to total vascular area [26]. Perivascular fibrosis was also assessed by using image J software. The evaluation of the histological sections was performed by an independent observer who was blinded to the experimental design.

Left ventricular mRNA expression analysis

The mRNA expression levels from LV samples ($n = 10-13$) were analyzed by quantitative real-time-PCR, according to the previously described protocol [27]. The following genes were investigated: *hypertrophy markers* – atrial natriuretic peptide (ANP), α myosin heavy chain (MHC) and β -MHC (ratio of β/α -MHC was calculated); *fibrosis marker* – connective tissue growth factor (CTGF). Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For sequences of the primers see the Suppl. Table 1 (<http://links.lww.com/HJH/B59>).

Left ventricular protein expression analysis

Left ventricular samples ($n = 6$) were homogenized in RIPA buffer (Sigma-Aldrich, Budapest, Hungary) containing Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) at 0°C. The tissue lysates were agitated at 4°C for 1 h. After that, centrifugation was applied for 20 min at 12000 r.p.m. at 4°C, and the supernatant was collected. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, Illinois, USA). Samples were mixed with Bio-Rad 2X Laemmli Sample Buffer (Sigma-Aldrich) containing reducing agent and subsequently boiled at 95°C for 5 min. An equal amount of protein (20 μ g) was loaded on a commercially available precast 4–12% SDS-PAGE gel (NuPAGE Novex Bis-Tris Mini Gel; Invitrogen, Carlsbad, California, USA) and separated by gel electrophoresis. Transfer of the separated proteins to a polyvinylidene difluoride membrane was carried

out under dry conditions by using an electroblotting system (iBlot Gel Transfer Device; Invitrogen). After transfer, the membranes were washed and blocked for 1 h in 5% of BSA in Tris-buffered saline-Tween 20 at room temperature to reduce the nonspecific bindings of the antibodies. Then the membranes were incubated overnight at 4°C with anti-CTGF primary antibody (38 kDa, 1:2000 dilution, Abcam #6992). The blots were washed to remove excessive primary antibody binding and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature (antirabbit IgG; Cell Signaling Technology, Danvers, Massachusetts, USA). GAPDH (1:5000, #5174, Cell Signaling Technology) housekeeping protein was used as loading control and protein normalization. Blots were developed by the enhanced chemiluminescence detection assay (SuperSignal West Pico PLUS Chemiluminescent Substrate; Thermo Scientific, Waltham, Massachusetts, USA), and the intensity of the bands was measured by the ChemiDoc Touch Imaging system (Bio-Rad, Budapest, Hungary).

Statistics

All values are expressed as mean $\pm$ standard error of the mean. The distribution of the datasets was tested by D'Agostino-Pearson omnibus and Kolmogorov–Smirnov normality tests. An unpaired two-sided Student's *t* test in case of normal distribution or Mann–Whitney U test in case of non-normal distribution was used to compare two independent groups. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Kruskal–Wallis test followed by Dunn's post-hoc test was carried out to compare three independent groups.

When data were available (in case of repeatable, noninvasive measurements: echocardiography) from the same animal at the time of the debanding surgery (*predebanding*: week 6 in case of early-debanded and week 12 in case of late-debanded) and at the end of the experimental period (*postdebanding*: week 12 in case of early-debanded and week 18 in case of late-debanded), a ratio of *postdebanding/predebanding* values was calculated. These values were used to directly compare the extent of regression between the early and the late-debanded groups.

When repetitive data were not available (in case of not repeatable measurements: post mortem organ measurements, pressure–volume analysis, histology and PCR) from the same animal, individual data were normalized to the mean value of the corresponding sham groups. These normalized values were used to compare hypertrophy-associated alterations among the aortic banding groups (AB-6wk, AB-12wk, AB-18wk groups) and between the debanded groups (early-debanded, late-debanded), respectively.

A *P* value less than 0.05 was used as a criterion of statistical significance.

RESULTS

Effect of early and late debanding on echocardiographic parameters

In the aortic banding groups, sustained pressure overload led to continuous increment in left ventricular mass, anterior wall thickness in diastole (AWTd) and posterior wall thickness in diastole (PWTd) (Fig. 1a and b).

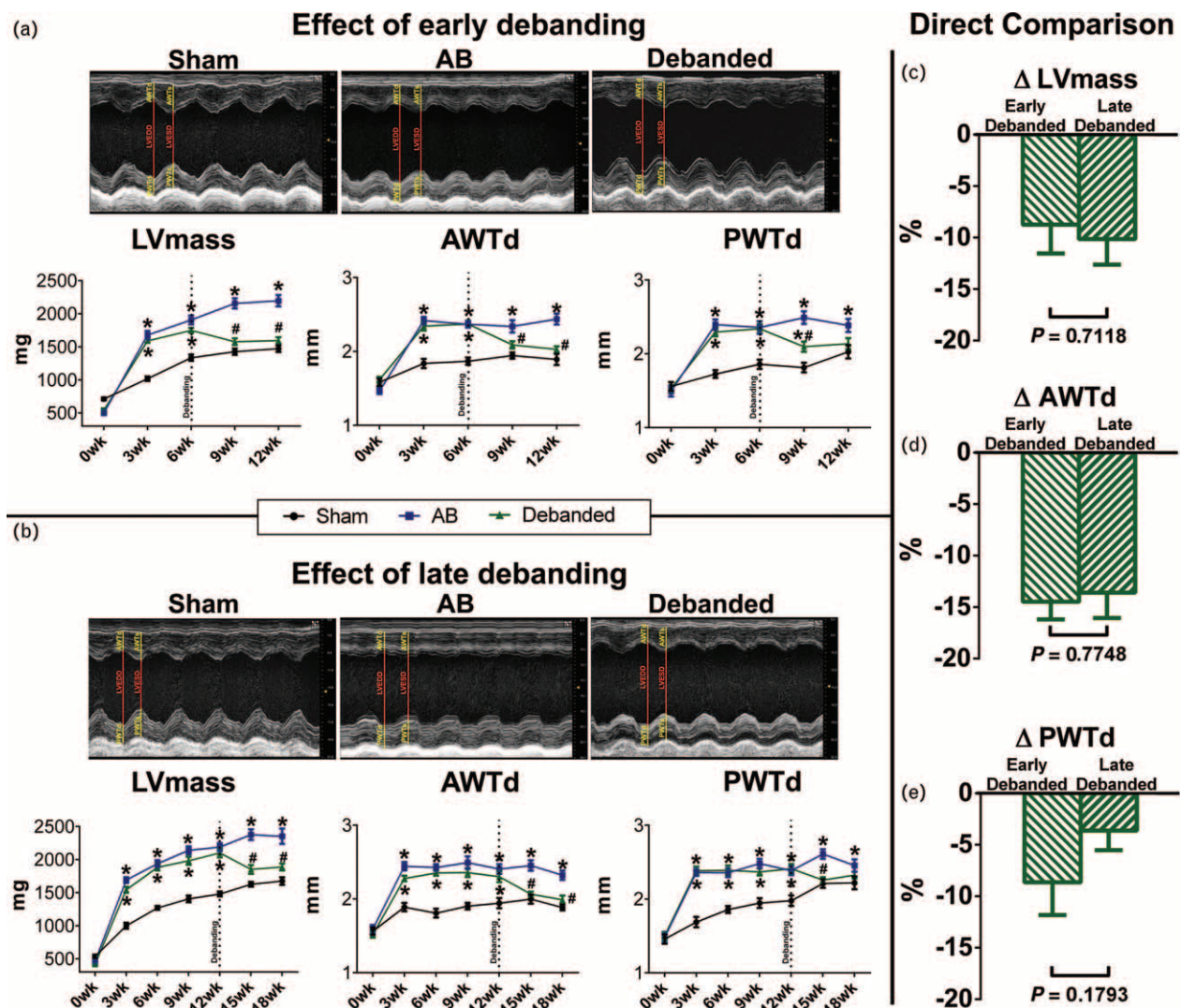


FIGURE 1 Echocardiographic follow-up during myocardial reverse remodeling from early and late-stage LVH. (a and b) Representative M-mode echocardiographic images at the midpapillary muscle level are shown at week 12 (in case of early debanding) and at week 18 (in case of late debanding), demonstrating the development and the regression of left ventricular (LV) myocardial hypertrophy. Both in the early and in the late-debanded groups, LV mass, AWTd and PWTd were decreased compared to the age-matched aortic banded (AB) groups. (c–e) To assess the extent of hypertrophy regression in the early and in the late-debanded groups, a ratio was calculated from the parameters measured at the postdebanding and the predebanding state. These calculated values were used to compare the effectiveness of reverse remodeling between the early and the late-debanded groups. No differences were found in the extent of LV mass, AWTd and PWTd regression between the early and the late-debanded groups. AWT, anterior wall thickness; d, diastole; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; PWT, posterior wall thickness; s, systole. * $P < 0.05$ vs. age-matched sham. # $P < 0.05$ vs. age-matched AB.

Both early (at week 6) and late debanding (at week 12) resulted in significant regression of the previously increased left ventricular mass, AWTd and PWTd (Fig. 1a and b). No difference was found in the extent of left ventricular mass (Fig. 1c), AWTd (Fig. 1d) and PWTd (Fig. 1e) regression between the early and late-debanded groups.

Effect of early and late debanding on pathological hypertrophy markers

In the aortic banding groups, cardiomyocyte diameter, heart weight/tibial length, β/α -MHC and ANP were increased compared to the age-matched control groups, confirming the development of pathological LVH (Fig. 2a, c, e and g and Supplementary Fig. 1a–d, <http://links.lww.com/HJH/B59>).

Both in the early and in the late-debanded groups, cardiomyocyte diameter, heart weight/tibial length, β/α -MHC

and ANP were decreased compared to the age-matched aortic banding groups (Fig. 2a, c, e and g). Regarding cardiomyocyte diameter (Fig. 2b), heart weight/tibial length (Fig. 2d) and β/α -MHC (Fig. 2f), no differences could be detected in the extent of regression between the early and the late-debanded groups. However, the relative mRNA expression level of ANP decreased to a greater extent in the early-debanded compared to the late-debanded group (Fig. 2h).

Effect of early and late debanding on myocardial fibrosis

Interstitial myocardial fibrosis, perivascular myocardial fibrosis and mRNA levels of connective tissue growth factor (CTGF) were significantly increased in the aortic banding groups compared to their age-matched sham groups (Figs. 3a and c and 4a and Supplementary Fig. 1e–g,

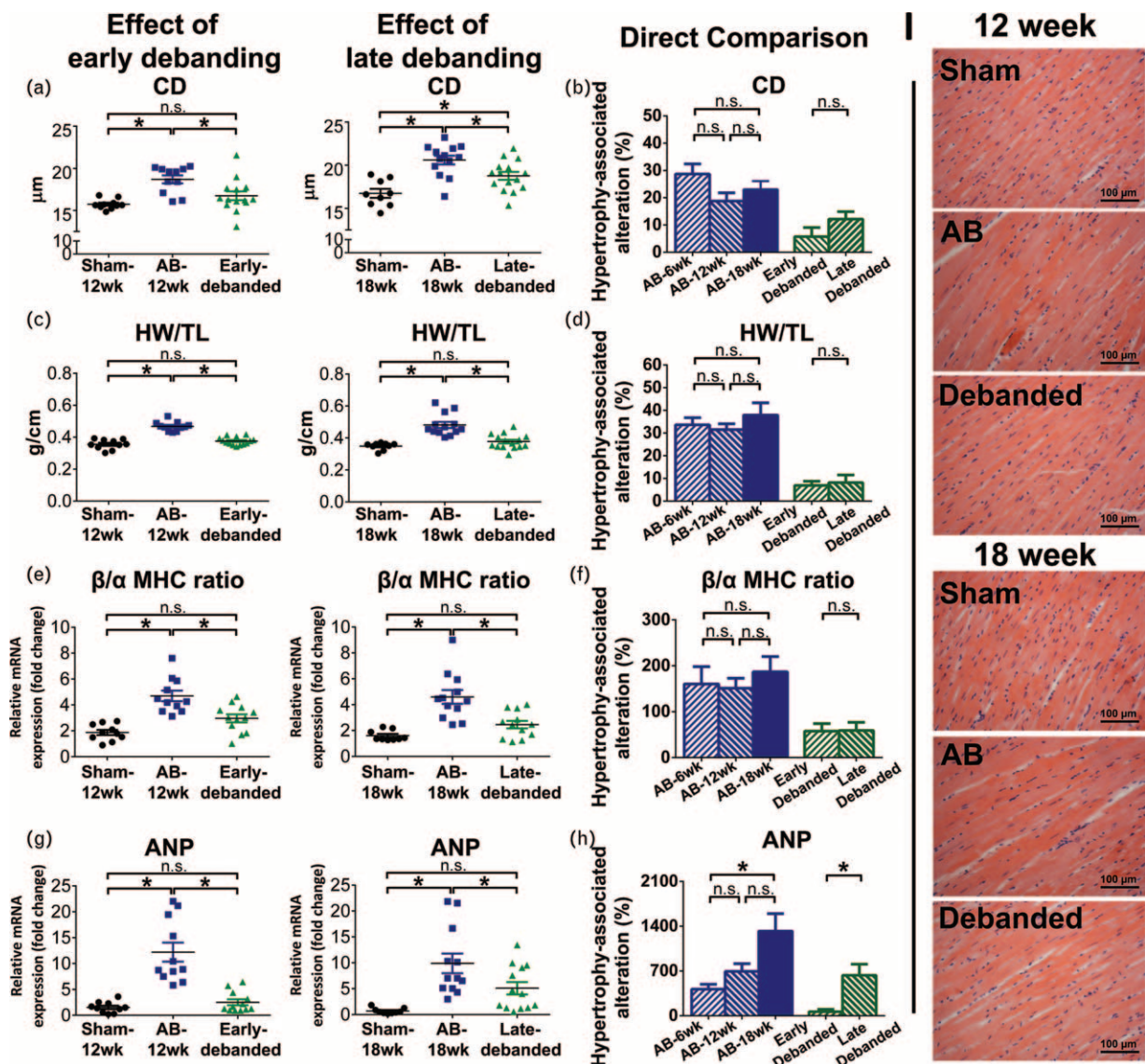


FIGURE 2 Effect of early and late debanding on pathological hypertrophy markers. (a, c, e, g) Increased cardiomyocyte diameter (CD), heart weight-to-tibial length ratio, beta-to-alpha myosin heavy chain ratio (β/α MHC) and atrial natriuretic peptide (ANP) expression level were observed in the aortic banded (AB) groups, indicating the development of pathological myocardial hypertrophy. Removing the aortic constriction at week 6 (early-debanded) and week 12 (late-debanded) effectively decreased the pathological hypertrophy-markers. (b, d, f, h) To directly compare the AB and the debanded groups, individual data were normalized to the mean value of the corresponding sham groups. The extent of CD, heart weight/tibial length and β/α MHC regression did not differ between the two debanded groups. However, the expression level of ANP decreased to a significantly greater extent in the early-debanded compared to the late-debanded group I: representative photomicrographs of hematoxylin and eosin staining are shown (magnification 200 $\times$, scale bar: 100 μ m), demonstrating enlargement of cardiomyocytes in the AB groups, and its regression in the debanded groups. * $P < 0.05$.

<http://links.lww.com/HJH/B59>). The protein level of CTGF showed a strong tendency towards increased values in the AB-wk12 group compared to the Sham-wk12 group ($P = 0.074$). Furthermore, in the AB-wk18 groups, the protein level of CTGF was significantly increased compared to the corresponding sham group (Fig. 4c).

In the early-debanded group, interstitial and perivascular myocardial fibrosis decreased compared to the age-matched aortic banding group. However, in the late-debanded group, interstitial and perivascular myocardial fibrosis remained increased, and it did not differ from the age-matched aortic banding group (Fig. 3a and c). Accordingly, the extent of both interstitial and perivascular myocardial fibrosis showed significantly higher levels in the late-debanded group compared to the early-debanded

group (Fig. 3b and d). In contrast, the increased mRNA/protein levels of CTGF effectively regressed in both debanded groups (Fig. 4a–d).

Effect of early and late debanding on hemodynamic parameters

Hemodynamic results obtained from pressure–volume analysis are summarized in Figs. 5 and 6, Tables 1 and 2 and Supplementary Table 2 (<http://links.lww.com/HJH/B59>).

Arterial loading and meridional wall stress

In the aortic banding groups, SBP, DBP, MAP, E_a and σ were increased compared to the age-matched sham groups (Table 1 and Fig. 5c). The extent of SBP, DBP, MAP and E_a

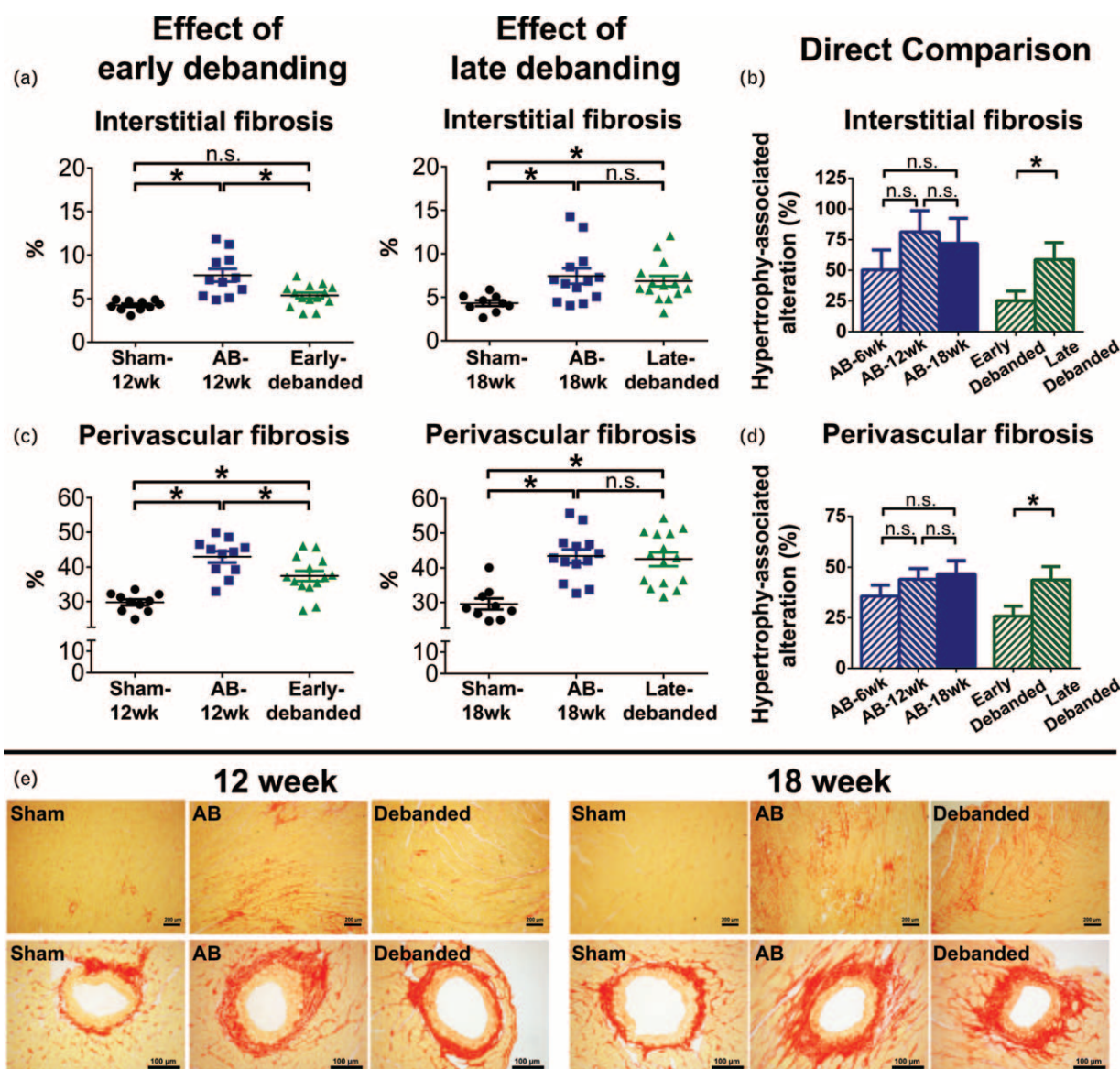


FIGURE 3 Effect of early and late debanding on myocardial fibrosis. (a and c) In the aortic banded (AB) groups increased interstitial and perivascular myocardial fibrosis (MF) was detected. In the early-debanded group, both interstitial and perivascular MF was significantly decreased when compared to the age-matched AB group. In contrast, in the late debanded group, interstitial and perivascular MF persisted, leading to significant differences between the early and the late-debanded groups (B). (e) Representative photomicrographs of picrosirius staining are shown demonstrating interstitial (magnification 50 $\times$, scale bar: 200 μ m) and perivascular (magnification 200 $\times$, scale bar: 100 μ m) MF. * $P < 0.05$.

increment was equal between the aortic banding groups at different time points (Table 2 and Fig. 5d). However, σ increased to a significantly greater degree in the AB-wk12 and AB-wk18 groups compared to the AB-wk6 group (Table 2).

In the early and late-debanded groups, SBP, DBP, MAP, E_a and σ were decreased compared to the age-matched aortic banding groups (Table 1 and Fig. 5c). The extent of afterload and wall stress reduction did not differ between the two debanded groups (Table 2 and Fig. 5d).

Load-dependent systolic parameters

In the AB-wk6 group, ejection fraction, SV and cardiac output (CO) did not differ from the age-matched sham group (Supplementary Table 2, <http://links.lww.com/HJH/B59>). In contrast, in the AB-wk12 and AB-wk18 groups,

ejection fraction was significantly reduced, whereas SV and CO showed a tendency towards decreased values when compared to their age-matched control groups (Table 1 and Fig. 6g).

Both in the early and in the late-debanded groups, ejection fraction, SV and CO were increased compared to the age-matched aortic banding groups (Table 1 and Fig. 6g). No differences were found regarding the extent of ejection fraction, SV and CO improvement between the early and late-debanded groups (Table 2 and Fig. 6h).

Load-independent systolic parameters

In the AB-wk6 group, ESPVR and PRSW were increased compared to the age-matched control group (Supplementary Table 2, <http://links.lww.com/HJH/B59>). In this particular group, the enhanced cardiac contractility effectively

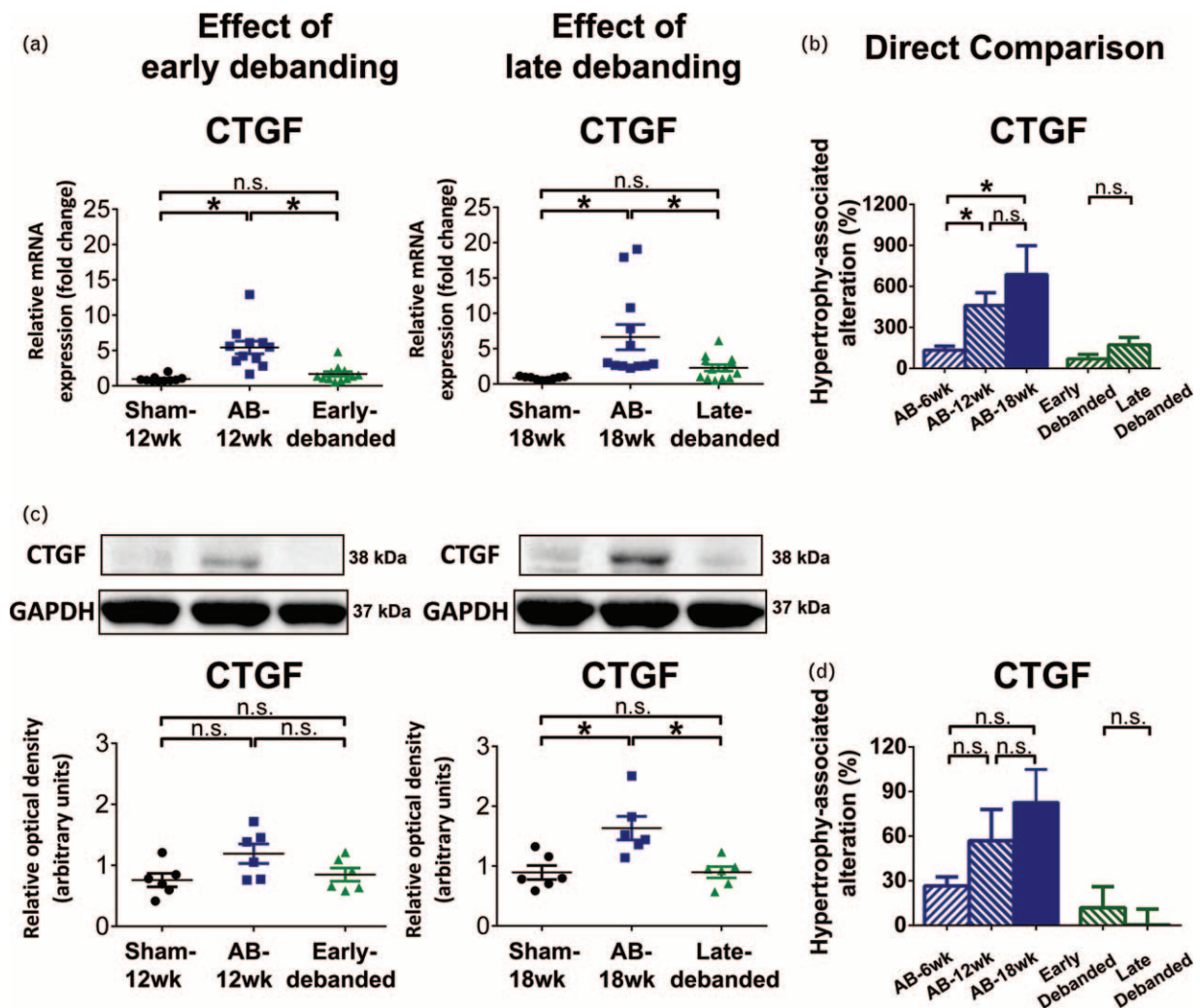


FIGURE 4 Effect of early and late debanding on connective tissue growth factor expression. In the aortic banded (AB) groups, increased mRNA levels of connective tissue growth factor (CTGF) were detected (a). The protein level of CTGF showed a strong tendency towards increased values in the AB-wk12 group compared to the age-matched sham group (c). Furthermore, in the AB-wk18 groups, the protein level of CTGF was significantly increased compared to the corresponding sham group (c). The enhanced expressional (mRNA and protein) level of CTGF was normalized in both the early and the late-debanded groups (a–d). * $P < 0.05$.

compensated the increased afterload (E_a) resulting in preserved VAC ratio (Supplementary Table 2, <http://links.lww.com/HJH/B59>). In contrast, in the AB-wk12 and AB-wk18 groups, ESPVR and PRSW did not differ from the control groups (Fig. 5a and e). Therefore, at these time points, increased E_a was not compensated by enhanced contractility, which led to impaired VAC (Fig. 5e). Both in the early and in the late-debanded groups, cardiac contractility and VAC were normalized to the age-matched sham groups' level (Figs. 5a–f, and 6e and f).

Diastolic parameters

Tau was prolonged in all the aortic banding groups compared to the corresponding control groups (Supplementary Table 2, <http://links.lww.com/HJH/B59> and Fig. 6a). Furthermore, the slope of EDPVR was increased in AB-wk12 and AB-wk18 groups when compared to the sham groups (Fig. 6c).

In the early-debanded group, the prolonged active relaxation was effectively shortened, and the values of Tau did not differ from that of healthy controls (Fig. 6a). In the late-debanded group, Tau was also decreased

compared to the age-matched aortic banding group. However, the extent of improvement was significantly smaller compared to the early-debanded group and the values of Tau still significantly differed from the control's level in the late-debanded group indicating maintained impairment of diastolic function (Fig. 6a and b).

Furthermore, in the early-debanded group, myocardial stiffness (reflected by EDPVR) was also substantially decreased when compared to the corresponding aortic banding group (Fig. 6c). In contrast, no significant differences could be observed in EDPVR between the late-debanded group and the AB-wk18 group (Fig. 6c). Therefore, the extent of improvement in myocardial stiffness was also significantly smaller in the late-debanded group than that observed in the early-debanded group (Fig. 6d).

DISCUSSION

The current study found that pressure unloading at both early and late-stage LVH led to left ventricular mass reduction and normalization of cardiomyocyte hypertrophy. In

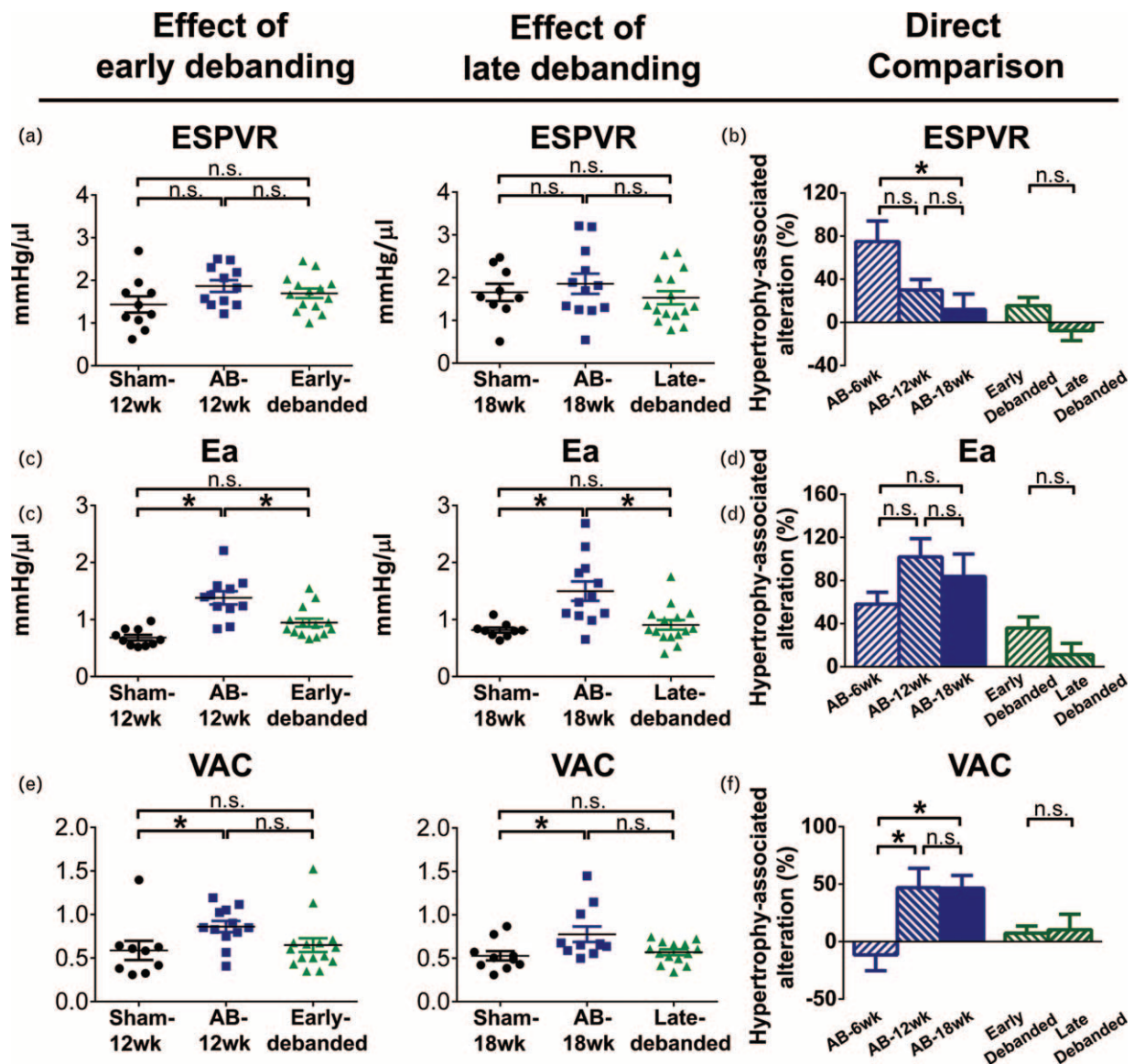


FIGURE 5 Effect of early and late debanding on ventriculo-arterial coupling. (a) Cardiac contractility (assessed as end-systolic pressure-volume relationship, ESPVR) was unchanged between the aortic banded (AB) and the debanded groups at week 12 and 18. (c) The integrative parameter of afterload, arterial elastance (E_a) was increased in the AB groups. Removing the aortic constriction at early and late time points equally normalized the increased E_a . (d) No differences could be detected in the extent of E_a reduction between the early and the late-debanded groups. (e) Ventriculo-arterial coupling (VAC) was significantly impaired in the AB groups at week 12 and 18 as well. In contrast, the afterload decreased in the early and late-debanded groups as well, and the values of E_a did not differ from the corresponding sham groups' level. (f) No differences could be observed in the extent of VAC improvement between the early and the late-debanded groups. * $P < 0.05$.

contrast, interstitial and perivascular myocardial fibrosis regressed to a greater extent when pressure unloading was performed at an earlier time point. The incomplete regression of LVH-associated structural abnormalities did not inhibit the improvement of VAC ratio and ejection fraction. However, a partial reverse remodeling process (reduced left ventricular mass, but persisting reactive myocardial fibrosis) mitigated the recovery of diastolic dysfunction after pressure unloading.

Recently, small animal models of aortic banding and debanding have been established [5,6,8,10,11,15,16]. These approaches provide unique opportunities to investigate the regression of LVH in ideal, homogenous population without the influence of any confounding variables such as different comorbidities, age or sex. A further advantage of

the aortic banding model is that pressure overload-induced structural (cardiomyocyte hypertrophy, myocardial fibrosis) and functional (diastolic and systolic dysfunction) alterations develop gradually. Therefore, by removing the aortic constriction at distinct time points, myocardial reverse remodeling from different stages of LVH can be investigated. In the present study, two particular stages of LVH were selected. At week 6, the aortic banding group was characterized by marked myocardial hypertrophy, relatively low level of interstitial myocardial fibrosis, preserved systolic function and impaired active relaxation. Overall, the parameters of the aortic banding group at week 6 met the criteria of an early-stage of pressure overload-induced LVH. On the contrary, in the aortic banding groups at week 12 and 18, we observed late-stage characteristics of LVH

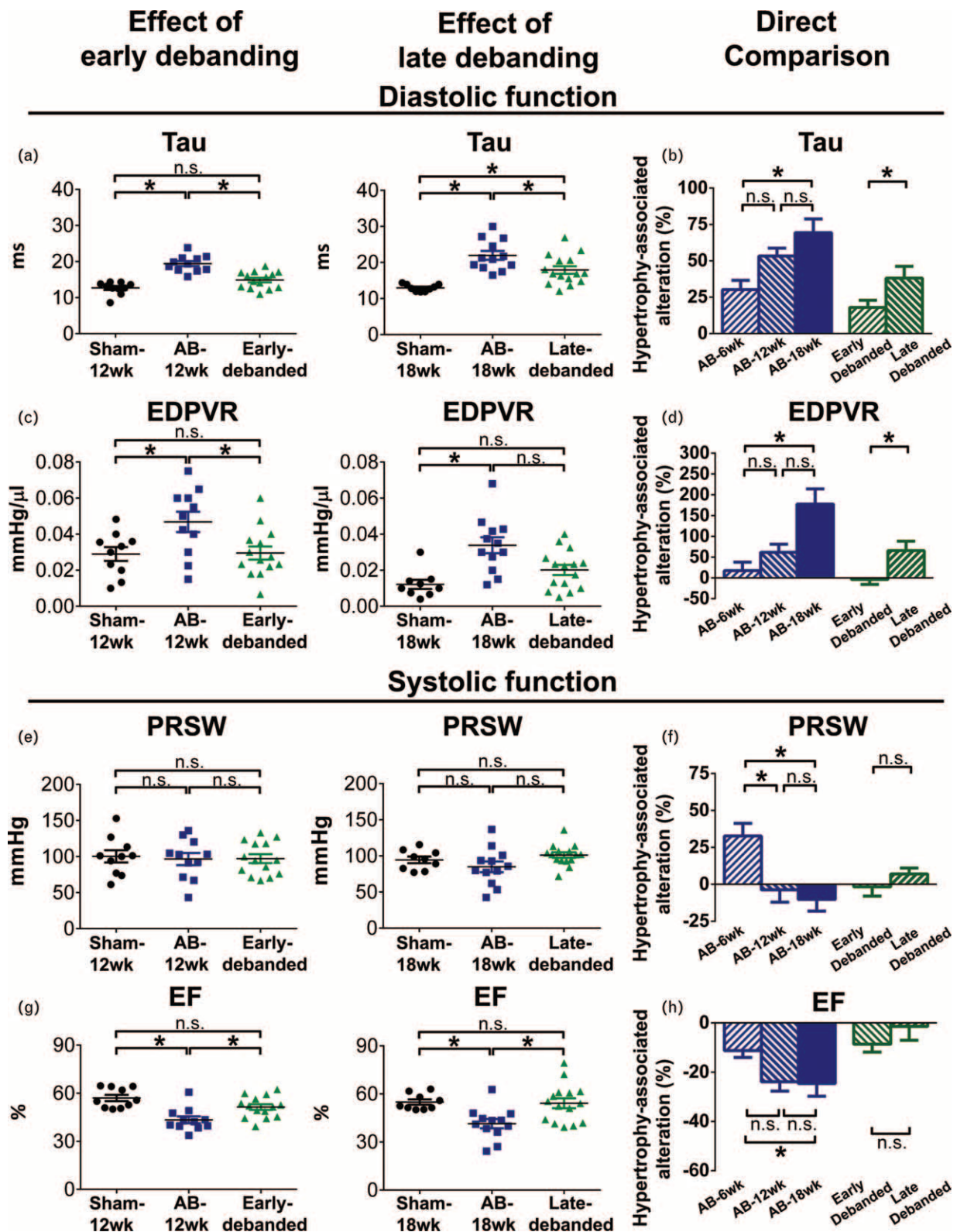


FIGURE 6 Effect of early and late debanding on diastolic and systolic LV function. (a) Prolongation of active relaxation time constant (Tau) was observed in the aortic banded (AB) groups at both time points. (c) Furthermore, in the AB-wk12 and AB-wk18 groups, increment in the slope of the end-diastolic pressure–volume relationship (EDPVR) revealed increased myocardial stiffness. In the early-debanded group, both aspects of diastolic function were normalized to the controls' level. In the late-debanded group, Tau was also decreased compared to the age-matched AB group. (a and b) However, the extent of improvement was significantly smaller compared to the early-debanded group and the values of Tau still significantly differed from the control's level in the late-debanded group. (c) Furthermore, no improvement could be detected in EDPVR in the late-debanded group when compared to the AB group. (e and f) Cardiac contractility (measured as preload recruitable stroke work, PRSW) was unchanged in the AB and in the debanded groups at weeks 12 and 18. (g) Ejection fraction (EF) was increased in the early- and the late-debanded groups as well, when compared to the age-matched AB groups. (h) No differences could be detected in the extent of improvement regarding EF between the early- and the late-debanded groups. * $P < 0.05$.

TABLE 1. Hemodynamic parameters in the aortic banded groups at different time points and in the early and the late-debanded groups

	12 weeks			18 weeks		
	Sham (n = 10)	AB (n = 11)	Early-debanded (n = 14)	Sham (n = 9)	AB (n = 12)	Late-debanded (n = 15)
SBP (mmHg)	139 ± 4	214 ± 5*	169 ± 3 ^{*,**}	152 ± 5	226 ± 5*	181 ± 4 ^{*,**}
DBP (mmHg)	110 ± 3	154 ± 5*	127 ± 3 ^{*,**}	121 ± 4	167 ± 4*	134 ± 2 ^{*,**}
MAP (mmHg)	120 ± 4	174 ± 4*	141 ± 3 ^{*,**}	132 ± 4	187 ± 4*	150 ± 3 ^{*,**}
σ (kdyn/cm ²)	13.9 ± 0.3	27.8 ± 1.6*	18.3 ± 1.1 ^{*,**}	18.9 ± 0.8	37.9 ± 2.0*	25.4 ± 1.5 ^{*,**}
HR (beats/min)	356 ± 6	365 ± 7	357 ± 7	381 ± 7.5	359 ± 4.7*	351 ± 5.1*
dP/dt _{max} (mmHg/s)	8291 ± 353	10579 ± 436*	9467 ± 483	9670 ± 359	11062 ± 505	9522 ± 267 ^{**}
dP/dt _{min} (mmHg/s)	−9220 ± 313	−9633 ± 277	−9887 ± 563	−10266 ± 328	−8838 ± 437*	−8907 ± 237*
LVEDP (mmHg)	4.0 ± 0.4	7.1 ± 0.9*	5.3 ± 0.7	5.4 ± 0.4	7.8 ± 1.0*	5.8 ± 0.4
LVESV (mmHg)	133 ± 3	202 ± 4*	161 ± 4 ^{*,**}	147 ± 5	209 ± 5*	168 ± 5 ^{*,**}
LVEDV (μl)	340 ± 19	357 ± 18	340 ± 16	329 ± 19	372 ± 19	362 ± 13
LVESV (μl)	148 ± 13	201 ± 11*	166 ± 10	150 ± 10	216 ± 12*	166 ± 13 ^{**}
SV (μl)	192 ± 10	156 ± 12	174 ± 10	178 ± 11	156 ± 17	196 ± 14
CO (ml/min)	68.6 ± 4.1	56.7 ± 4.3	61.4 ± 3.0	68.3 ± 4.4	55.9 ± 5.8	68.8 ± 5.0

Values are expressed as mean ± standard error of the mean.

σ , meridional wall stress; AB, aortic banding; CO, cardiac output; DBP, diastolic blood pressure; dP/dt_{max}, maximal slope of systolic pressure increment; dP/dt_{min}, maximal slope of diastolic pressure decrement; HR, heart rate; LVEDP, left ventricular (LV) end-diastolic pressure; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic pressure; LVESV, LV end-systolic volume; MAP, mean arterial pressure; SBP, systolic blood pressure; SV, stroke volume.

*P < 0.05 vs. age-matched Sham.

**P < 0.05 vs. age-matched AB.

with robust myocardial hypertrophy, severe interstitial myocardial fibrosis, impaired systolic function, prolonged active relaxation and increased myocardial stiffness. Thus, in the present investigation, debanding was performed at week 6 and week 12 to study myocardial reverse remodeling from early and late stages of LVH.

Myocardial hypertrophy

The majority of clinical and experimental studies have shown that cardiomyocyte hypertrophy remains reversible even after a long period of sustained pressure overload [5,12,28,29]. In concordance with earlier findings, the present study also found that removal of the aortic constriction led to massive regression of myocardial hypertrophy from early and late-stage LVH. No differences could be found in the extent of hypertrophy regression between the early and the late-debanded groups either on macroscopic (left ventricular mass, AWTd, PWTd, heart weight/tibial length), microscopic (cardiomyocyte diameter) or gene expression

level (β/α -MHC ratio). The comparable degree of hypertrophy regression in the early and late-debanded group could be explained by the fact that the same degree of afterload (Δ SBP, Δ DBP, Δ MAP, ΔE_a) and meridional wall stress ($\Delta\sigma$) reduction was achieved in both cases.

Myocardial fibrosis

The regression of myocardial fibrosis after pressure unloading is generally considered to be a less efficient process compared to the regression of cardiomyocyte hypertrophy [5,9,12]. Importantly, myocardial fibrosis can be subcategorized into reactive (including perivascular and interstitial myocardial fibrosis) and replacement myocardial fibrosis. These two forms of myocardial fibrosis differ in their plasticity. Weidemann *et al.* [9] reported that the latter type (replacement fibrosis) shows no reversibility after AVR. In contrast, it has been demonstrated that interstitial myocardial fibrosis is plastic and able to regress in the long term [1]. However, the first histopathological evidence for the

TABLE 2. Comparison of pressure overload-induced alterations in the aortic banded groups at different time points and during myocardial reverse remodeling after early and late-debanning

	AB			Debanded		P value
	6 weeks (n = 10)	12 weeks (n = 11)	18 weeks (n = 12)	Early (n = 14)	Late (n = 15)	
Δ SBP (%)	50 ± 3	55 ± 4	49 ± 3	21 ± 2	19 ± 3	0.53
Δ DBP (%)	32 ± 2	39 ± 4	38 ± 3	14 ± 3	11 ± 2	0.38
Δ MAP (%)	39 ± 2	45 ± 4	42 ± 3	17 ± 3	14 ± 2	0.40
$\Delta\sigma$ (%)	33 ± 11	100 ± 12*	101 ± 11*	31 ± 8	36 ± 8	0.65
Δ LVEDP (%)	51 ± 21	79 ± 23	46 ± 19	31 ± 16	9 ± 7	0.21
Δ LVESV (%)	52 ± 4	53 ± 3	43 ± 3	22 ± 3	15 ± 3	0.10
Δ LVEDV (%)	7 ± 6	5 ± 5	13 ± 6	1 ± 5	10 ± 4	0.13
Δ LVESV (%)	23 ± 8	35 ± 7	44 ± 8	11 ± 7	11 ± 8	0.98
Δ SV (%)	−5 ± 6	−19 ± 6	−13 ± 9	−8 ± 5	10 ± 8	0.07
Δ CO (%)	−4 ± 4	−17 ± 6	−18 ± 9	−8 ± 5	1 ± 7	0.31

The values of the aortic banded (AB) and the debanded groups were normalized to the corresponding sham groups. These calculated values are expressed as mean ± standard error of the mean.

CO, cardiac output; DBP, diastolic blood pressure; E_a , arterial elastance, σ , meridional wall stress, LVEDP, left ventricular (LV) end-diastolic pressure; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic pressure; LVESV, LV end-systolic volume; MAP, mean arterial pressure; SBP, systolic arterial blood pressure; SV, stroke volume.

*P < 0.05 vs. AB-week 6.

resorption of interstitial collagen fibers is often observed only years after AVR [2,3,12,13]. In the present study, removing the aortic constriction at week 6 (when LVH was associated with relatively low level of interstitial myocardial fibrosis) efficiently attenuated the accumulation of reactive myocardial fibrosis in the myocardium during the 6-week-long observation period. These results are in agreement with our previous findings using this model [15,19]. On the contrary, removal of the aortic constriction at week 12 (when LVH was associated with severe interstitial myocardial fibrosis) did not lead to the regression of reactive myocardial fibrosis during the 6-week-long follow-up. In line with our findings, in a previous preclinical study with a similar experimental approach (comparing early and late debanding), normalization of perivascular collagen content also occurred to a greater extent in the early-debanded group compared to the late-debanded group [29]. Surprisingly, CTGF, which is one of the master regulators of cardiac fibrosis, showed decreased mRNA and protein expression in the late-debanded group. This is of particular interest, as it has been previously demonstrated that inhibition of CTGF expression can induce the regression of pre-established tissue fibrosis [30]. Thus, the observed robust difference on the molecular level may indicate that in case of a longer follow-up period, regression of reactive myocardial fibrosis would also have occurred in the late-debanded group.

Systolic function

Removal of the chronic pressure overload often induces a rapid improvement in systolic function even before the normalization of left ventricular structural abnormalities (especially fibrosis) is completed [14]. This phenomenon can be underpinned by the fact that at certain stages of pressure overload-induced LVH, the increased arterial loading condition (due to aortic stenosis or arterial hypertension) and not the depressed left ventricular contractility is primarily responsible for the contractility–afterload mismatch and thus for the reduced systolic function [31]. Therefore, it can be hypothesized that in these cases, the acute relief of afterload can lead to sudden normalization of VAC and thereby increment in ejection performance. In the present study, pressure–volume analysis revealed that at early stage of LVH, augmentation in left ventricular contractility (shown by the load-independent indices ESPVR, PRSW) counterbalanced the increased afterload (E_a). Thus, the AB-wk6 group was associated with preserved VAC and maintained ejection fraction. In contrast, at later stages of LVH (week 12 and week 18), the progression of pressure overload-induced structural and molecular alterations diminished the compensatory increment in left ventricular contractility. Therefore, in the AB-wk12 and AB-wk18 groups impaired VAC and reduced ejection fraction were detected. However, it is important to highlight that even in these groups the increased arterial afterload (E_a) and not the depressed left ventricular contractility was the source of the observed contractility–afterload mismatch. In correspondence, reduction in E_a after pressure unloading successfully normalized the VAC ratio in the late-debanded group similarly to the early-debanded group.

Diastolic function

Contrary to systolic dysfunction, amelioration of diastolic dysfunction occurs in a slow fashion and usually continues over several years following AVR [2,3]. Interestingly, clinical experience suggests that the two aspects of diastolic function, namely active relaxation and passive filling, differ in their reversibility after pressure unloading [2].

Impairment of active ventricular relaxation is a common and early feature of pressure overload-induced LVH [25,27]. Importantly, it has been demonstrated that an acute increase in systolic peak pressure is sufficient to prolong the active relaxation time constant (Tau) independently from the development of LVH [32]. Furthermore, Ikonomidis *et al.* [33] showed that patients undergoing AVR experienced a significant improvement in myocardial relaxation early after surgery, at a time point where regression of left ventricular wall thicknesses had not yet occurred. The findings of acute prolongation of Tau in response to increased hemodynamic overload, and its fast regression after pressure unloading, together indicate that active relaxation is extremely sensitive to the alterations in afterload. In accordance, we also found that termination of the sustained pressure overload by removing the aortic constriction effectively decreased the prolonged Tau in both debanded groups. However, the extent of Tau improvement proved to be inferior in the late-debanded group compared to the early-debanded group. This can partly be explained by the higher persistent stretch on the single cardiomyocytes in the late-debanded group, which was reflected by increased ANP expression in our investigation [34]. The enhanced tension at the myofibrillar level might have originated from ultrastructural differences between the early and the late-debanded groups, because at the ventricular level, meridional wall stress was reduced to the same degree after early and late debanding. In accordance with our findings, prior investigations identified persistent interstitial myocardial fibrosis and incomplete normalization of cardiomyocyte enlargement as potential sources of increased myofibrillar tension following AVR [12]. In our study, cardiomyocyte diameter decreased to the same extent in the early and the late-debanded groups. Therefore, it can be inferred that the persistently elevated interstitial myocardial fibrosis was mainly responsible for the increased tension on the single cardiomyocyte level and thereby for the incomplete normalization of impaired active relaxation in the late-debanded group. Furthermore, it has been suggested that severe perivascular myocardial fibrosis can reduce the oxygen supply of the cardiomyocytes [35], thereby decreasing the energy production of the myocardium. Considering the fact that active relaxation is an ATP-dependent process, an indirect connection might indeed exist between increased perivascular fibrosis and impaired ventricular relaxation in the hypertrophied LV. Correspondingly, in the late-debanded group, the persistent perivascular fibrosis could have also contributed to the ineffective normalization of prolonged active relaxation in our investigation.

The second part of diastole, passive filling, is predominantly determined by the structural properties of the LV. In case of pressure overload-induced LVH, enhanced interstitial collagen accumulation is one of the most important

ultrastructural alterations that increase the stiffness of the LV. Therefore, it is not surprising that previous investigations observed improvement in myocardial compliance only years after AVR, when regression of interstitial myocardial fibrosis had already occurred [2,3]. In our study, pressure–volume derived EDPVR values also followed the alterations in interstitial myocardial fibrosis after pressure unloading. Accordingly, in the early-debanded group, the relatively low level of myocardial fibrosis was associated with normalized EDPVR values. However, the debanding surgery at the later time point failed to reduce the extent of interstitial myocardial fibrosis and in parallel could not improve the increased passive stiffness during the 6-week long follow-up period. Therefore, our hemodynamic data were in accordance with previous clinical findings, demonstrating that regression of interstitial myocardial fibrosis is inevitable for the normalization of the increased myocardial stiffness.

In conclusion, the present study provided the first experimental evidence that pressure unloading from both early and late stages of LVH effectively reduces the increased left ventricular mass and cardiomyocyte hypertrophy. However, removing the aortic constriction at a later time point does not induce the regression of interstitial and perivascular myocardial fibrosis during a relatively short follow-up period. The incomplete regression of LVH-associated structural alterations does not inhibit the improvement of systolic function, as the reduction in afterload proves to be sufficient to harmonize the relation (VAC) between the contractile status of the LV (ESVPR) and the arterial system (E_a). In contrast, the persisting myocardial fibrosis impairs both aspects of diastole (active relaxation and myocardial stiffness) after pressure unloading.

Limitations

The interpretation of results from the present study is limited to young male rats. Therefore, further experimental studies are required to investigate whether age and/or female sex affects the process of reverse remodeling under standard laboratory conditions.

Moreover, it has to be noted that a relatively short postdebanding observational period (6-week-long) was utilized in the current study. Hence, it is important to further investigate whether myocardial fibrosis (both interstitial and perivascular) and diastolic dysfunction would also regress in case of a longer follow-up period in the late-debanded group.

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Conflicts of interest

There are no conflicts of interest.

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