Right Ventricular Diastolic Impairment in Patients with Pulmonary Arterial Hypertension

Running title: Rain et al.; RV diastolic impairment in PAH

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Abstract

Background—The role of right ventricular (RV) diastolic stiffness in pulmonary arterial hypertension (PAH) is not well-established. Therefore, we investigated the presence and possible underlying mechanisms of RV diastolic stiffness in PAH-patients.

Methods and Results—Single-beat RV pressure-volume analyses were performed in 21 PAH-patients and 7 controls to study RV diastolic stiffness. Data presented as mean±SEM. RV diastolic stiffness (β) was significantly increased in PAH-patients (PAH: 0.050±0.005 vs. control: 0.029±0.003; p<0.05) and closely associated to disease severity. Subsequently, we searched for possible underlying mechanisms, using RV tissue of PAH-patients undergoing heart-lung transplantation and non-failing donors. Histological analyses revealed increased cardiomyocyte cross-sectional areas (PAH: 453±31 vs. control: 218±21 μm²; p<0.001), indicating RV hypertrophy. In addition, the amount of RV fibrosis was enhanced in PAH tissue (PAH: 9.6±0.7 vs. control: 7.2±0.6%; p<0.01). To investigate the contribution of stiffening of the sarcomere (the contractile apparatus of RV cardiomyocytes) to RV diastolic stiffness, we isolated and membrane-permeabilized single RV cardiomyocytes. Passive tension at different sarcomere lengths was significantly higher in PAH compared to controls (+200%; pinteraction<0.001), indicating stiffening of RV sarcomeres. An important regulator of sarcomeric stiffening is the sarcomeric protein titin. Therefore, we investigated titin isoform composition and phosphorylation. No alterations were observed in titin isoform composition (N2BA/N2B ratio PAH: 0.78±0.07 vs. control 0.91±0.08), but titin phosphorylation in RV-tissue of PAH-patients was significantly reduced (PAH: 0.16±0.01 vs. control 0.20±0.01 a.u.;p<0.05).

Conclusions—RV diastolic stiffness is significantly increased in PAH-patients, with important contributions from increased collagen and intrinsic stiffening of the RV cardiomyocyte sarcomeres.

Key words: pulmonary hypertension, right ventricular failure, sarcomere physiology, diastolic dysfunction
Introduction

Idiopathic pulmonary arterial hypertension (PAH) is a rare but fatal disease with a survival rate of 58% in 3 years. Present therapy is unable to normalize pulmonary arterial pressures and PAH-patients ultimately develop right heart failure.

Previous studies have demonstrated that PAH-patients have reduced systolic function as measured by RV ejection fraction. However, the knowledge on the role of RV diastolic stiffness in PAH is limited. Measuring RV diastolic stiffness has been hindered until now, because non-invasive techniques (echocardiography, magnetic resonance imaging) provide only information on relaxation velocities and not on diastolic stiffness per se. In addition, these measures are highly sensitive to the confounding effects of increased pre- and afterload, and are therefore not reliable in the setting of PAH. On the other hand, the gold standard of measuring load-independent diastolic stiffness by pressure-volume (PV) analysis is not without risk in PAH-patients, since it requires temporal preload reduction. In left heart failure this was circumvented by the development of single-beat analyses of diastolic PV relationship. However, it is unclear whether this analysis could also be used for the right ventricle in PAH.

There are several possible contributing factors explaining RV diastolic stiffness in PAH. Hypertrophy and fibrosis are known to increase ventricular stiffness. But RV diastolic stiffness could also be caused by changes in the contractile apparatus of RV cardiomyocytes: the sarcomeres. Sarcomeric stiffness is tightly regulated by the giant sarcomeric protein titin. Titin consists of two isoforms: the stiff N2B isoform and the compliant N2BA isoform. Besides changes in isoform composition, titin compliance is regulated by phosphorylation. Whether these factors are altered in human PAH pathophysiology is unknown.

Therefore, the aim of this study is to determine the presence of RV diastolic stiffness in
PAH-patients and explore the contribution of collagen formation, sarcomeric stiffening and post-translational modifications of titin in RV tissue of PAH-patients.

**Methods**

**Assessment of RV diastolic stiffness**

Hemodynamic data was obtained from digitally stored routine clinical measurements. Patients eligible for this study were referred to the VU University Medical Center for evaluation of pulmonary hypertension and patients with PAH undergoing follow-up analysis between September 2001 and November 2011. Standard clinical care included right heart catheterization (balloon-tipped flow-direct 7F Swan-Ganz catheter - 131HF7, Baxter Healthcare Corporation, Irvine, CA) and cardiac MRI (1.5-T whole-body system, Siemens Sonata, Siemens Medical Solutions, Erlangen, Germany). During right heart catheterization, radial or femoral blood samples were collected and standard laboratory tests including N-terminal pro-brain natriuretic peptide level (NT-proBNP) were performed. New York Heart Association (NYHA) class and six-minute-walk-distance (6MWD) were registered during the same clinical evaluation. All patients were evaluated in stable hemodynamic condition, lying supinely and breathing at normal frequencies.

Patients were selected based on the following criteria: good-quality recordings of right heart catheterization pressure curves with cardiac MRI performed within the same hospital admission and under the same hemodynamic condition (n=28). PAH was diagnosed according to the PAH diagnostic guidelines (n=21). Controls were selected from referred patients suspected with PAH but in whom the condition was ruled out after recording normal pulmonary pressures during right heart catheterization (n=7).
**Right heart catheterization**

The following invasive variables were recorded: right atrial pressure (RAP), RV pressure, mean pulmonary artery pressure (mPAP) and pulmonary capillary wedge pressure (PCWP). Cardiac output (CO) was determined by Fick method and pulmonary vascular resistance (PVR) was calculated using $PVR=(mPAP-PCWP)/CO$. Diastolic filling pressures were measured at minimum pressure point (recorded after tricuspid valve opening) and noted as begin-diastolic pressure (BDP). End-diastolic pressure (EDP) was recorded at maximal diastolic filling pressure point before the onset of isovolumic contraction (Fig. 1A).

**Cardiac MRI**

RV volumes were calculated using Mass software (MEDIS, Medical Imaging Systems, Leiden, the Netherlands) from multiple short axial-slice MRI analysis. End-systolic volumes (ESV) were considered to correspond to BDP and is further referred as begin-diastolic volumes (BDV), while end-diastolic volumes (EDV) corresponded to EDP (Fig. 1A). Stroke volume was calculated from MRI-derived pulmonary artery flow and used to accurately determine RV BDV.

RV volumetric filling curves were obtained from the stack of short-axis cine images for the quantification of RV early (E) and atrium (A) induced peak filling rate (E/A ratio) as previously described.

**Single-beat pressure-volume analysis**

PV relations were constructed by fitting a non-linear exponential curve through the diastolic PV points using the following formula:

$$P = \alpha \left( e^{\beta V} - 1 \right) \text{ (Equation 1)},$$

where $P$: pressure; $\alpha$: curve-fitting constant; $\beta$: diastolic stiffness constant; $V$: volume.
The slope of the curve was characterized by the exponential term $\beta$ and the curve constant $\alpha$, which were further used to quantify RV diastolic stiffness. The first two points used to construct the PV relation were the BDP-BDV point and the EDP-EDV point. The third PV point used to construct the diastolic PV relation was set at $0_{pressure, 0_{volume}}$, since prolonging the PV curves to volumes lower than the intercept volume does not modify the exponential term $\beta$ and the curve constant $\alpha$ (see supplemental data). Due to large variation in EDV in control patients compared to PAH-patients, the EDV was normalized to the maximal EDV recorded among the patients. Consecutively, the BDV was calculated by subtracting the corresponding stroke volume from the normalized EDV. To avoid measurement errors due to the positioning of the RV catheter, BDP was normalized at 1mmHg, while the EDP was calculated with the following formula: $\text{EDP}_{\text{normalized}} = 1 + (\text{EDP}_{\text{initial}} - \text{BDP}_{\text{initial}})$. To account for covariance in $\alpha$ and $\beta$, derived $\alpha$ and $\beta$ of each individual subject were used to calculate the $V$ at a common $P$ of 20mmHg ($V_{20}$).\textsuperscript{12}

Experimental support for the use of single-beat instead of multiple-beat RV diastolic PV relation was obtained in rats with PAH-induced right heart failure undergoing right heart catheterization with a conductance catheter and echocardiography. For details see the online supplement.

**Assessment of RV end-systolic elastance**

The slope of the end-systolic pressure volume relation (end-systolic elastance, Ees) was calculated as previously described: $\text{Ees} = (P_{\text{iso}} - \text{mPAP})/\text{SV}$.\textsuperscript{13} The isovolumic pressure ($P_{\text{iso}}$) was obtained by fitting an inverted cosine wave over the RV pressure curve using the isovolumic contraction period (from end-diastole to the point of maximal rate of pressure rise (dP/dt$_{\text{max}}$)) and the isovolumic relaxation period (from minimal dP/dt to start diastole) by a semi-automatic
Matlab R2008a program (The MathWorks, Natick, MA).14

**RV histological analyses.**

Explanted RV tissue samples were collected from PAH-patients undergoing heart/lung transplantation (n=10). Control RV tissue was obtained from non-failing donors (n=9). Written informed consent was obtained and the study protocol was approved by the local ethics committees. All samples were immediately frozen and stored in liquid nitrogen.

The degree of RV hypertrophy was analyzed on 5µm-thick tissue sections stained with antibodies against the extracellular protein Laminin (1:200; L9393, Sigma-Aldrich). Minimally 40 cells per sample were used in order to calculate cross-sectional area. Cardiomyocytes with non-transversal cross-sections were not included in the analysis.15-18

RV fibrosis was determined on 5µm-thick tissue sections stained with picrosirius red and analyzed under double-polarized light.16,17 Images were collected by the use of a Leica DMRB microscope (Wetzlar, Germany), a Sony XC-77CE camera (Towada, Japan) and a LG-3 frame grabber (Scion, Frederick MD). For each PAH and control sample, a minimum of 10 pictures obtained from different areas was analyzed. ImageJ for Windows 1.42 software (National Institutes of Health, Bethesda MD) was used for image analysis, taking the pixel-to-aspect ratio into account. Collagen content was quantified as area percentage of the recorded images under a microscopy magnification of 20x.

**RV cardiomyocyte force measurements**

Tissue pieces were defrosted in relaxing solution and single cardiac cells were isolated mechanically as described before (7 control and 7 PAH samples).19 A minimum of 3 cells per sample were measured and the average total, active and passive tension were calculated. Cardiomyocytes were incubated for 5 minutes in relaxing solution containing 1% Triton X-100
to premeabilize membranes. To remove Triton, the cardiomyocyte solution was washed six times with relaxing solution, after which a single cell was attached with silicone adhesive between a force transducer and a piezoelectric motor. Force measurements were performed at 1.8 and 2.2 μm sarcomere length in activating solutions with maximal and submaximal calcium concentrations ranging from 1 to 30 μmol/L. After maximal force development in activating solutions, the cell was shortened to 70% of its original length in order to determine total force development (F_{total}). A similar shortening was performed in the relaxing solution to record passive tension (F_{passive}). Active force (F_{active}) was calculated by subtracting F_{passive} from F_{total}. Force values at submaximal [Ca^{2+}] were normalized to the maximal force value obtained at 30μmol/L Ca^{2+} to determine Ca^{2+}-sensitivity of the myofilaments expressed as EC_{50}, i.e. the [Ca^{2+}] at which 50% of maximal force was obtained. Steady-state F_{passive} measurements were performed at increasing sarcomere lengths (1.8 – 2.6μm).

To determine tension, we corrected for differences in RV cross-sectional area between control and PAH. Individual force values were normalized for the cardiomyocyte width and depth recorded at 2.2μm sarcomere length.

Contribution of actomyosin interaction to passive tension was determined by incubating skinned cardiomyocytes with actomyosin inhibitor 2,3-butanedione monoxime (BDM; 25mM) at 15°C, for 10 minutes. After 10 minutes active tension was measured in maximal activation solution to determine the efficiency of the compound. Subsequently, passive tension was recorded at increasing sarcomere lengths (1.8 – 2.4μm) and compared to passive tension recorded before BDM incubation.

**Titin isoform composition and phosphorylation**

Frozen RV tissue samples were weighed and pulverized in liquid nitrogen using a mortar and
pebble. Tissue powder was solubilized in 8M urea buffer with DTT and 50% glycerol solution with protease inhibitors (4X Leupeptin, E-64 and PMSF). Equal dilutions were calculated based on Myosin Heavy Chain (MHC) content; protein homogenate samples were loaded on custom-made 1% agarose gels. Solubilized human soleus muscle was used as reference. Gels were washed overnight in presoak solution, stained with Coomassie Blue and destained. Protein composition was determined using 1D-Scan software program. Titin N2B, N2BA, degradation products and MHC were quantified and Titin N2B/N2BA ratio was determined.

To quantify titin phosphorylation, gels were stained for 2 hours with ProQ diamond (Molecular Probes). Thereafter, the gels were washed and subsequently stained with SYPRO Ruby (Molecular Probes).22

Statistical analyses

Statistical analyses were performed using Prism 5 for Windows (GraphPad Software Inc, San Diego, CA). Normal distribution was tested and logarithm transformation was performed if necessary. P-values lower than 0.05 were considered significant.

Changes in patient characteristics and diastolic stiffness were tested for significance with unpaired Student t-tests or non-parametric Mann-Whitney U test (RAP, NT-proBNP). The relation between diastolic stiffness and several variables for disease severity (SV, 6MWD, RAP and NT-proBNP levels) was tested with Pearson’s correlation. To adjust for possible confounding by body surface area, age, treatment duration and pulmonary vascular resistance, multivariable regression analyses was performed. Histological data were analyzed using multilevel analysis to correct for non-independence of successive measurements per patient (MLwiN 2.02.03, Center for Multilevel Modeling, Bristol, UK).15-18,23 Changes in cardiomyocyte maximal tension, Ca²⁺-sensitivity and passive stiffness were tested for significance by repeated
measures ANOVA followed by Bonferroni post-hoc test.

**Results**

**Assessment of RV diastolic stiffness**

RV diastolic stiffness was calculated in PAH-patients (n=21) and controls (n=7). The clinical characteristics of the patients enrolled in this part of the study are described in **Table 1**. PAH-patients were in majority women (20 of 21) with an average age of 45 years. Control patients matched in terms of age, sex and body mass index. Compared to controls, PAH-patients had significantly increased mPAP and PVR and normal PCWP. RAP and NT-proBNP levels were significantly increased mPAP and PVR and normal PCWP. RAP and NT-proBNP levels were significantly higher in PAH-patients compared to controls. Furthermore, RV ejection fraction was lower in PAH-patients, as well as CO. PAH-patients were in a relatively good functional state (NYHA class II 17 of 21; comparable 6MWD to control), presumably related to intensive treatment (multiple therapy: 16 of 21).

As can be observed in the online supplement, multiple-beat (β\textsubscript{multiple}) and single-beat (β\textsubscript{single}) derived RV diastolic stiffness were closely correlated in rats (R\textsuperscript{2}=0.94; p<0.001). Therefore, we used the single-beat method to calculate RV diastolic stiffness in PAH-patients and controls. The average PAH and control diastolic curves are presented in **Fig. 1B**. Compared to the control curve, the steeper PAH-curve indicates increased stiffness of the myocardium. On average, PAH-patients had an almost two-fold increase in RV diastolic stiffness parameter β (**Fig. 1C**), and a reduced curve constant α (**Table 2**). After controlling for the covariance of α and β, RV diastolic stiffness remained significant in PAH-patients in comparison to control (V\textsubscript{20}; **Table 2**). Non-invasive assessment of diastolic dysfunction by measures of MRI-obtained E/A ratio confirmed the observed increase in RV diastolic stiffness in PAH-patients (**Fig. 1D**). In
addition, RV diastolic stiffness measurements $\beta$ and $V_{20}$ were both modestly correlated to E/A ratio ($r_{E/A \ vs. \ \beta} = -0.41$; $r_{E/A \ vs. \ V_{20}} = 0.48$; both $p<0.05$). Increased RV diastolic stiffness coincided with increased RV Ees in the same PAH-patients (Fig. 1E).

To investigate whether RV diastolic stiffness is also present in other forms of pulmonary hypertension, we included an additional group of patients with chronic thrombo-embolic pulmonary hypertension (CTEPH; $n=24$). Similar to PAH, RV diastolic stiffness was significantly increased in CTEPH-patients in comparison to control ($\beta$ CTEPH: $0.054\pm0.005$, PAH: $0.050\pm0.005$ vs. control: $0.029\pm0.003$; $p<0.05$).

RV diastolic stiffness, characterized by the curve constant $\beta$, significantly correlated with PAH disease severity. Stroke volume and 6MWD were significantly and inversely correlated with RV diastolic stiffness, suggesting that increased RV cardiomyocyte stiffness is associated with reduced cardiac function and exercise capacity (Fig. 2A,B). In addition, RV diastolic stiffness was closely correlated to RAP and NT-proBNP, both markers of increased RV stiffness and wall stress (Fig. 2C,D). These correlations remained significant after correction for the possible confounding effects of age, gender, body surface area, treatment duration and PVR (Table 3).

**RV histology analyses**

To perform histological analyses, RV tissue samples were obtained from PAH-patients ($n=10$) and controls ($n=9$). Patient characteristics are shown in Table 4. A two-fold increase of RV cardiomyocyte cross-sectional area in PAH was found compared to control cardiomyocytes (PAH: $531\pm34$ $\mu m^2$ vs. control: $256\pm24$ $\mu m^2$, $p<0.001$; Fig.3A). In addition, a significant increase in collagen content was found in PAH tissue sections compared with controls (PAH: $9.6\pm0.7\%$ vs. control: $7.2\pm0.6\% \ p<0.01$; Fig.3B).
RV cardiomyocyte force measurements

To investigate the contribution of sarcomeric stiffening on RV diastolic stiffness in PAH, we isolated and membrane-permeabilized single RV cardiomyocytes of RV tissue from PAH-patients (n=7) and controls (n=7). The advantage of the single RV cardiomyocyte approach is that RV sarcomeric function (the contractile apparatus of the RV cardiomyocytes) can be investigated in detail, without the confounding effects of hypertrophy, fibrosis or calcium handling. First, we investigated overall sarcomeric function in PAH and control RV cardiomyocytes. A similar length-dependent increase in $F_{\text{active}}$ was found in both groups with increasing sarcomere lengths from 1.8 to 2.2μm. Interestingly maximal $F_{\text{active}}$ was higher in PAH-patients compared to control cardiomyocytes at both 1.8 and 2.2μm, although the difference was only significant at 2.2 μm sarcomere length (Fig.4A). Normalized tension–calcium relations were constructed in order to determine myofilament Ca$^{2+}$-sensitivity. The length-dependent increase in myofilament Ca$^{2+}$-sensitivity (ΔEC$_{50}$, i.e. difference between EC$_{50}$ values at 1.8 and 2.2μm) did not differ between PAH and controls, indicating preserved Frank-Starling mechanism in PAH-patients (Fig.4B). No significant changes in Ca$^{2+}$-sensitivity were observed between PAH and controls, although the averaged tension-calcium curve was slightly shifted to the left in PAH (Fig. 4C). Overall, RV cardiomyocytes in PAH had a significantly higher total tension compared to control cardiomyocytes over a broad range of calcium concentrations (Fig.4D).

Second, we determined cardiomyocyte passive tension (measure of sarcomeric stiffness) in relaxing solution at increasing sarcomere lengths (1.8 to 2.6 μm). A significantly higher cardiomyocyte passive tension at different sarcomere lengths was observed in PAH compared to control cardiomyocytes (+200%; Fig.5A). The relative increase in passive tension observed in
PAH compared to control is shown in Fig. 5B. To determine the role of the actin-myosin interaction component in generating passive tension, RV cardiomyocytes were incubated with BDM and passive tension was measured before and after incubation: no change in passive tension was observed (Fig. 6A-C), only a reduction in total tension (Fig. 6D). This indicates that the increase in RV passive tension in PAH cardiomyocytes is not a consequence of residual actin-myosin interactions, but a consequence of increased RV sarcomeric stiffness derived from passive structures (titin).

**Titin isoform expression and phosphorylation**

To investigate the underlying molecular mechanism accounting for RV diastolic stiffness in PAH, we analyzed titin isoform composition and phosphorylation. Titin is a giant sarcomeric protein which regulates sarcomere compliance. Titin consists of 2 isoforms, the stiff N2B isoform and the compliant N2BA isoform. In RV samples of PAH-patients and controls, we did not observe a difference in the ratio between N2B and N2BA isoform expression (Fig. 7A). But, we did observe reduced titin phosphorylation in RV samples of PAH-patients (Fig. 7B), indicating that the observed RV sarcomeric stiffening was associated with reduced titin phosphorylation.

**Discussion**

By combining in vivo measurements of RV function in PAH-patients with functional and histological analyses of RV tissue derived of PAH-patients, we were able to demonstrate that:

1. RV diastolic stiffness is increased in PAH-patients and closely associated with markers of disease severity.

2. RV hypertrophy and collagen deposition are increased in RV tissue of PAH-patients in
comparison to controls.

3. RV cardiomyocyte passive tension at different sarcomere lengths was significantly higher in PAH-cardiomyocytes than in controls; RV cardiomyocytes exhibited preserved length-dependent activation and generated higher total tension in comparison to control RV cardiomyocytes over a broad range of calcium concentrations.

4. Titin phosphorylation was significantly reduced in RV tissue of PAH-patients in comparison to controls.

**RV diastolic stiffness in PAH**

Diastolic dysfunction is characterized by altered filling patterns, prolonged relaxation and intrinsic diastolic stiffness. Several epidemiological studies have demonstrated elevated RAP in PAH-patients. In concordance, RV imaging studies revealed altered RV filling patterns characterized by increased atrial-induced filling ("atrial kick"). In addition, prolonged RV isovolumic relaxation time has been described in PAH-patients. However, previously used measurements of diastolic function are all highly load-dependent, therefore it is still unclear whether PAH-patients suffer from true RV diastolic impairment or that the observed changes in filling and relaxation are merely a reflection of increased RV afterload.

Therefore, we investigated the presence of RV diastolic impairment in PAH-patients both *in vivo* by single-beat PV analyses, as well as by measuring RV diastolic stiffness directly in RV cardiomyocytes. Diastolic stiffness is ideally quantified from the diastolic PV relationship constructed from multiple PV loops at different loading conditions. Due to cardio-pulmonary compromise, this procedure is highly undesirable and considered too invasive in PAH. Therefore, we used the single-beat approach, a technique that has been used successfully in left heart failure studies. In our experimental PAH-model, we observed an excellent correlation
between RV diastolic stiffness derived by single- and multiple-beat approach, and therefore considered the single-beat approach as an appropriate, less invasive alternative for our patients. In addition, the finding of altered early and atrial-induced RV peak filling rate further confirmed increased RV diastolic stiffness in PAH.

**RV hypercontractility**

Interestingly, RV diastolic stiffness in PAH coincided with increased RV contractility (Ees) and force generating capacity of RV cardiomyocytes (active force). This finding is somewhat unexpected, since it is well known that PAH is associated with severe RV systolic dysfunction. It is also in contrast to earlier observations of diastolic left heart failure (or heart failure with preserved ejection fraction), where increased passive stiffness was accompanied by reduced active tension. In a previous study in PAH-rats, we did observe an increase in both diastolic stiffness and RV contractility, consistent with our findings in cardiomyocytes of PAH-patients. However, the increase in RV contractility in rats did not result in an improved RV-arterial coupling in rats, suggesting that the increase in RV contractility was insufficient to cope with the higher increase in RV afterload. Therefore, the observed increase in force generating capacity may be a compensatory mechanism attempting to cope with the increased RV afterload.

This compensatory mechanism might negatively affect the normal relaxation pattern. The “hypercontractile” sarcomeres, which are evident after combining the increase in maximal force generating capacity with higher myofilament Ca\(^{2+}\)-sensitivity and increased passive stiffness (Fig.5D), may limit myocardial relaxation during the diastolic phase and contribute to impaired diastolic function in PAH-induced right heart failure.

**Possible mechanisms causing RV diastolic stiffness in PAH**
RV diastolic stiffness was not only observed in idiopathic PAH-patients, but was also prevalent in patients with CTEPH. This indicates that RV diastolic stiffness is not specific for PAH, but could also be expected in other syndromes with increased RV pressures. Thus increased RV pressure overload could be an initial trigger for RV diastolic impairment in PAH. Nevertheless, also other factors could explain RV diastolic stiffness in PAH in vivo. We observed a 3-fold higher RV sarcomeric stiffness over the whole range of sarcomere lengths in PAH-patients compared to controls. By repeating RV sarcomeric stiffness measurements after incubation with the cross-bridge inhibitor BDM, we could rule out a contribution of remaining cross-bridge interactions on RV diastolic stiffness. A remaining factor that is likely to contribute to the high cardiomyocyte stiffness is the sarcomeric protein titin. Titin is a molecular spring that spans the half sarcomere and determines muscle stiffness in diastole. Phosphorylation and isoform composition of titin determine the elasticity of the protein and thereby passive (diastolic) stiffness of the cardiomyocytes. In this study, we revealed that titin isoform composition was unaltered in PAH-cardiomyocytes, but titin phosphorylation was significantly reduced in PAH in comparison to controls. Also extracellular factors such as RV collagen deposition might contribute to diastolic impairment, though we observed only a relatively modest increase in RV collagen deposition, which is in line with previous preclinical studies.\textsuperscript{16,17,27}

**Clinical implications**

RV diastolic stiffness was closely associated with markers of disease progression. This finding suggests that RV diastolic stiffness may represent a contributing factor involved in disease worsening and not a benign compensatory mechanism associated with increased afterload. Future therapeutic strategies targeting the reduced titin phosphorylation and increased RV collagen deposition will reveal the clinical implication of increased RV diastolic stiffness.
Limitations of the study

RV diastolic stiffness only weakly correlated with RV peak filling rate. This is comparable to earlier data in patients with heart failure with preserved ejection fraction where the direct comparison of E/A ratio (echo) with the diastolic stiffness parameter $\beta$ (conductance catheterization), showed a similar weak correlation. A possible explanation for this finding is that E/A measurements by echo or MRI are highly sensitive to the confounding effects of increased pre- and afterload. This also indicates that other factors besides RV myocardial stiffness are associated with a reduction in E/A ratio.

The majority of RV samples used in this study were from patients with PAH secondary to congenital heart disease (CHD). RV samples of patients with idiopathic PAH are difficult to procure since these patients often undergo only lung transplantation. There may be important differences in myocardial structure and function between the right ventricle of a formerly normal adult who develops idiopathic PAH and that from CHD-patients. However, both idiopathic PAH and CHD-patients were in end-stage right heart failure at time of heart/lung transplantation (NYHA IV). More importantly, subgroup analyses revealed that the increase in active force and cardiomyocyte stiffness were comparable between RV samples of idiopathic PAH and congenital heart disease.

The sample size of this study was relatively small, which may have lead to type I errors, and therefore nominal significant p-values should be interpreted with caution. However, our main finding has been confirmed by several clinical and experimental observations. Therefore, RV diastolic stiffness in PAH is not only a statistically significant finding but also physiologically plausible.
Conclusion

We demonstrated that patients with PAH have increased RV diastolic stiffness. Furthermore, we observed significant correlations between increased diastolic stiffness and disease severity. We revealed that alterations in the extracellular matrix and cardiomyocyte sarcomeres are both important contributors to increased RV diastolic stiffness in PAH-patients and may represent future treatment targets.

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Conflict of Interest Disclosures: None.

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Table 1.

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<td>PCWP (mmHg)</td>
<td>8 ± 3</td>
<td>7 ± 3</td>
<td>0.83</td>
</tr>
<tr>
<td>RAP (mmHg)</td>
<td>7 ± 6</td>
<td>3 ± 2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>86 ± 15</td>
<td>71 ± 7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NT-proBNP (pg/L)</td>
<td>1603 ± 2332</td>
<td>125 ± 155</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Years on treatment</td>
<td>4.2 ± 2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monotherapy</td>
<td>5/21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple drug therapy</td>
<td>16/21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Treatment strategies**

- Sildenafil 16/21
- Bosentan 14/21
- Epoprostenol 5/21
- Terguride 3/21
- Treprostenil 4/21
- Sitaxentan 1/21

Data presented as mean ± SD. PAH: idiopathic pulmonary arterial hypertension. BMI: body mass index. NYHA: New York Heart Association. 6MWD: six-minute-walk-distance. mPAP: mean pulmonary arterial pressure; CO: cardiac output; PVR: pulmonary vascular resistance; RVEF: right ventricular ejection fraction; PCWP: pulmonary capillary wedge pressure; RAP: right atrial pressure; HR: heart rate. NT-proBNP: N-terminal pro-hormone brain natriuretic peptide.

Table 2.

<table>
<thead>
<tr>
<th></th>
<th>PAH (n=21)</th>
<th>Controls (n=7)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Alpha (α)</td>
<td>0.003 ± 0.001</td>
<td>0.007 ± 0.002</td>
<td>0.048</td>
</tr>
<tr>
<td>Beta (β)</td>
<td>0.050 ± 0.005</td>
<td>0.029 ± 0.003</td>
<td>0.034</td>
</tr>
<tr>
<td>V₂₀ (ml)</td>
<td>281 ± 7</td>
<td>308 ± 3</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM. Alpha, curve-fitting constant; beta, diastolic stiffness constant; V₂₀, calculated volume at a common pressure of 20 mmHg based on individual derived α and β (Equation 1).
### Table 3.

<table>
<thead>
<tr>
<th>Diastolic stiffness</th>
<th>95% CI for B</th>
<th>R²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke volume</td>
<td>-2.92</td>
<td>0.71</td>
<td>0.001</td>
</tr>
<tr>
<td>6MWD</td>
<td>-11.8</td>
<td>0.59</td>
<td>0.009</td>
</tr>
<tr>
<td>RAP</td>
<td>1.01</td>
<td>0.62</td>
<td>0.001</td>
</tr>
<tr>
<td>Ln_NT-proBNP</td>
<td>0.15</td>
<td>0.59</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Multivariable linear regression corrected for body surface area, age, gender, treatment duration, pulmonary vascular resistance.

Regression coefficients presents 0.005 unit change in RV diastolic stiffness. CI, confidence interval; 6MWD, six-minute-walk-distance; RAP, right atrial pressure; Ln_NT-proBNP, log-transformed N-terminal pro-hormone brain natriuretic peptide.

### Table 4.

<table>
<thead>
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<th>RV sample</th>
<th>Diagnosis</th>
<th>NYHA Class</th>
<th>Gender</th>
<th>Age</th>
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<tr>
<td>1</td>
<td>Idiopathic PAH</td>
<td>IV</td>
<td>Female</td>
<td>38</td>
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<tr>
<td>2</td>
<td>Idiopathic PAH</td>
<td>IV</td>
<td>Female</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>Idiopathic PAH</td>
<td>IV</td>
<td>Female</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>PAH-Eisenmenger</td>
<td>IV</td>
<td>Female</td>
<td>46</td>
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<tr>
<td>12</td>
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<tr>
<td>20</td>
<td>Donor</td>
<td></td>
<td>Male</td>
<td>37</td>
</tr>
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</table>

Figure Legends:

Figure 1. Increased RV diastolic stiffness in PAH-patients. A. Illustration of the methodology: End-diastolic volume (EDV) was combined with end-diastolic pressures (EDP), and end-systolic volume (ESV) with begin-diastolic pressures (BDP). PV relations were constructed by fitting a non-linear exponential curve through the diastolic PV points using the following formula: \( P = \alpha (e^{\beta V} - 1) \), where \( P \): pressure; \( \alpha \): curve-fitting constant; \( \beta \): diastolic stiffness constant; \( V \): volume. B. Average diastolic PV relations in control and PAH-patients show a steeper curvature in PAH compared to controls. C. Diastolic stiffness coefficient (\( \beta \)) measured by the single-beat protocol is significantly increased in PAH-patients compared to controls. D. Non-invasive assessment of RV early (E) and atrial (A) induced peak filling rate (E/A ratio) confirmed increased RV diastolic dysfunction in PAH-patients in comparison to controls. E. RV diastolic stiffness coincided with increased RV end-systolic elastance (Ees) in PAH-patients. Data presented as mean±SEM, n=21 PAH, n=7 controls. *: \( p<0.05 \); ***: \( p<0.001 \).

Figure 2. Diastolic stiffness correlates with disease severity. RV diastolic stiffness was inversely correlated to stroke volume (A) and six-minute-walk-distance (B). In addition, a close correlations were found between RV diastolic stiffness and right atrial pressure (C) and NT-proBNP (D). 6MWD, six-minute-walk-distance; RAP, right atrial pressure; Ln_NT-proBNP, log-transformed N-terminal pro-hormone brain natriuretic peptide.

Figure 3. RV hypertrophy and fibrosis. A. RV hypertrophy was significantly increased in PAH compared to controls. Representative images of histological sections of RV specimens are shown
of a control subject and a PAH-patient, stained for laminin (green) and DAPI (nuclei, blue). B. A significant increase in RV fibrosis was found in RV tissue sections of PAH-patients compared to controls. Representative images of histological sections of RV specimens are shown of a control subject and a PAH-patient, stained for collagen (picrosirius red, under double-polarized light). Data presented as mean ± SEM, n=10 PAH, n=9 controls. **: p<0.01, ***: p<0.001. CSA, cross-sectional area.

**Figure 4.** RV cardiomyocyte force measurements. A. Active tension at maximal calcium concentration was higher in PAH-patients compared to control cardiomyocytes at both 1.8 and 2.2 μm, although the difference was only significant at 2.2 μm sarcomere length (2 comparisons). B. Stretching RV cardiomyocytes from a sarcomere length of 1.8 to 2.2 μm resulted in a similar increase in myofilament Ca\(^{2+}\)-sensitivity in both PAH and control samples, indicating that the Frank-Starling mechanism was preserved in PAH (2 comparisons). C. In PAH, averaged tension-Ca\(^{2+}\) curve was slightly shifted to the left, indicating a small increase in myofilament Ca\(^{2+}\)-sensitivity (7 comparisons). D. At different Ca\(^{2+}\)-concentration, RV cardiomyocyte total tension was significantly higher in RV cardiomyocytes of PAH-patients than controls (7 comparisons). Data presented as mean ± SEM, n=7 PAH, n=7 controls. *: p<0.05, ***: p<0.001, Bonferroni corrected. Con, control; EC\(_{50}\), calcium concentration at which 50% of force is produced; [Ca\(^{2+}\)], calcium-concentration.

**Figure 5.** RV cardiomyocyte passive stiffness. A. Passive tension was significantly higher in RV cardiomyocytes of PAH-patients than controls (5 comparisons). B. A significant increase in passive tension in PAH compared to controls (set at 100%) was found at all sarcomere lengths (5
comparisons). Data presented as mean ± SEM, n=7 PAH, n=7 controls. *: p<0.05, **: p<0.01, ***: p<0.001, Bonferroni corrected.

Figure 6. Role of actin-myosin interaction in RV cardiomyocyte passive stiffness. No change in RV passive tension was observed upon 2,3-butanedione monoxime (BDM) incubations in controls (A&C) and PAH (B&C), indicating that the higher passive tension in PAH cardiomyocytes is not a consequence of residual actin-myosin interactions. BDM incubation resulted in a significant reduction of total tension in both PAH and control (D). Data presented as mean ± SEM, n=7 PAH, n=7 controls. *: p<0.05, ***: p<0.001

Figure 7. Titin isoform composition and phosphorylation. A. Expression of the stiff N2B and compliant N2BA titin isoform was similar in RV tissue of PAH-patients and controls, indicating no alterations in titin isoform composition. B. Reduced titin phosphorylation in RV tissue of PAH-patients in comparison to controls. The typical example of the gel electrophoresis illustrates reduced titin phosphorylation in RV tissue of PAH-patients. Data presented as mean ± SEM, n=10 PAH, n=9 controls. *: p<0.05.
Figure 1
Figure 2
Figure 3

(A) RV hypertrophy

(B) RV fibrosis

Control  PAH

CSA (μm²)

% Collagen

Control  PAH

500

***

250

0

Control  PAH

15

***

10

**

5

0

100μm

50μm

100μm

50μm
Figure 4

A. Active Tension

- Interaction sarcomere length * disease
  - p = 0.18

B. Ca²⁺-sensitivity

- Interaction disease * sarcomere length
  - p = 0.10

C. Relative force-calcium relation (sarcomere length 2.2 μm)

- Interaction [Ca²⁺] * disease
  - p = 0.054

D. Total tension-calcium relation (sarcomere length 2.2 μm)

- Interaction [Ca²⁺] * disease
  - p < 0.001
Figure 5

A. RV cardiomyocyte stiffness

B. RV cardiomyocyte stiffness

Interaction sarcomere length*disease
p<0.05

Passive tension (kN/m)

Relative passive tension (% of control)

Sarcomere length (μm)

1.8 2.0 2.2 2.4 2.6

Con PAH

1.8μm 2.0μm 2.2μm 2.4μm 2.6μm

Con Con Con Con Con

PAH PAH PAH PAH PAH

*** *** *** *** ***

**
**Figure 6**

A. **RV Cardiomyocyte Stiffness in Control after BDM Incubation**

B. **RV Cardiomyocyte Stiffness in PAH after BDM Incubation**

C. **Effect of Cross Bridge Inhibitor BDM on RV Cardiomyocyte Stiffness**

D. **RV Cardiomyocyte Total Tension After BDM Incubation**

Interaction disease*BDM incubation p=0.57
Figure 7

A. Titin isoform composition

B. Titin Phosphorylation

[Graphs and data showing comparisons between control and PAH groups for Titin isoform composition and Titin Phosphorylation.]
Right Ventricular Diastolic Impairment in Patients with Pulmonary Arterial Hypertension


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SUPPLEMENT MATERIAL

Right Ventricular Diastolic Impairment in Patients with Pulmonary Arterial Hypertension

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Supplemental METHODS

Single-beat method

Experimental Model. To experimentally support the single-beat method we performed studies in a rat model of PAH. All animal experiments were approved by the Institutional Animal Care and Use Committee of the VU University Amsterdam, The Netherlands. The study was performed in 15 Male Wistar rats. Pulmonary Arterial Hypertension (PAH) was induced by a single dose monocrotaline (60mg/Kg) subcutaneously injected (n= 9). Rats used as controls received a saline injection (n=6). The study was ended 31 days after monocrotaline or saline injection or after development of manifest right heart failure.51

Echocardiography. Transthoracic Doppler Ultrasound (ProSound SSD-4000 system equipped with 13MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) was performed in all spontaneously breathing rats under general anesthesia at the end of the study (isoflurane 2.0% in 1:1 O₂/air mix, Pharmachemie, Haarlem, The Netherlands).

Right ventricular function was measured by the following parameters: Doppler derived stroke volume, cardiac output and tricuspid annular plane systolic excursion (TAPSE). Right ventricular morphology was assessed by the RV end diastolic diameter and RV wall thickness.52

Invasive RV pressure-volume analysis. After transthoracic echocardiography, cardiac function was assessed invasively by performing right heart catheterization with dual pressure – volume catheters. Rats underwent general anesthesia by isoflurane inhalation (induction: 4.0% in 1:1 O₂/air mix; maintenance: 2.0% in 1:1 O₂/air mix), were intubated (16G Teflon tube) and mechanically ventilated with a frequency of 75/min, at a pressure of 9/0 cmH₂0 and 1:1 inspiratory/expiratory ratio (Micro-Ventilator, UNO, Zevenaar, The Netherlands). During the procedure body temperature was maintained at normal values by placing the rats on warming pads. The thorax was then open and the inferior vena cava was encircled by performing a loose ligature around its trunk. The apex of the heart was then pierced with a needle (23G), a cotton swap was used to stop the hemorrhage and the combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle.53
Pressure-volume loops were recorded at rest and after preload reduction secondary to vena cava gradual occlusion (VCO). Analysis was made off line using custom-made algorithms (programmed in MATLAB 2007b, The MathWorks, Natick MA). Doppler ultrasound derived stroke volume was used to convert catheter volume units in milliliters. One catheterization unit was calculated to its corresponding volume (ml) by dividing the ultrasound obtained stroke volume (ml) by the catheterization stroke volume (units). Catheterization stroke volume was previously obtained from subtracting the end systolic volume (units) from the end diastolic volume (units). Due to procedure limitations to record absolute RV volumes (End Systolic Volume and End Diastolic Volume), only changes in volume could be measured, all end diastolic volumes were normalized at 1.5ml and end systolic volumes were calculated by subtracting the stroke volume (ml) from the reference point (1.5ml).

To quantify RV diastolic stiffness, multiple pressure-volume loops were recorded with the pressure-volume catheter placed in the right ventricle, both at steady state and during vena cava occlusion. The diastolic pressure-volume relation was then constructed using an exponential fit (Equation 1: \( P = a(e^{\beta v} - 1) \)) through the decreasing pressure-volume points (after vena cava occlusion) and the diastolic stiffness factor \( \beta_{\text{multiple}} \) was calculated. The same equation was used to calculate RV diastolic stiffness \( \beta_{\text{single}} \) from a single beat pressure-volume loop (recorded before vena cava occlusion was started, at steady-state). For this exponential fit only 3 points were used: 1) 0\text{pressure},0\text{volume} point, 2) begin diastolic point and 3) end diastolic point.

The classical pressure-volume relation implies the construction of an exponential pressure-volume curve through decreasing pressure-volume points. Furthermore, the pressure-volume relation is considered to intersect the volume axes at pressure=0mmHg and a certain intercept volume \( (V_d) \). To calculate \( \beta_{\text{single}} \), \( V_d \) was set to 0. Although physiologically inexact, we considered the 0\text{pressure},0\text{volume} point as a satisfactory substitute for the intercept since:

1. 0\text{volume} is always lower or equal to \( V_d \)
2. Extending the diastolic exponential pressure-volume curve to volumes lower than the $V_d$ (undetermined value) does not modify the exponential term $\beta$ (further used to quantify RV diastolic stiffness). See also Fig.S1

**Supplemental RESULTS**

**RV diastolic stiffness**

RV Diastolic stiffness obtained with the classical method using multiple pressure-volume loops ($\beta_{\text{multiple}}$) was compared to diastolic stiffness obtained from a single beat steady-state loop ($\beta_{\text{single}}$). Since no significant difference was found between the methods (Fig. S2), we further used the single beat approach for the clinical setting, where vena cava occlusion and multiple pressure-volume loops recording are contraindicated.

**Right ventricular cardiomyocyte force measurements**

To investigate whether resting sarcomere length was different between PAH and control tissue samples, we randomly selected 10 isolated RV cardiomyocytes for each control and PAH tissue sample. Resting sarcomere length was optically determined in at least two distinct areas of the cell and the average cellular sarcomere length was calculated. No significant difference in resting sarcomere length was found between control and PAH cardiomyocytes (Fig. S3)

To investigate whether our findings of increased RV active force and cardiomyocyte stiffness differ between RV samples obtained from patients with idiopathic PAH or PAH secondary to congenital heart disease, we performed a subgroup analyses. As can be observed in Fig. S4, the increase in RV active force and RV cardiomyocyte stiffness was comparable among the groups.
**Supplemental FIGURE LEGENDS**

**Figure S1**
A&C. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (after vena cava obstruction). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points.

B&D. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (after vena cava obstruction). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points and the pressure=0mmHg – volume=0ml point.

**Figure S2**
A&B. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (continuous line) and from a single-beat steady-state pressure-volume loop (dotted line). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points ($\beta_{\text{multiple}}$), respectively through the end-diastolic, end-systolic and pressure=0-volume=0 points ($\beta_{\text{single}}$).

C. Method correlation between diastolic stiffness $\beta_{\text{multiple}}$ and $\beta_{\text{single}}$ in control rats and PAH rats.

**Figure S3**
Right ventricular resting sarcomere length determined in control and PAH isolated skinned cardiomyocytes. p=0.56

**Figure S4**
Measures of right ventricular active force and stiffness do not differ between right ventricular cardiomyocytes obtained from patients with idiopathic pulmonary arterial hypertension or congenital heart disease.

Data presented as mean ± SEM. iPAH, idiopathic pulmonary arterial hypertension; CHD, congenital heart disease.
Supplemental FIGURES
Figure S1

Control Rat

A. 

Pressure (mmHg)

Volume (ml)

β=1.22±0.07
R²=0.97

B. 

Pressure (mmHg)

Volume (ml)

β=1.22±0.07
R²=0.99

PAH Rat

C. 

Pressure (mmHg)

Volume (ml)

β=6.68±0.01
R²=0.97

D. 

Pressure (mmHg)

Volume (ml)

β=6.68±0.01
R²=0.99
Figure S2

A. **Diastolic Pressure Volume Relation in Control Rat**

B. **Diastolic Pressure Volume Relation in PAH Rat**

C. **Diastolic Pressure Volume Relation**

- \[ \beta_{\text{multiple}} = 2.13 \]
- \[ \beta_{\text{single}} = 2.08 \]

- \[ \beta_{\text{multiple}} = 10.75 \]
- \[ \beta_{\text{single}} = 10.10 \]

- \[ R^2 = 0.94 \]
- \[ \text{Slope} = 1.1 \ (0.95 - 1.29) \]
- \[ p < 0.001 \]
Figure S3

RV cardiomyocyte
resting sarcomere length

<table>
<thead>
<tr>
<th>Control</th>
<th>PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.74 ± 0.01</td>
<td>1.72 ± 0.02</td>
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</tbody>
</table>
Figure S4

**Active Tension CHD vs. iPAH**

- Interaction sarcomere length*disease
  - p=0.417

**RV cardiomyocyte stiffness iPAH vs. CHD**

- Interaction sarcomere length*disease
  - p=0.997
Supplemental REFERENCES


