Supplementary material

Pressure overload induced right ventricular remodeling is not attenuated by the anti-fibrotic agent Pirfenidone

Expanded methods

Echocardiography

During anesthesia (Sevoflurane, induction: 7.0% in 2:1 O_2/air mix; maintenance: 3.5% in 2:1 O_2/air mix) and spontaneous respiration, echocardiographies were performed in all rats at baseline and at end of study using a Vevo 2100 echocardiographic system (Visual Sonics, Canada) with a 21-MHz linear array transducer. RV systolic function was estimated by measurement of TAPSE (tricuspid annular plane systolic excursion) i.e. the shortening between lateral tricuspid annulus and the apex in M-mode. Tricuspid regurgitation was assessed by color Doppler in the apical four chamber view. Pulsed wave Doppler measurements were obtained from three systematic locations of the pulmonary artery just distal to the pulmonary valves in the parasternal long-axis view. Heart rate was recorded through ECG electrodes during the whole procedure. Analyses were performed offline (Vevo® 2100; Fujifilm VisualSonics Inc., Amsterdam, The Netherlands) with the observer blinded to the source of the samples. Cardiac output (CO) was calculated by the formula:

$$CO = \left(\frac{PA_{diameter}}{2}\right)^2 \cdot \pi \cdot VTI \cdot HR$$

Where $PA_{diameter}$ = pulmonary artery diameter, *VTI* = velocity time integral, and *HR* = heart rate. For every analyzed parameter, we analyzed three consecutive cycles outside respiration and used the mean used as a representative value.

Magnetic resonance imaging

For evaluation of RV and LV volumes and cardiac output all rats underwent a cardiac magnetic resonance imaging (MRI) scan. We used a 9.4 Tesla Agilent MRI system and a trigger system from SAII (New York, USA) to synchronize image acquisition to the heart beat and respiration of the rats. During the scan, the rats were anaesthetized (Sevoflurane, induction: 7.0% in 2:1 O_2/N_2O mix; maintenance: 3.5% in 2:1 O_2/N_2O mix) and kept with spontaneous respiration. Body temperature was monitored and kept above 36°C during the

whole scan. For best image quality, a dedicated rat heart receive coil was used (Rapid Biomedical, Rimpar, Germany). To assess RV and LV volumes, a series of short axis cine images covering the ventricles from apex to basis was acquired. Slice thickness was 1.5 mm, and in-plane resolution was 0.2 mm. A phase contrast flow measurement allowed for assessment of blood flow in the pulmonary artery. RV and LV volumes were assessed from the short axis images using segment (http://segment.heiberg.se, Medviso) cardiac analysis software. The endocardium of both ventricles was traced manually during a whole heart cycle to obtain end diastolic and end systolic volume. Ejection fraction was calculated using the equation:

$$Ejection\ fraction\ (\%) = \frac{End\ diastolic\ volume\ (mL) - End\ systolic\ volume\ (mL)}{End\ diastolic\ volume\ (mL)}$$

Flow values were obtained from the phase contrast sequences using specific analysis software (Siswin) and used to calculate cardiac output. All data analyses were performed with the observer blinded to the source of the sample.

Pressure-volume measurements

Rats were anesthetized (Sevoflurane, induction: 7.0% in 2:1 O_2/air mix; maintenance: 3.5% in 2:1 O_2/air mix), intubated and put on a ventilator. An intramuscular injection of 50 units of heparin (Heparin, Leo Pharma A/S, Ballerup, Denmark) was given before the neck was shaved and a midline incision was made. Through careful dissection of the underlying tissue the left carotid artery was exposed and a Millar catheter (SPR-869; Millar Instruments, Houston, TX) was installed in the artery through a small incision after calibration. The catheter was fixated by a ligature. Systemic blood pressure was sampled by an MPVS Ultra (Millar Instruments) and processed in Powerlab 16/35 (AD Instruments, UK). Afterwards, the thorax was opened and a conductance catheter (SPR-869, Millar Instruments, Houston, TX) was installed in the RV

through the apex. After allowing for stabilization, steady state RV pressures were recorded. Pressure-volume loops were recorded during occlusion of the inferior vena cava by slowly pulling the two ends of a ligature placed under the vessel. All data was analyzed using LabChart Software (AD Instruments, UK) by a blinded observer. End-systolic and end-diastolic volumes from the MRI scans were used to calibrate the conductance signal.

Euthanasia

During anesthesia and after obtainment of pressure-volume measurements, the abdominal cavity was opened through a midline incision. Blood was drawn from the abdominal aorta into a clean tube and left at room temperature for 30 min to cloth. The sample was then centrifuged at 5000 rpm for 10 minutes at 4°C, the supernatant transferred to new tubes and the centrifugation repeated. The serum was transferred to a clean tube and stored at -80°C for subsequent analyses of serological biomarkers of fibrosis.

The heart was excised, and the RV and LV plus septum were separated, weighed and immersion fixated in phosphate-buffered 4% formaldehyde. The left tibia bone was isolated and the weight of the RV corrected for the length of the tibia was used as a measure of RV hypertrophy. The presence of ascites and pleural fluid was assessed by weighing cotton swaps before and after wiping the abdominal and pleural cavity. A surplus of >1g was used as a cut of for the presence of ascites and pleural fluid. Dark discoloration of the liver (nutmeg liver) was noted as a sign of congestion and backward failure.

Stereology

The figure below shows the work flow of sampling and principles of quantification of fibrosis by stereology.

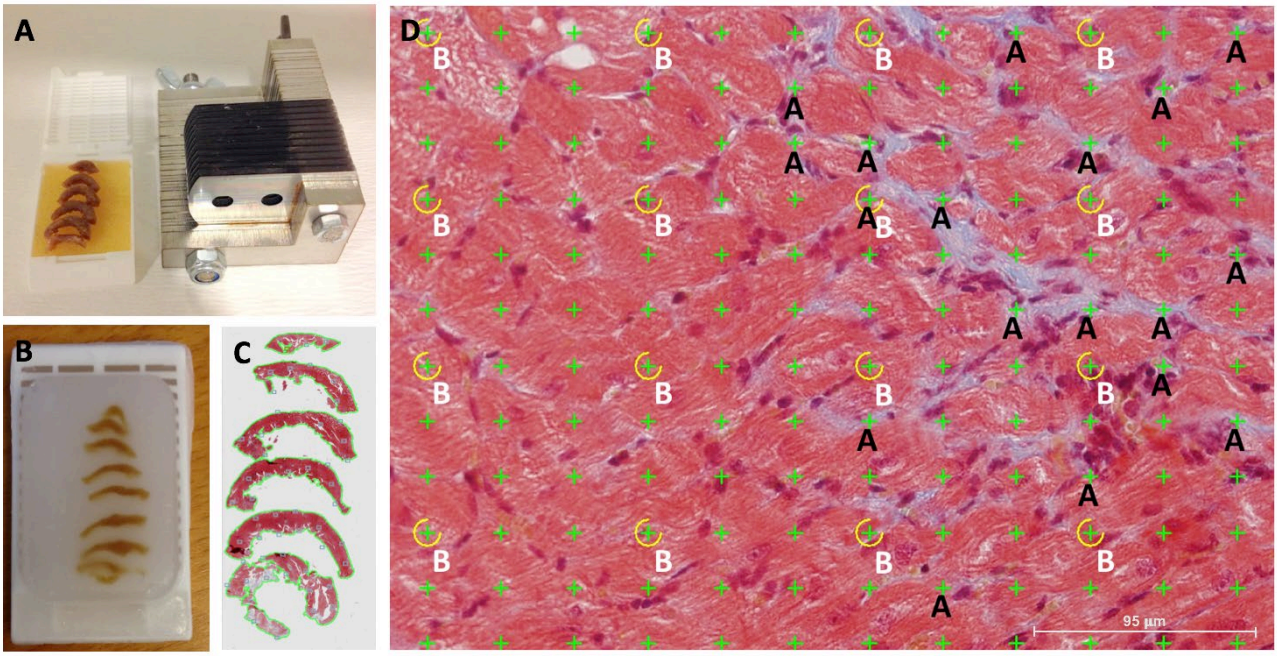

Figure S1.

After 24 h fixation in formalin, the RV was cut into 2 mm slaps transverse to the apex-basis axis (Figure S1-A). The slaps were embedded in paraffin with the apical cutting face facing the surface of the paraffin block. The most apical slab with no apical cutting face was excluded (Figure S1-B). 2 µm sections were stained with Masson's Trichrome. Analyses were performed using an Olympus BX51 light microscope with Olympus DP70 camera, a x20 dry lens (Olympus, UPlanFI, NA 0.50), prior motorizedstage and newCAST software (version 2017.7.1.3832, Visiopharm, Hørsholm, Denmark). The RV, LV and septum were detected by drawing regions of interest (ROIs) along the outer borders of the tissue, and fields of view (FOVs) were systematically sampled by the software within the ROIs for analyses (Figure S1-C). For estimation of volume of fibrosis step length was 2195 µm corresponding to 3% of the tissue being analyzed. To take the variation in tissue area of the sections, especially for the RV, into consideration, 4% (step length 1901 µm) were analyzed for sections with an estimated small RV tissue area. Per animal, an average of 35 ± 11 FOVs were analyzed for the RV, 37 ± 8 FOVs were analyzed for the septum, while 44 ± 9 FOVs were evaluated for the

LV. The volume fraction of fibrosis was evaluated in each FOV using two point grids: one for fibrosis (green plus signs) and one for the RV (yellow circles). Fibrosis (black A) and RV (white B) were counted in each FOV (Figure S1-D) and the average of all FOVs for each rat used as a representative value. Samples were prepared and analyzed similarly for evaluation of RV, septal and LV fibrosis.

Analyses of cardiomyocyte hypertrophy were performed with the above-mentioned microscope and a x40 dry lens (Olympus, PlanApo, NA 0.95). We used a 2D IUR nucleator probe to assess cardiomyocyte size. The RV, the septum and the LV were evaluated separately. Approximately 120 fields of views were analyzed per rat per ventricle/septum corresponding to an average step length of 1150 μm for the RV, 1180 μm for the septum, and 1380 μm for the LV. Only cardiomyocyte profiles with a visible characteristic nucleus sampled by the unbiased counting frame were analyzed. Approximately 50 cardiomyocyte profiles were analyzed per rat for the RV, the septum and the LV, respectively. Both cross sectional and longitudinal cut cardiomyocytes were included in the analysis.

Gene expression

RNA was extracted from 8 sections of 10 μm thickness of formalin fixated paraffin embedded RV tissue. The tissue was the deparaffinized and rehydrated in xylene/ETOH followed by Proteinase K treatment. RNA was extracted using the Qiagen miRNeasy Kit Cat. No. 217504, and RNA concentration determined by the Nanoquant. Using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), total RNA was reverse transcribed into complimentary DNA (cDNA) following a standard protocol. First strand cDNA was diluted, and RT-qPCR performed using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific). Specific primers for the following genes were used: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-smooth muscle actin (α-SMA), β-myosin heavy chain (β-MHC), transforming growth factor-β receptor

type 2 (TGF-β-R2), galectin-3, Forkhead box subfamily O type 1 (FoxO1), collagen I, collagen III and collagen IV. Measured mRNA expression levels were normalised to the housekeeping gene GAPDH, log transformed, and made relative to the normalised mRNA levels of Sham rats.

Table 1. Primer sequences.

Gene	**Forward (5´ → 3´)**	**Reverse (5´ → 3´)**
GAPDH:	TTAAGGGCATCCTGGGCTACACT	TTACTCCTTGGAGGCCATGTAGG
ANP:	TAGATCTGCCCTCTTGAAAA	TCCAATCCTGTCAATCCTAC
BNP:	GCTGGAGCTGATAAGAGAAA	TTGAACTATGTGCCATCTTG
α-SMA:	CACGGCATTATCACCAACTG	AACAATGCCTGTGGTTCTCC
β-MHC:	GAGCTTCTAGACATGCTGCT	TGGAGTTCTTCTCTTCTGGA
TGF-β-R2:	TCACTAGGCACGTCATCAGC	AGGACAACCCGAAGTCACAC
Collagen I:	TCAAGATGGTGGCCGTTACT	CATCTTGAGGTCACGGCATG
Collagen III:	ATGAATTGGGATGCAACTAC	TCTAGTGGCTCATCATCACA
Collagen IV	AACCTGGCAGTGATGGAATC	TCACCCTTGGAACCTTTGTC
Galectin-3	TCGCCTTCCACTTTAACCCC	GGCTTCAACCAGGACCTGTA
FoxO1	AGCTGCATCCATGGACAACA	TCATCATTGCTGTGGGACCC

Biomarker measurements by enzyme-linked immunosorbent assay (ELISA)

The non-invasive fibrosis biomarkers C3M, PRO-C3, C4M and α-SMA were measured by competitive ELISAs (Nordic Bioscience, Herlev, Denmark). The procedure for the different ELISAs is detailed in the references in Table 2. Briefly a 96-well ELISA plate coated with streptavidin (Roche, Hvidovre, Denmark), was coated with the synthetic peptide at 20^{o}C for 30 minutes, by constant shaking at 300 rpm. The plate was then washed

five times in washing buffer (20mM Tris, 50mM NaCl, pH 7.2). Thereafter, 20 µL of the standard peptide or sample diluted according the protocol were added, followed by 100 µL of peroxidase conjugated mAb against the sequence of interest. The plate was incubated for 1 h, 3h or overnight at 20°C or 4 °C while shaking at 300 rpm (according to the specifications for the individual assay). Afterwards, the plate was washed five times in washing buffer, 100 µL TMB (Kem-En-Tec, Taastrup, Denmark) were added and the plate was incubated for 15 minutes in the dark while shaking at 300 rpm. The reaction was stopped by addition of 100 µL stopping solution (1% H_2SO_4), and the plate was analyzed on an ELISA reader at 450 nm with 650 nm as the reference. Samples below lower limit of quantification (LLOQ) were assigned the value of LLOQ, while samples above upper limit of quantification (ULOQ) were assigned the value of ULOQ.

Table 2. Overview of measured biomarkers to assess ECM turnover in serum

Biomarker	**Target**	**Reference**
C3M	Neo-epitope of MMP-9 mediated degradation of type III collagen (collagen type III degradation)	[1]
PRO-C3	Released N-terminal pro-peptide of type III collagen (collagen type III formation)	[2]
C4M	Neo-epitope of MMP-2,9,12 mediated degradation of type IV collagen alpha 1 chain (collagen type IV degradation)	[3]
α-SMA	Acetylated N-terminal of alpha-smooth muscle actin	[4]

References

1. Barascuk N, Veidal SS, Larsen L, et al. A novel assay for extracellular matrix remodeling associated with liver fibrosis: An enzyme-linked immunosorbent assay (ELISA) for a MMP-9 proteolytically revealed neo-epitope of type III collagen. *Clin Biochem*. 2010; 43: 899-904.

2. Nielsen MJ, Nedergaard AF, Sun S, et al. The neo-epitope specific PRO-C3 ELISA measures true formation of type III collagen associated with liver and muscle parameters. *Am J Transl Res*. 2013; 5: 303-15.

3. Sand JM, Larsen L, Hogaboam C, et al. MMP mediated degradation of type IV collagen alpha 1 and alpha 3 chains reflects basement membrane remodeling in experimental and clinical fibrosis--validation of two novel biomarker assays. *PLoS ONE*. 2013; 8: e84934.

4. Holm Nielsen S, Willumsen N, Leeming DJ, et al. Serological Assessment of Activated Fibroblasts by alpha-Smooth Muscle Actin (alpha-SMA): A Noninvasive Biomarker of Activated Fibroblasts in Lung Disorders. *Transl Oncol*. 2018; 12: 368-74.

Supplementary results

Supplementary tables

Table S1. Data at randomization one week after sham or PTB surgery.

	Sham (n=12)	**PTB** (n=13)	**PTB+pirf** (n=10)
Body weight (g)	154 ± 13	145 ± 11	147 ± 11
TAPSE (mm)	2.23 ± 0.60	1.51 ± 0.41**	1.56 ± 0.33
Cardiac output (mL/min)	82 ± 21	63 ± 24*	62 ± 8
Heart rate (bpm)	353 ± 41	329 ± 31	306 ± 37
Stroke volume (mL)	0.24 ± 0.07	0.19 ± 0.06	0.20 ± 0.01

TAPSE: Tricuspid annular plane systolic excursion. Data are presented as mean ± SD. *p<0.05, **p<0.01 vs sham.

Table S2. Supplementary autopsy data.

	Sham (n=11)	**PTB** (n=11)	**PTB+pirf** (n=10)
RV/body weight (g/kg)	0.53 ± 0.04	1.27 ± 0.15***	1.38 ± 0.12
LV+S/body weight (g/kg)	2.07 ± 0.17	2.25 ± 0.20	2.17 ± 0.17
Tibia length (mm)	41.2 ± 1,2	40.9 ± 0.8	40.0 ± 0.9
Lungs (g)	1.65 ± 0.16	1.69 ± 0.21	1.48 ± 0.17^
Liver (g)	15.6 ± 2,0	15.9 ± 4,0	15.6 ± 4,0
Nutmeg liver, n (%)	0 (0)	6 (55)*	4 (40)
Spleen (g)	1.02 ± 0.25	0.97 ± 0.20	0.99 ± 0.21
Kidneys (g)	2.62 ± 0.32	2.22 ± 0.18**	2.18 ± 0.29
Ascites/pleural fluid, n (%)	0 (0)	3 (27)	2 (20)

Data are presented as mean ± SD or n. *p<0.05, **p<0.01, ***p<0.001 vs sham; ^p<0.05 vs PTB.

Table S3. Total fibrosis in the RV, the septum and the LV.

	Sham (n=11)	**PTB** (n=11)	**PTB+pirf** (n=10)
Right ventricle			
V(RV) (cm^3)	0.21 ± 0.02	0.46 ± 0.06***	0.47 ± 0.09
V_V(total fib/RV) (%)	5.9 ± 1,5	13.0 ± 2,7***	13.3 ± 1.6
V(total fib,RV) (cm^3)	0.012 ± 0.003	0.060 ± 0.014***	0.062 ± 0.010
Septum			
V(septum) (cm^3)	0.32 ± 0.05	0.41 ± 0.05**	0.34 ± 0.08^
V_v(total fib/septum) (%)	2.7 ± 0.4	7.0 ± 0.8***	7.3 ± 1.1
V(total fib,septum) (cm^3)	0.009 ± 0.002	0.028 ± 0.004***	0.025 ± 0.004
Left ventricle			
LV volume (cm^3)	0.53 ± 0.04	0.44 ± 0.07**	0.38 ± 0.05
V_V(total fib/LV) (%)	3.1 ± 0.4	6.0 ± 1.7***	5.2 ± 1.1
V(total fib,LV) (cm^3)	0.016 ± 0.003	0.026 ± 0.009**	0.020 ± 0.006

Total fibrosis = interstitial fibrosis plus the epicardium and the endocardium. V: volume; V_v: volume fraction. Data are presented as mean ± SD. **p<0.01, ***p<0.001 vs sham; ^p<0.05 vs PTB.

Supplementary figures

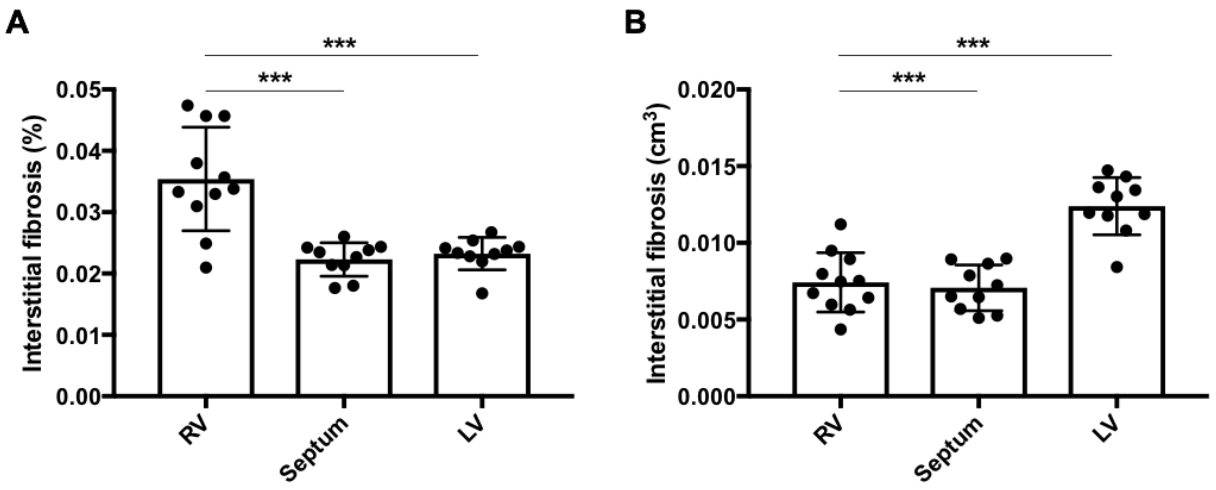

Figure S2. Fibrosis in the healthy RV, septum and LV.

(A) Volume fraction of interstitial fibrosis and (B) the absolute volume of fibrosis in the RV, the septum and the LV from healthy rats. Data are presented as mean ± SD. ***p<0.001.

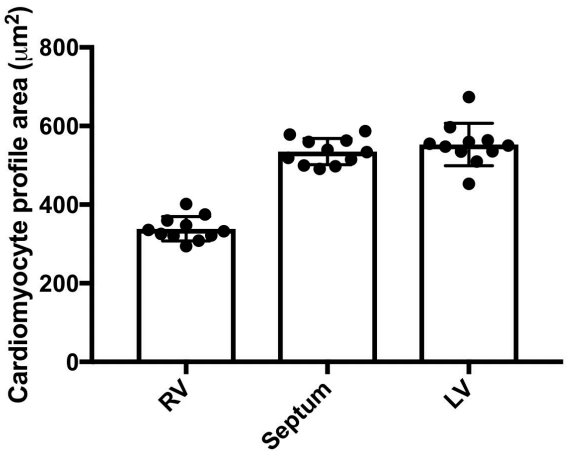

Figure S3. Cardiomyocyte profile area in the healthy RV, septum and LV.

Average cardiomyocyte profile area in the RV, the septum and the LV from healthy rats. Data are presented as mean ± SD. ***p<0.001.