Altered proteasome function in right ventricular hypertrophy

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ONLINE SUPPLEMENT MATERIAL

Material and methods

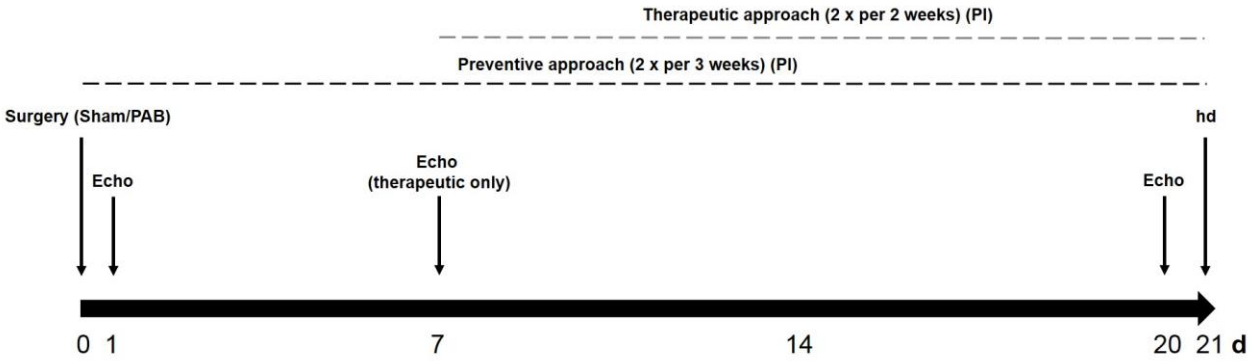
In vivo studies

Pathogen-free male C57/Bl6N mice, 20-25g, 12 weeks, by Charles River, were housed in the local animal facility five days before the start of experiments. Pulmonary artery banding (PAB) was performed to provoke right ventricular hypertrophy (RVH) by reducing the cross-sectional area (CSA) of the artery to 0.3 mm (about 66% of original CSA). During thoracotomy a titanium clip was placed around the pulmonary trunk as described previously[1–5]. The mice received either six (preventive approach) or four (therapeutic approach) drug treatments during the next three weeks (Supplementary figure 1). Placebo-treated animals served as sick control group (*PAB*). Sham surgery was performed the same way as described for PAB surgery without placing a titanium clip. These mice served as healthy control group (sham). All mice were checked daily according to a score sheet with special focus on the development of clinical signs for right heart failure (inactivity, ruffled fur, dyspnoea, ascites)[6].

One day before and three weeks after surgery echocardiography was performed as described previously[2,3,7–10]. During the therapeutic approach, drug administration only started when hypertrophy had already developed as determined by an additional echocardiography one week after PAB surgery. The images were acquired with a VEVO2100 high resolution imaging system (Visual Sonics, Toronto, Canada) using a 40 MHz MicroScan linear array transducer MS550D. Calculations were performed offline with the according software Vevo LAB. The following parameters were derived: internal diameter of the right ventricle (RVID, [mm]), wall thickness of its free wall (RVWT, [mm]), tricuspid annular plane systolic excursion (TAPSE, [mm]) and

cardiac index (CI, $[l/(min*g)]$). Hemodynamic measurements via Millar-catheter were performed three weeks after PAB surgery in a previously reported manner[4,11,12]. Subsequently the mice were sacrificed; tibia, lung, heart, liver, blood and pulmonary artery were harvested and RV mass to tibia length (RV/TL, $[g/mm*10^{-4}]$) was determined.

A



Bortezomib ip 0.5 mg/kg ONX po 50 mg/kg

Supplementary figure 1: Scheme for the preventive and therapeutic approaches

A: Each preventive and therapeutic approach took three weeks during which pulmonary artery banding (**PAB**) or sham surgery, two (preventive approach) or three (therapeutic approach) echocardiographies (**echo**), and hemodynamic measurements (**hd**) were performed. During the preventive approach, the mice received six treatments with the proteasome inhibitors (**PI**) Bortezomib or ONX-0912. In the therapeutic approach, the treatment started after right ventricular hypertrophy (RVH) had already developed.

d day, **ip** intraperitoneal, **po** oral application.

Proteasome inhibitors and solvent controls

Bortezomib (BTZ, Velcade by Millennium, Cambridge, USA), a class-III proteasome inhibitor[13], was diluted in isotonic saline solution in a concentration of 0.5 mg/kg bodyweight (BW). Mannitol was mixed in a 0.9 fold amount of original BTZ sample weight in saline and served as placebo. Both compounds were applied intraperitoneally (IP). ONX-0912 (ONX, Onyx Pharmaceuticals, South San Francisco, USA) is an epoxyketone and a successor to carfilzomib[14]. ONX was suspended in 1% carboxyl methylcellulose (CMC) at a concentration of 50 mg/kg BW. Furthermore, CMC served as a vehicle for placebo treatment and was administered at 1% orally. The different administration routes (IP or orally) and solvents (mannitol solution or CMC) made it necessary to obtain a healthy (sham) and sick (PAB) control group for each BTZ and ONX treatment.

Histology and morphometry

After fixating the tissue[7], different staining were performed on paraffin-embedded RV slices of 3 µm. Staining with Picrosirius Red determined collagen (red, [%]), a marker for fibrosis, as previously described[4,7,9,11,12]. In this staining, collagen is stained red whereas the other tissue appears yellow. The area of stained interstitial collagen was measured with the help of Leica QWin software, a well-established morphometric method in our research group[4,7,9,11,12]. At least twenty fields of view per slide were analysed meander-like at 400× magnification. Analysis was performed blinded. Collagen content per RV was calculated as the mean of all measured images.

To assess the cross-sectional area of cardiomyocytes (CM CSA, [µm]), a parameter for hypertrophy, RV sections were stained with wheat germ agglutinin (WGA),

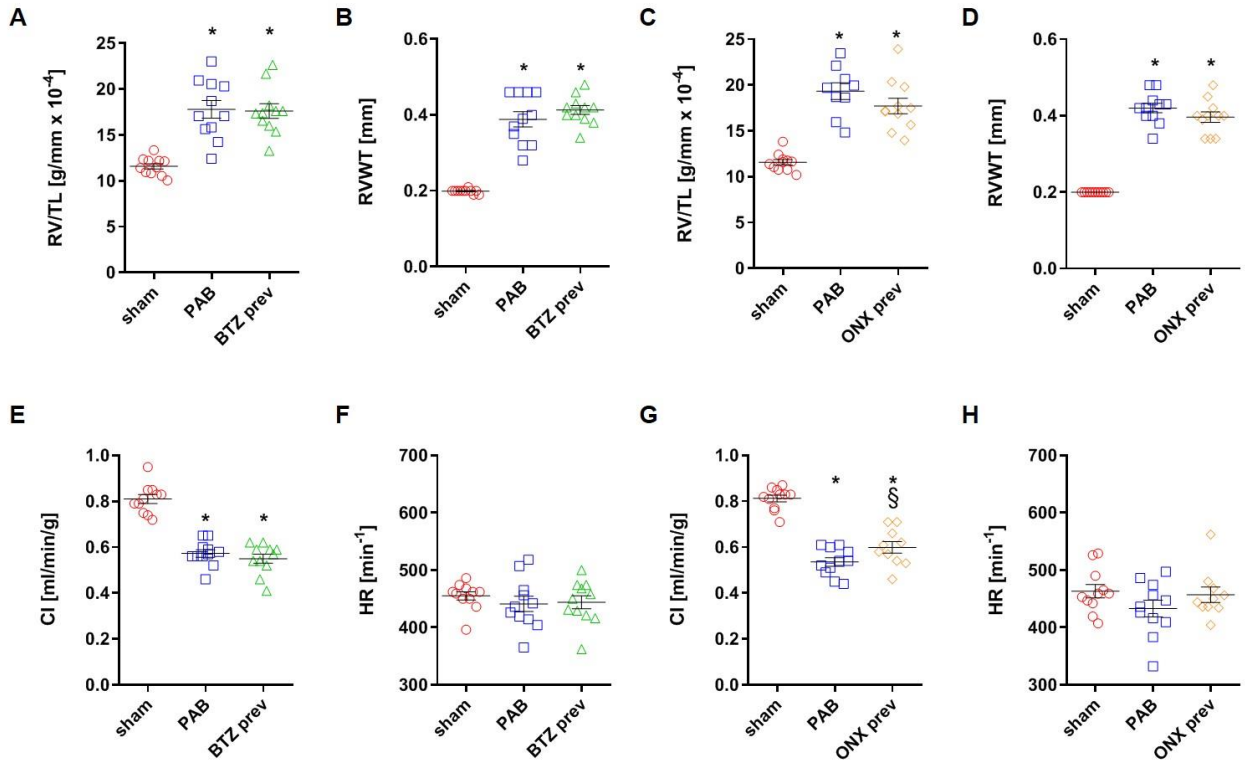
fluorescein isothiocyanate (FITC) and 4′,6-diamidino-2-phenylindole (DAPI). This staining is well established at our institute as reported before[2,3,7]. CSA of at least 100 cardiomyocytes was measured with the help of Leica QWin software before calculating the mean CSA per RV. IHC-staining for Rpn6 and tropomyosin were performed according to a previously described protocol[15]. Each analysis was performed in a blinded manner. Semi-quantitative analysis of Rpn6 expression in all human RV samples was performed using the scoring system: score **0** was given when there was no expression, and the following scores were given when the stained signal occupied from 1-30% (**2**), 31-60% (**4**) and more than 60% (**6**) of the tissue in the given field.

Protein and mRNA analysis

Protein biochemistry and mRNA analysis was performed as described elsewhere[15]. Tissue was lysed in TSDG buffer containing 0.2% detergent (NP-40). Tissue was homogenized and protein concentration was determined using the Pierce bicinchoninic acid assay. To determine the activity of the catalytic subunits of the proteasome, the Proteasome-Glo Assay kit (Promega, Fitchburg, USA) and the activity based probe MV151 were used as described before[16]. In-gel proteasome activity and protein bands were quantified using the volume tool of Image Lab software and normalised to sham mice.

For quantitative analyses of the mRNA level via real time quantitative polymerase chain reaction (qRT-PCR), the SYBR Green LC480 System was used. The expression of the target genes were normalised to the 60S ribosomal protein L19 (RPL19) as a housekeeping gene.

Results



Supplementary figure 2: Right ventricular structure and function upon preventive proteasome inhibition

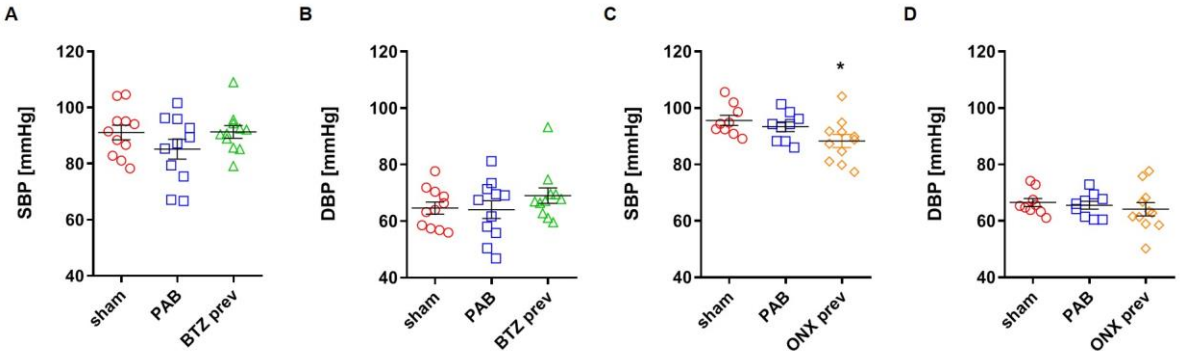
A, C: Three weeks after pulmonary artery banding (PAB), the mass of the right ventricle (**RV/TL**) increased in PAB groups (**PAB**, n=11), indicating the development of right ventricular hypertrophy. Preventive proteasome inhibition by Bortezomib (**BTZprev**, n=11) or ONX (**ONXprev**, n=11) did not influence the mass. **TL** tibia length

B, D: RV wall thickness (**RVWT**) was not influenced by proteasome inhibition.

E-F: Preventive proteasome inhibition by Bortezomib (BTZprev) altered neither cardiac index (**CI**) nor heart rate (**HR**) when compared to the placebo-treated control group PAB.

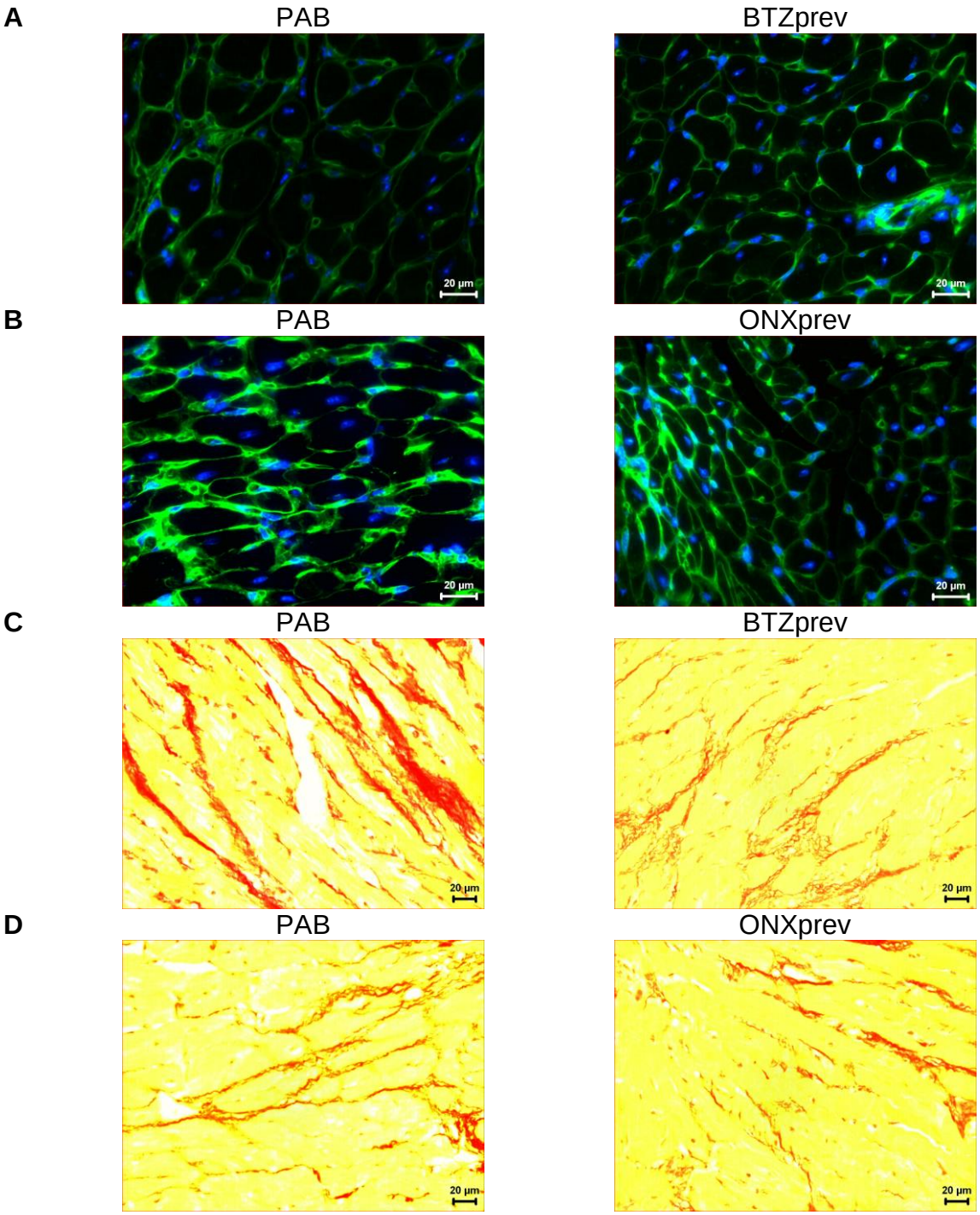
G-H: Preventive proteasome inhibition by ONX (ONXprev) improved CI significantly while HR remained constant in comparison to PAB. §Significance compared to

placebo-treated PAB group, *significance compared to healthy control group (**sham**, n=11); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 3: Hemodynamic parameters upon preventive proteasome inhibition

Three weeks after pulmonary artery banding (PAB), hemodynamic measurements were derived by right heart catheterization in mice. Preventive treatment with proteasome inhibitors (**A, B:** BTZprev, **C, D:** ONXprev n=9-11) did not change the systemic blood pressure (**SBP, DBP**) in comparison to placebo-treated PAB controls (**PAB**, n=9-11). *Significance compared to healthy control group (**sham**, n=11); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

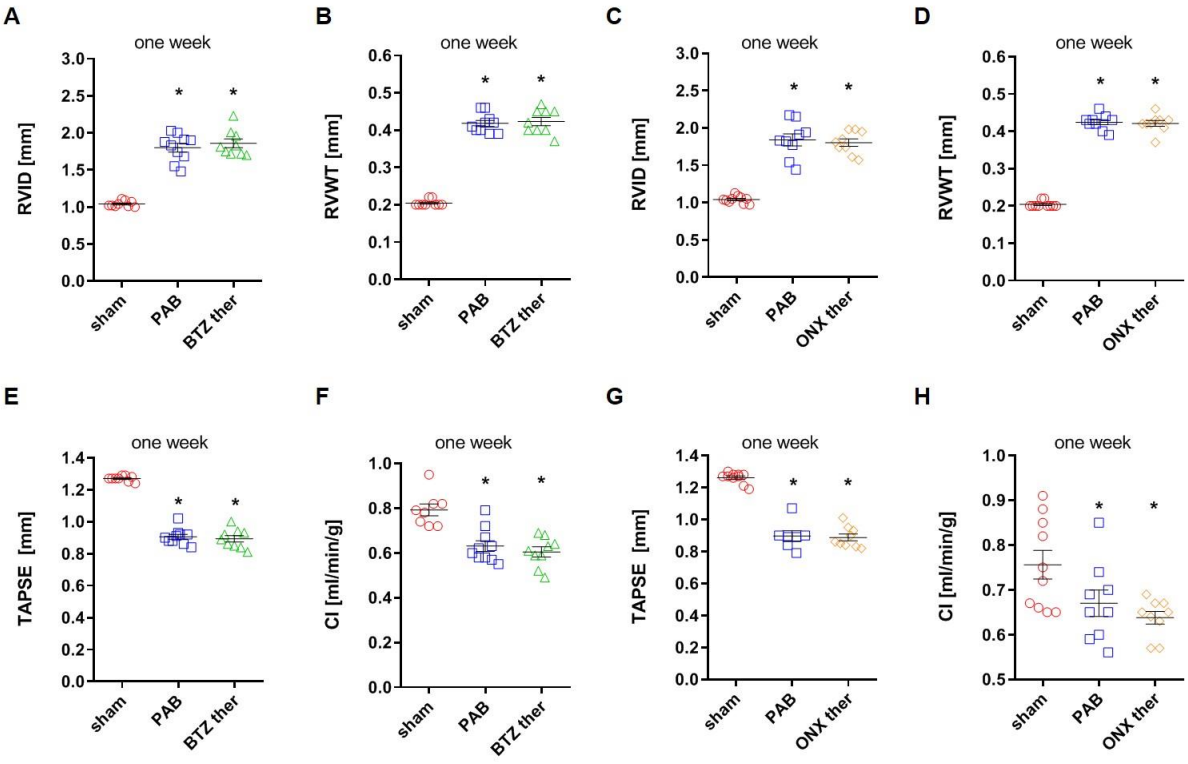


Supplementary figure 4: Effects of preventive treatment on cardiomyocyte size and collagen content

A, B: Three weeks after pulmonary artery banding (PAB), murine right ventricles (RV) were stained with WGA (wheat germ agglutinin), FITC (fluorescein isothiocyanate) and DAPI (4′,6-diamidino-2-phenylindole) to assess the size of cardiomyocytes (green).

Preventive proteasome inhibition with Bortezomib (**BTZprev**, n=9-11), but not with ONX (**ONXprev**, n=9-11), led to a decrease in cardiomyocyte cross-sectional area (**CM CSA**) in comparison to placebo-treated PAB controls (**PAB**, n=9-11).

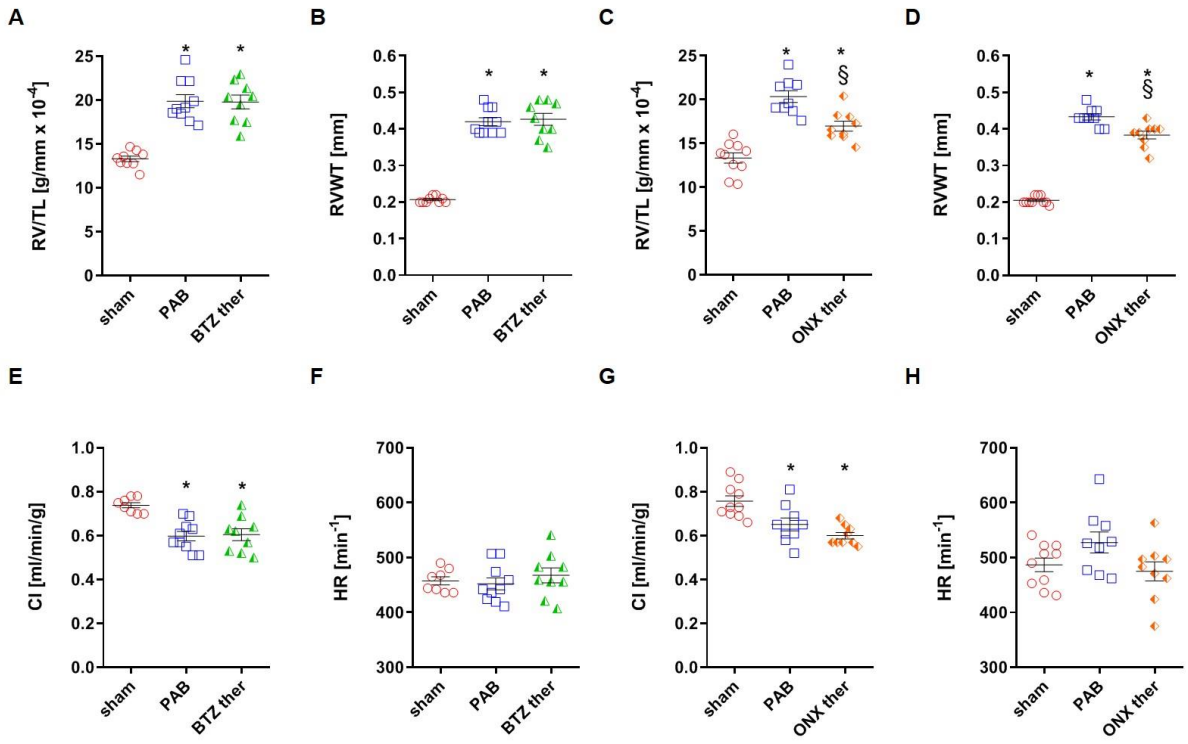
C, D: Staining with Sirius red for collagen detection (red) revealed a slight decrease of the collagen content in RV of mice that had been treated with proteasome inhibitors. Scale bars = 20 µm.



Supplementary figure 5: Development of RVH one week after PAB

A-D: During the therapeutic approach, echocardiography was performed one week after pulmonary artery banding (PAB), but before treatment started. Operated mice (**PAB**, **BTZther**, **ONXther**, n=7-10) had developed right ventricular hypertrophy (**RVID**, **RVWT**) compared to the healthy control group (**sham**, n=7-10).

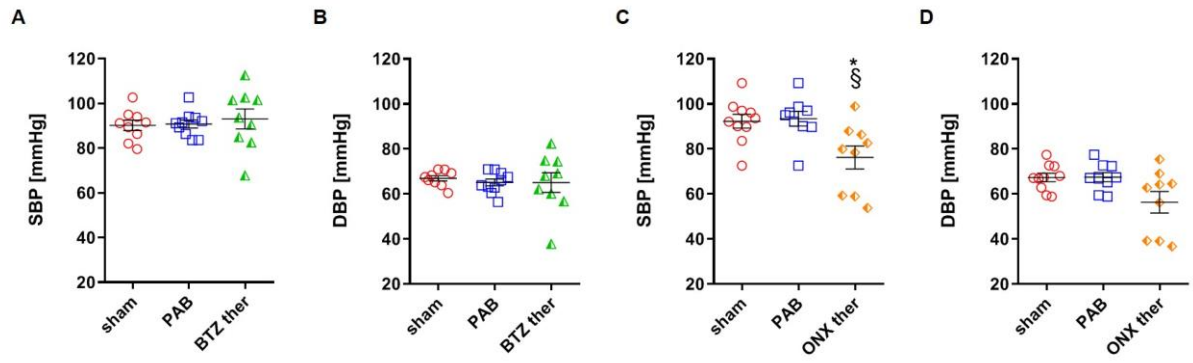
E-H: Furthermore, there was a decrease in (right) heart function (**TAPSE**, **CI**) in mice with PAB (**PAB**, **BTZther**, **ONXther**, n=7-10). The heart rate remained constant (data not shown). Afterwards, treatment with the proteasome inhibitors Bortezomib (BTZ) or ONX started. **RVID** right ventricular internal diameter, **RVWT** right ventricular wall thickness, **TAPSE** tricuspid annular plane systolic excursion, **CI** cardiac index. *Significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 6: Right ventricle structure and function upon therapeutic approach

A-D: Three weeks after pulmonary artery banding (**PAB**), the mass of the right ventricle in relation to tibia length (**RV/TL**) and its free wall (**RVWT**) increased in PAB groups (**PAB**, n=9-11), indicating the development of right ventricular hypertrophy. Therapeutic proteasome inhibition by ONX (**ONXther**, n=9-11) significantly reduced RV/TL and RVWT.

E-H: Therapeutic proteasome inhibition by Bortezomib (**BTZther**) and ONX (**ONXther**) altered neither cardiac index (**CI**) nor heart rate (**HR**) when compared to the placebo-treated control group PAB. **ther** therapeutic administration of drugs. §Significance compared to placebo-treated control group (**PAB,** n=8-10), *significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

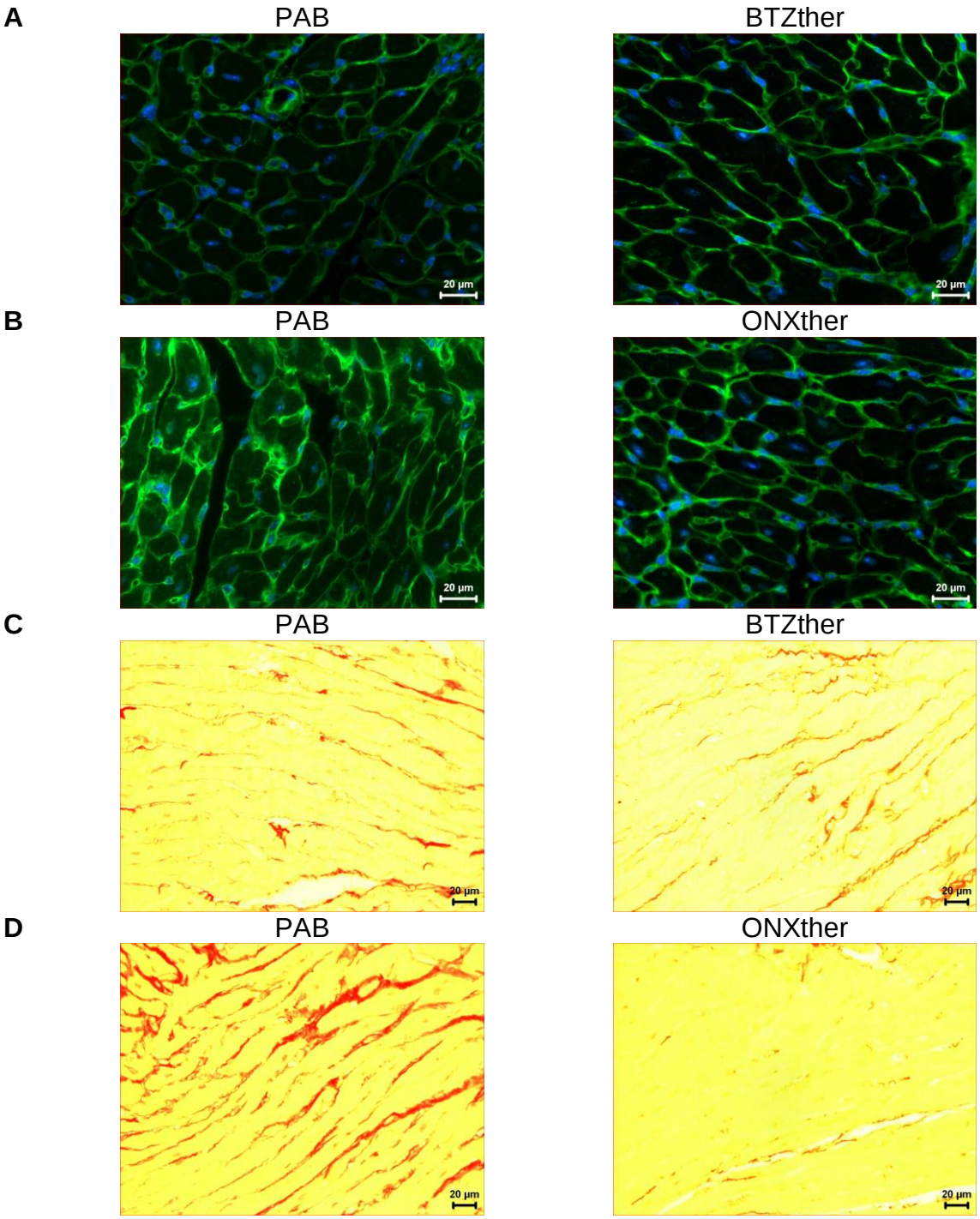


Supplementary figure 7: Hemodynamic parameters upon therapeutic proteasome inhibition

Three weeks after pulmonary artery banding (PAB), hemodynamic measurements were derived by right heart catheterization.

A, B: Therapeutic treatment of mice with the proteasome inhibitor Bortezomib (**BTZther**, n=8-10) changed neither systolic nor diastolic blood pressure (**SBP, DBP**) compared to placebo-treated PAB controls (**PAB**, n=8-10).

C, D: Mice that had received the proteasome inhibitor ONX (**ONXther**, n=8-10) showed a decrease in systolic blood pressure (SBP). §Significance compared to placebo-treated control group (**PAB,** n=8-10), *significance compared to healthy control group (sham); One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

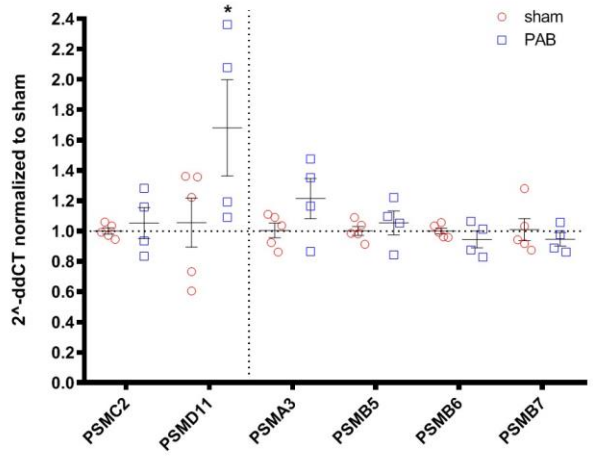


Supplementary figure 8: Effects on cardiomyocyte size and collagen content by therapeutic proteasome inhibition

A, B: Three weeks after pulmonary artery banding (PAB), murine right ventricles (RV) were stained with WGA (wheat germ agglutinin), FITC (fluorescein isothiocyanate) and DAPI (4′,6-diamidino-2-phenylindole) to assess the size of cardiomyocytes (green). Therapeutic proteasome inhibition with ONX (**ONXther**, n=8-10) led to a significant decrease in cardiomyocyte cross-sectional area (**CM CSA**) compared to

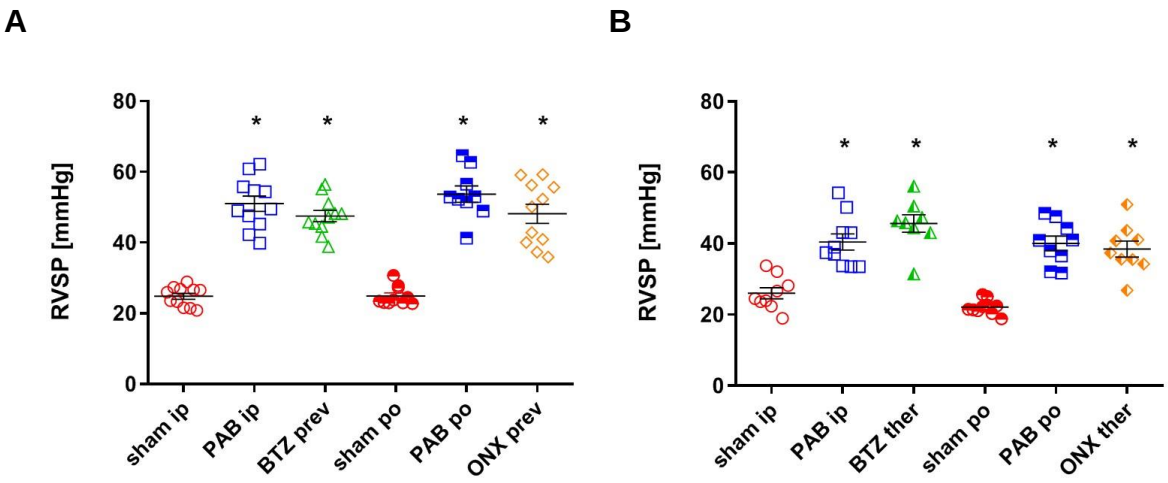
placebo-treated PAB controls (**PAB**, n=8-10). This did not apply for the treatment with proteasome inhibitor BTZ (**BTZther**, n=8-10).

C, D: Staining with Sirius red for collagen detection (red) revealed a significant decrease of collagen content in RV that had been treated therapeutically with ONX (**ONXther**). Scale bars = 20 µm.



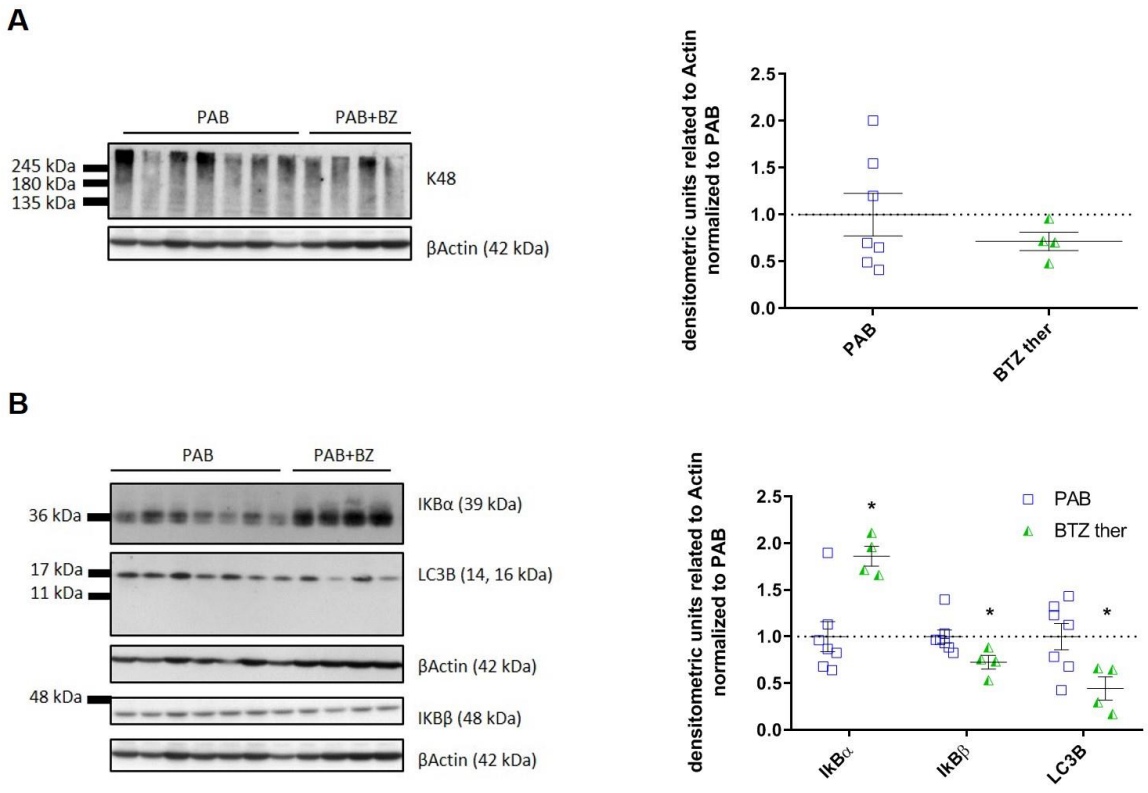
Supplementary figure 9: mRNA expression of the Rpn6 encoding gene PSMD11 increased in RVH

qRT-PCR analysis showed no alterations in mRNA expression of proteasomal subunits in RVH except for PSMD11; PSMD11 encodes for Rpn6, a subunit of the 19S proteasome which is involved in the assembly of the 26S proteasome. **PSMC2** gene name for Rpt1; **PSMA3** gene that encodes subunit α3; **PSMB5**, **PSMB6**, **PSMB7** encode subunit β5, β6 and β7, respectively. *****Significance compared to control group (sham); Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/- SEM.



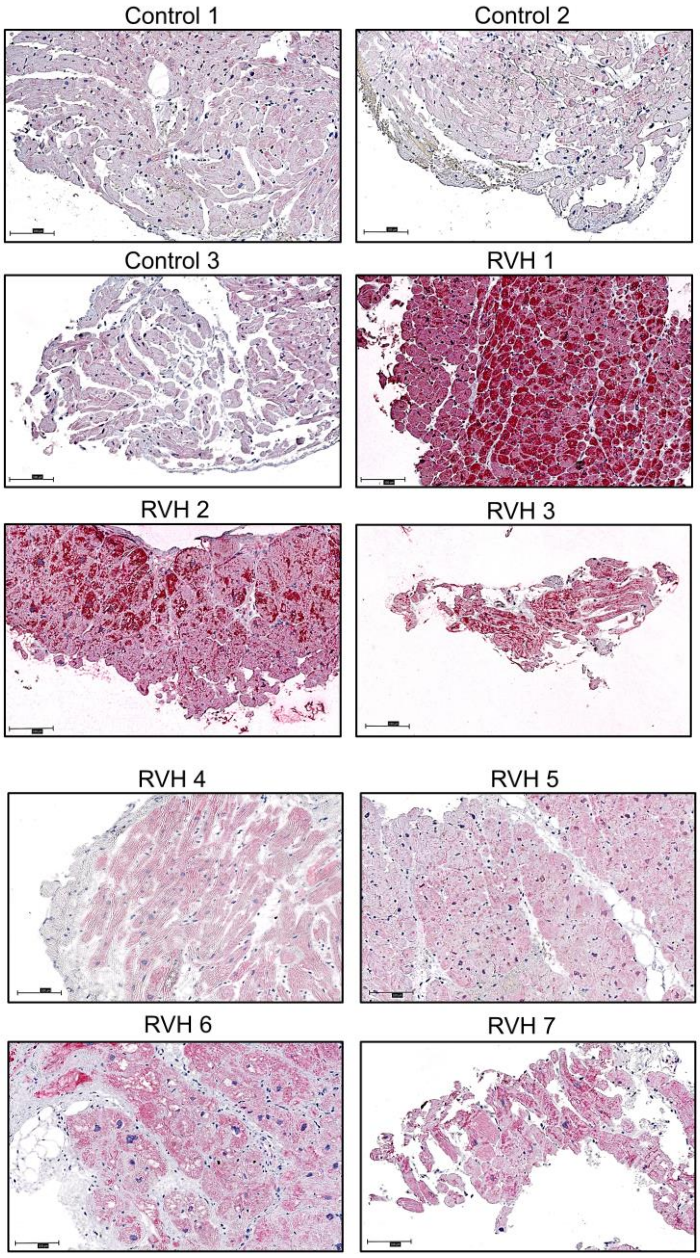
Supplementary figure 10: Right ventricular systolic blood pressure (RVSP)

Hemodynamic measurements revealed a significant difference of the PAB groups (PAB ip, PAB po) and the treated groups (BTZprev / ther, ONXprev / ther) in comparison to the healthy control (sham). **Ip** intraperitoneal injection, **po** per os; n=8-11, One-way ANOVA with Newman-Keuls-test ($p \leq 0.05$). Results are presented as mean+/-SEM.

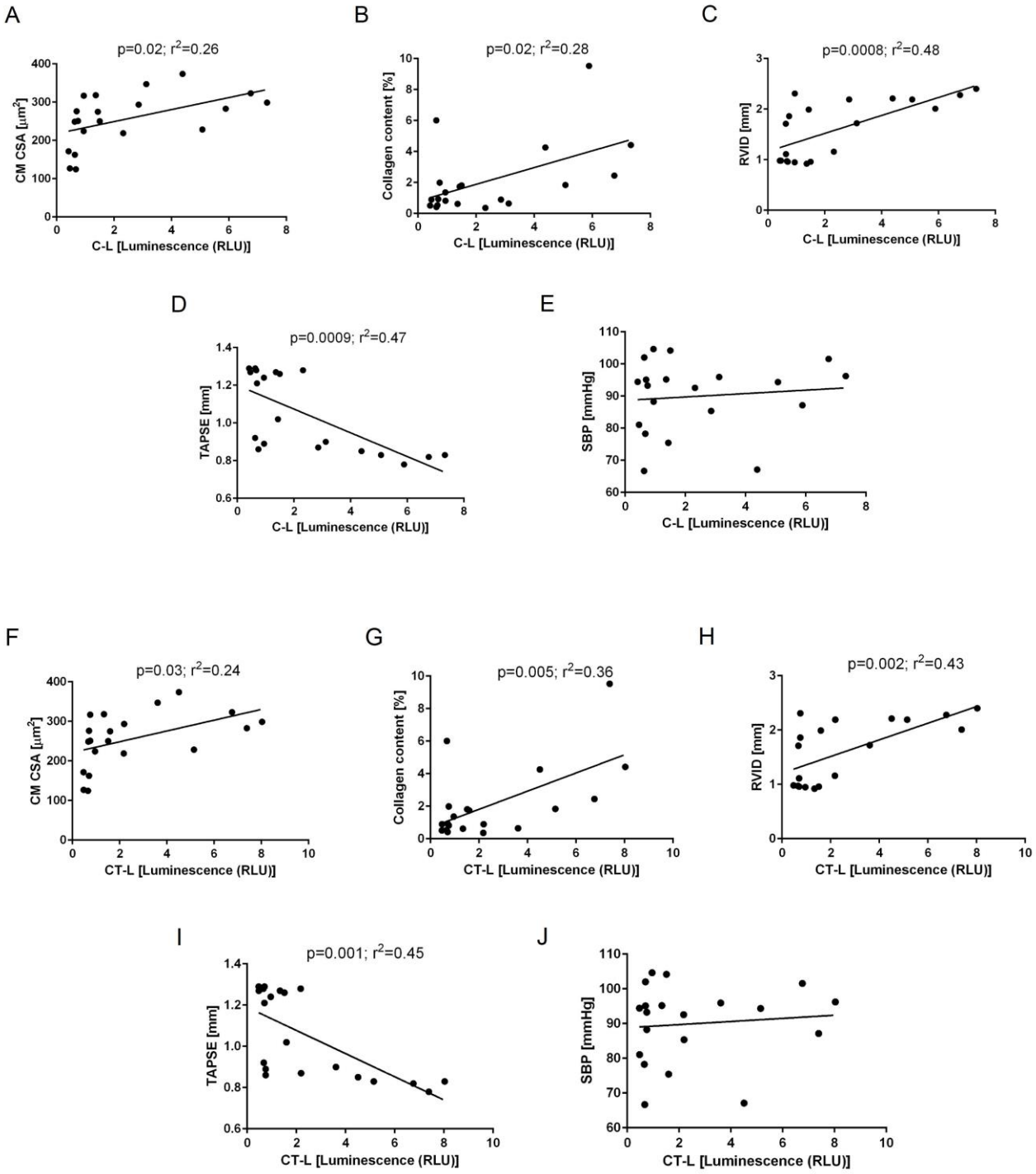


Supplementary figure 11: Proteostasis and inflammatory signaling upon bortezomib (BTZ) treatment in PAB mice

A: Western blotting of murine right ventricle tissues of BTZ-treated PAB mice (**BTZther**, n=4) for K48-linked polyubiquitinated proteins (K48-Ubi) compared to the control group (**PAB**, n=7). **B:** Western botting of the same tissue for IκBα, IκBβ and the autophagy marker LC3B. For quantification see densitometric analysis of subunit expression, normalised to the respective β-Actin loading control. Unpaired t-test ($p \leq 0.05$). Results are presented as mean+/-SEM.



Supplementary figure 12: Examples of Rpn6 expression in right ventricles derived from each patient with RVH and control subjects are presented. Scale bars = 100 µm.



Supplementary figure 13: Correlations between the proteolytic activities of the proteasome (C-L and CT-L) and important histological, functional, hemodynamic and echocardiographic parameters. A, F: cardiomyocyte size; B, G: collagen content; C, H: RVID; D, I: TAPSE and E, J: SBP (n=20).

Supplementary table 1: Patients' data for immunohistochemical analysis of right ventricle biopsies

Year of Birth	Sex	Diagnosis
controls		
1934	m	RV endomyocardial biopsies were taken to exclude myocarditis, normal LV function. Histological findings: mild lipomatosis cordis in the right ventricular myocardium. No myocarditis or cardiomyopathy. No cardiac virus infection.
1954	f	RV endomyocardial biopsies were taken to exclude myocarditis. No pathological findings, no myocarditis, no cardiomyopathy.
na	na	RV endomyocardial biopsies were taken to exclude myocarditis. No pathological findings, no myocarditis, no cardiomyopathy.
PH		
1951	m	RV endomyocardial biopsies. Case history: LVEF 38%; pulmonary hypertension, AV block I°, obesity. Histological and immunohistochemical investigations revealed reactive hypertrophy of myocytes due to pulmonary hypertension, interstitial fibrosis and a mild inflammation. No arrhythmogenic RV cardiomyopathy. No cardiac virus infection.
1940	m	RV endomyocardial biopsies. Case history: LVEF 29%, moderate pulmonary hypertension, DCM of uncertain aetiology, no coronary heart disease.

		Histological and immunohistochemical investigations revealed hypertrophic myocytes, a few apoptotic myocytes and interstitial fibrosis. No myocarditis. No cardiac virus infection.
2010	f	RV endomyocardial biopsies. Case history: LVEF normal, pulmonary hypertension, status post occlusion of ASD II, suspected non-compaction cardiomyopathy of RV, secondary hypertrophy. Histological and immunohistochemical investigations revealed a moderate reactive hypertrophy due to pulmonary hypertension. No non-compaction cardiomyopathy, no myocarditis. No cardiac virus infection.
1943	m	RV endomyocardial biopsies to exclude potential storage disorders or a restrictive cardiomyopathy. Case history: LVEF 60%, pulmonary hypertension, NYHA II-III, atrial fibrillation. Histological and immunohistochemical investigations revealed findings consistent with a hypertensive heart disease, with hypertrophic myocytes and small arterioles with hypertrophied walls. No cardiac virus infection.
1959	m	RV endomyocardial biopsies to exclude myocarditis. Case history: LVEF 20%, pulmonary hypertension, NYHA II-III. Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with DCM.

		No cardiac virus infection. No acute or chronic myocarditis, no amyloidosis.
1963	m	RV endomyocardial biopsies to exclude myocarditis. Case history: LVEF 15%, pulmonary hypertension, manifested DCM Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with DCM. No cardiac virus infection. No acute or chronic myocarditis, no amyloidosis.
1964	f	RV endomyocardial biopsies to exclude amyloidosis. Case history: LVEF < 20%, restrictive cardiomyopathy with left ventricular NYHA III-IV. Histological and immunohistochemical investigations revealed chronic myocardial damage consistent with restrictive cardiomyopathy with reactive hypertrophy and diffuse interstitial fibrosis. Mild inflammation consistent with suspected chronic lymphocytic myocarditis.

Supplementary table 2: Deterioration of RV structure and function after pulmonary artery banding (PAB)

Groups	RV/TL	RVID	RVWT	TAPSE	CI	CM CSA	CC
	*g/mm*10^{-4}*	*mm*	*mm*	*mm*	*ml/min/g*	*µm²*	*%*
sham	11.59 ± 0.29	0.98 ± 0.01	0.19 ± 0.00	1.25 ± 0.01	0.81 ± 0.02	193.9 ± 20.2	0.86 ± 0.13
PAB	17.79* ± 0.97	2.08* ± 0.07	0.38* ± 0.02	0.85* ± 0.02	0.57* ± 0.02	308.9* ± 13.6	4.08* ± 1.00

RV/TL right ventricular mass to tibia length **RVID** right ventricular internal diameter **RVWT** right ventricular wall thickness **TAPSE** tricuspid annular plane systolic excursion **CI** cardiac index **CM CSA** cardiomyocyte cross-sectional area **CC** collagen content. *Significance compared to control group (sham,n=11); Unpaired t-test (p≤0.05). Results are presented as mean+/-SEM.

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