Neuregulin-1 improves right ventricular function and attenuates monocrotaline-induced pulmonary arterial hypertension

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Abstract:	Aims: Pulmonary arterial hypertension (PAH) is a serious disease that affects both the pulmonary vasculature and the right ventricle (RV). Current treatments options are insufficient. The cardiac neuregulin (NRG)-1/ErbB system is deregulated during heart failure, and treatment with recombinant human NRG-1 (rhNRG-1) has been shown to be beneficial in animal models and in patients with left ventricle (LV) dysfunction. This study aimed to evaluate the effects of rhNRG-1 in RV function and pulmonary vasculature in monocrotaline-induced PAH and RV hypertrophy (RVH). Methods and Results: Male wistar rats (7-8 week old, n=78) were injected with monocrotaline (MCT, 60 mg/kg, s.c.) or saline and treated with rhNRG-1 (40 µg/kg/day) or vehicle for 1 week, starting 2 weeks after MCT administration. Another set of animals was submitted to pulmonary artery banding (PAB) or sham surgery, and followed the same protocol. MCT administration resulted in the development of PAH,

	pulmonary arterial and RV remodelling and dysfunction, and increased RV markers of cardiac damage. Treatment with rhNRG-1 attenuated RVH, improved RV function and decreased RV expression of disease markers. Moreover, rhNRG-1 decreased pulmonary vascular remodelling and attenuated MCT-induced endothelial dysfunction. The anti-remodelling effects of rhNRG-1 were confirmed in the PAB model, were rhNRG-1 treatment was able to attenuate PAB-induced RVH. Conclusion: rhNRG-1 treatment attenuates pulmonary arterial and RV remodelling and dysfunction in a rat model of monocrotaline-induced PAH, and has direct anti-remodelling effects on the pressure-overloaded RV.

Neuregulin-1 improves right ventricular function and attenuates monocrotaline-induced pulmonary arterial hypertension

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ABSTRACT

Aims: Pulmonary arterial hypertension (PAH) is a serious disease that affects both the pulmonary vasculature and the right ventricle (RV). Current treatments options are insufficient. The cardiac neuregulin (NRG)-1/ErbB system is deregulated during heart failure, and treatment with recombinant human NRG-1 (rhNRG-1) has been shown to be beneficial in animal models and in patients with left ventricle (LV) dysfunction. This study aimed to evaluate the effects of rhNRG-1 in RV function and pulmonary vasculature in monocrotaline-induced PAH and RV hypertrophy (RVH).

Methods and Results: Male wistar rats (7-8 week old, n=78) were injected with monocrotaline (MCT, 60 mg/kg, s.c.) or saline and treated with rhNRG-1 (40 µg/kg/day) or vehicle for 1 week, starting 2 weeks after MCT administration. Another set of animals was submitted to pulmonary artery banding (PAB) or sham surgery, and followed the same protocol. MCT administration resulted in the development of PAH, pulmonary arterial and RV remodelling and dysfunction, and increased RV markers of cardiac damage. Treatment with rhNRG-1 attenuated RVH, improved RV function and decreased RV expression of disease markers. Moreover, rhNRG-1 decreased pulmonary vascular remodelling and attenuated MCT-induced endothelial dysfunction. The anti-remodelling effects of rhNRG-1 were confirmed in the PAB model, were rhNRG-1 treatment was able to attenuate PAB-induced RVH.

Conclusion: rhNRG-1 treatment attenuates pulmonary arterial and RV remodelling and dysfunction in a rat model of monocrotaline-induced PAH, and has direct anti-remodelling effects on the pressure-overloaded RV.

Porto, September 22nd 2015

Dear Editor,

Please find enclosed the **revised manuscript - MS # CVR-2015-820** now entitled **"Neuregulin-1 improves right ventricular function and attenuates experimental pulmonary arterial hypertension"** (previously entitled "Neuregulin-1 improves right ventricular function and attenuates monocrotaline-induced pulmonary arterial hypertension") by Mendes-Ferreira P, Maia-Rocha C, Adao R, Mendes MJ, Santos-Ribeiro D, Alves BS, Cerqueira RJ, Castro-Chaves P, Lourenço AP, De Keulenaer GW, Leite-Moreira AF, Brás-Silva C, submitted to Cardiovascular Research. This study aimed to evaluate the effects of neuregulin (NRG)-1 on right ventricular function and the pulmonary vasculature in a rat model of pulmonary arterial hypertension. We found that NRG-1 has an important role in pulmonary arterial hypertension and right ventricular dysfunction and that NRG-1 treatment improves both cardiopulmonary function and structure. NRG-1 decreases pulmonary artery remodelling, improves endothelial function, and restores right ventricular function. These beneficial effects may improve outcome in pulmonary arterial hypertension.

Please see the attached file with the point-by-point reply to the reviewers.

Concerning author contribution: Carmen Brás-Silva (CBS), Gilles De Keulenaer (GDK), Pedro Mendes-Ferreira (PMF), and Adelino Leite-Moreira (ALM) designed research; PMF, Carolina Maia-Rocha (CMR), Rui Adão (RA), Maria José Mendes (MJM), Diana Santos-Ribeiro (DSR), Bárbara Silvana Alves (BSA), Rui João Cerqueira (RJC), Paulo Castro-Chaves (PCC), André Pedro Lourenço (APL), and CBS conducted research; PMF, CMR, RA, CBS analyzed data; PMF, GDK and CBS wrote the paper.

The manuscript, or part of it, has neither been published (except in form of abstract or thesis) nor is currently under consideration for publication by any other journal. All authors have read the manuscript and approved its submission to Cardiovascular Research.

We agree to pay the costs of printing the colour figures present in this manuscript, as required by Cardiovascular Research.

Sincerely yours,
Carmen Bras Silva

ANSWER TO REVIEWER COMMENTS

(Manuscript MS # CVR-2015-820)

We thank the reviewers for their bright and detailed analysis of our work, which helped us to improve the manuscript. All the comments were taken into account in the revised version of the manuscript.

REVIEWER 1:

Reviewer #1 Comments:

The authors addressed the concerns.

Minor comment:

The authors appropriately point out in the title - this study is about "monocrotaline-induced pulmonary arterial hypertension" in the rat.

I would ask for the authors to add this text to all conclusions in the Title, Abstract, Text, and Figures "monocrotaline-induced pulmonary arterial hypertension in the rat".

ANSWER:

We agree with the reviewer and have adapted the text accordingly, except in the title once we followed the suggestion of the Editor to rewrite the title.

REVIEWER 3:

Reviewer #3 Comments:

The revised manuscript is significantly improved. The authors have done significant work to condense findings into a much more logical story and have removed redundant and conflicting data. I have only 2 remaining comments:

1) In your rebuttal to Reviewer 3, answer 3 - you state that "with our echocardiographic analysis, were not able to quantify the tricuspid regurgitation gradient (only its presence), and therefore we could not quantify PASP." However, in Figure 1, panel F, you have reported tricuspid insufficiency in m/sec, not by grade, degree or volume. The trans-tricuspid velocity is a reflection of the trans-tricuspid gradient, not the degree of tricuspid insufficiency. Furthermore, this gradient can be calculated from the peak velocity by 4V^2. Therefore, you should clarify both the language used to describe this data (i.e.. you cannot say that tricuspid insufficiency improved, but you can say that the trans-tricuspid gradient decreased, likely reflecting a decrease in RVSP and, by extrapolation, PASP). For example, you cannot say (as on p. 14):

"Dilation of the tricuspid

264 annulus resulted in tricuspid regurgitation (Figure 1 - F), which, together with the

265 aforementioned pathological heart remodelling (RV and RA increase and IVS thickening),

266 observed in MCT animals through echocardiography, was restored by rhNRG-1 treatment." This implies that you have quantitated the degree or amount of TI.

Please discuss this data further with your local ultrasound expert and revise text appropriately.

2) Some small additional discussion of the findings of lung edema is warranted. You state in your discussion:

"Our data (Figure S2) shows that,

435 despite maintained RV function, 14 days after MCT administration animals develop RV

436 hypertrophy, lung oedema, and compromised pulmonary flow, which is not different from

437 MCT animals at the end of the protocol."

It is unclear what mechanism might be at play to cause this lung edema - cardiac or pulmonary vascular. Though you have done a nice job attempting to explain that the inflammatory markers do not point to lung inflammation as a primary mediator of the improvement after NRG treatment, the absence of RV dysfunction (which alone should not cause pulmonary edema anyway) and therefore unlikely LV filling (preload) issues to cause high LVEDP seem to point to primary lung inflammation as a cause for this edema.

ANSWER:

1) We fully agree with the reviewer. In fact by measuring peak velocity we cannot state that the degree of tricuspid regurgitation was decreased, but as suggested, we can only state that the gradient was decreased. The intention of the statement cited by the reviewer above was to indicate that a decrease in RVEDD (as measured by the tricuspid annulus, shown in Figure 1) in MCT animals treated with NRG-1 (MN group), and therefore a decrease in RV dilation might account for the decrease in the tricuspid regurgitation gradient.

Due to a lack of attention, the tricuspid insufficiency quantified in m/sec was mistakenly interpreted as percentage of tricuspid regurgitation. The echocardiographic machine used at the moment, the Acuson Sequoia 512 with a 15MHz (15L8) probe, does not have continuous wave Doppler capabilities and therefore does not allow us to quantify high velocity flows, as did the machine used when the evaluations presented in this manuscript were performed, the GE Vivid 7, which unfortunately is not at our disposal at the moment. Despite that fact, it

does not excuse the mistake presented in the previous version of the manuscript and reply to the reviewers, and we apologize for that.

The reviewer is completely correct in stating that the trans-tricuspid velocity is a reflection of the trans-tricuspid gradient, and therefore can be used to estimate PASP, through the Bernoulli equation (4V^2). Despite this observation, taking the data presented in Figure 1, panel F, the tricuspid retrograde flow quantified by our echocardiographic analysis was 2.14 ± 0.53 m/sec in the MCT group, and 0.6 – 0.62 m/s in all the other groups, which would account for approximately (applying the equation) 18 mmHg in the MCT group, and 1.5 mmHg in all the other groups, which is not realistic and does not reflect the catheterization data.

Taking this into account, and previously published data by others (Koskenvuo et al, Int J Cardiovasc Imaging (2010) 26:509–518) showing that no detectable tricuspid regurgitation is present at baseline before MCT administration, we believe that tricuspid regurgitation quantification was not properly quantified, resulting in an underestimation of tricuspid regurgitation, and a misinterpretation of the data. Previous Figure 1-panel F was thus removed from the revised version of the manuscript.

2) The statement has been rephrased for the sake of clarification. Only pulmonary flow measurements were not different between 14 and 21 days after MCT. All the other parameters presented (i.e. RV hypertrophy and lung oedema) are further increased one week later.

Again the reviewer is correct and we agree with what was suggested. An early inflammatory response to MCT is most likely the mechanism responsible for the increased lung oedema observed at 14 days after MCT administration. Despite this fact, we believe that "inflammation-induced oedema" is already established at 14 days after MCT administration, and therefore the aggravation of this oedema observed at 21 days after MCT might be accounted to RV dysfunction with higher LV filling pressures. While NRG-1 treatment does not attenuate lung inflammation, as described in our data, it improves both RV and LV function, leading to a decrease in the Lung/TL ratio in the MCT animals treated with rhNRG-1.

In fact the Lung/TL ratio is significantly higher 3 weeks after MCT when compared to 2 weeks after MCT administration (M14D vs M21D: 0.55 ± 0.03 vs 0.71 ± 0.03, p=0.0039), while treatment with rhNRG-1 decreases the Lung/TL ratio to the same value as in 2 weeks after MCT administration (M14D vs MN: 0.55 ± 0.03 vs 0.61 ± 0.02, p=0.1762). This further corroborates that the role of rhNRG-1 in attenuating lung oedema is due to an overall improvement in cardiac function, and not due to an attenuation of the inflammatory response observed in the MCT animals, which seems to be established prior to the beginning of treatment (2 weeks after MCT administration).

EDITOR:

Editor Comments:

With regard to the title, the journal does not request inclusion of animal species and we'll accept the general statement. In this regards, some re-considering of the title would be good as now the PAB is barely noticeable while these data enhance the broader relevance of the study.

ANSWER:

The title was rewritten as suggested by the editor.

CVR-2015-820

Neuregulin-1 improves right ventricular function and attenuates experimental pulmonary arterial hypertension

Short title: NRG-1 improves pulmonary arterial hypertension

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ABSTRACT

Aims: Pulmonary arterial hypertension (PAH) is a serious disease that affects both the pulmonary vasculature and the right ventricle (RV). Current treatments options are insufficient. The cardiac neuregulin (NRG)-1/ErbB system is deregulated during heart failure, and treatment with recombinant human NRG-1 (rhNRG-1) has been shown to be beneficial in animal models and in patients with left ventricle (LV) dysfunction. This study aimed to evaluate the effects of rhNRG-1 in RV function and pulmonary vasculature in monocrotaline-induced PAH and RV hypertrophy (RVH).

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Conclusion: rhNRG-1 treatment attenuates pulmonary arterial and RV remodelling and dysfunction in a rat model of monocrotaline-induced PAH, and has direct anti-remodelling effects on the pressure-overloaded RV.

Key words: pulmonary hypertension, right ventricular function, neuregulin, endothelial dysfunction, cardiac hypertrophy

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by pulmonary arterial remodelling, elevated pulmonary vascular resistance, increased right ventricular (RV) afterload and RV failure[1]. RV adaptation to loading and RV function are main predictors of outcome in PAH[2].

Current treatment of PAH consists of prostanoids, endothelin-1 antagonists and phosphodiesterase inhibitors. These therapeutic interventions target pulmonary vascular endothelial dysfunction and pulmonary arterial vasoconstriction. Despite some clinical successes with these therapies, PAH remains a severe disease[3]. Thus, new therapies for PAH should protect against RV maladaptation and failure[4]. The mechanisms of RV dysfunction in PAH are, however, complex and multifactorial. Most likely, these mechanisms go beyond a mechanical overload[5] and may be more systemic[6].

The NRG-1/ErbB system is critical for cardiac development and is activated at an early stage of compensated heart failure, in conditions of myocardial stress, and decreases with disease progression and decompensation[7, 8]. NRG-1 acts through transmembrane tyrosine kinase receptors of the ErbB family that dimerize upon binding of NRG-1 to ErbB3 or ErbB4, leading to phosphorylation and downstream signalling. NRG-1 is released from cardiac endothelial cells, whereas ErbB2 (co-receptor), ErbB3 and ErbB4 receptors are expressed in cardiomyocytes and cardiac fibroblasts[9, 10].

Administration of NRG-1 ameliorates cardiac dysfunction and reduces the mortality in several models of left ventricular (LV) failure[11]. Treatment with NRG-1 improves LV function in volume overload[12] , doxorubicin-induced LV dysfunction[13], and in ischemic[14] and diabetic cardiomyopathy[15]. These findings have led to clinical trials, that showed efficacy and safety of NRG-1 in improving LV function in patients with heart failure[16, 17].

Apart from its role in endothelium-cardiomyocyte cross-talk[18], NRG-1 also reduces neointimal hyperplasia following vascular injury and inhibits proliferation of vascular smooth muscle cells[19], having a protective role in both smooth muscle and endothelial cells[20]. These observations are relevant, since neointima formation and smooth muscle cell proliferation in pulmonary vessels are a hallmark of PAH[1].

Based on the actual knowledge described above, we hypothesize that by treating monocrotaline (MCT)-induced PAH animals with exogenous NRG-1 we might protect not only lung vessels, but also the RV and thus attenuate MCT-induced PAH and improve RV and overall myocardial function. In the present study, we evaluated the functional and structural effects of the administration of recombinant human NRG-1 (rhNRG-1) on the heart and pulmonary vessels in MCT-induced PAH in rats. In order to distinguish cardiac-specific actions from its effects on the pulmonary vasculature, rhNRG-1 treatment was also studied in an experimental model of pressure overload by pulmonary artery banding (PAB), which results in RV loading without PAH.

MATERIALS AND METHODS

All the procedures in this work followed the recommendations of the Guide for the Care and Use of Laboratory Animal, published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 2011), are certified by the Portuguese Veterinary Governmental Association, approved by the Portuguese Foundation for Science and

Technology (PTDC/SAU-FCF/100442/2008) and approved by the faculty ethical committee (0420/000/000/2010). All animal handling was performed by trained researchers, certified with a Laboratory Animal Sciences course according to the Federation of European Laboratory Animal Science Associations. A detailed description of methods is presented in Supplementary material.

Animal models and experimental design

Seven-to-eight week-old male Wistar rats (Charles River Laboratories) weighing 180–200g, were randomly assigned to receive a subcutaneous injection of 60mg/kg monocrotaline (MCT, Sigma-Aldrich) or an equal volume of vehicle. Two weeks (14 days) after administration, rats were assigned to receive 40μg/kg rhNRG-1 i.p. (Peprotech) or vehicle daily during 1 week, resulting in 4 groups: Ctrl+vehicle (Group C, n=16); Ctrl+rhNRG-1 (Group CN, n=14); MCT+vehicle (Group M, n=24); MCT+rhNRG-1 (Group MN, n=24). In order to determine if MCT-induced PAH was already present prior to treatment, an additional group underwent the same experimental protocol and was evaluated at an earlier time point (14 days).

Another group of animals was submitted to pulmonary artery banding (PAB), and submitted to the same randomization, time points, and chronic treatment protocol (see supplementary methods), resulting in 4 groups: Sham+vehicle (Group S, n=8); Sham+rhNRG-1 (Group SN, n=7); PAB+vehicle (Group B, n=8); PAB+rhNRG-1 (Group BN, n=10). Applying a 1.65 mm pulmonary artery constriction resulted in a degree of hypertrophy and RV overload identical to the MCT-induced PAH model (see Figure S1).

Three weeks (21 days) after MCT and PAB, rats were submitted to echocardiographic (MCT protocol) and hemodynamic evaluation, with subsequent sample collection for *in vitro* functional studies, morphological, histological and molecular analysis.

Echocardiography

Rats were anesthetized with an i.p. injection of ketamine/xylazine (75mg/kg and 10 mg/kg, respectively). Echocardiographic evaluation was performed using a 12 MHz probe (GE Healthcare) and a General Electrics Vivid 7 echocardiograph (GE Healthcare). The echocardiographic parameters assessed included: PA acceleration and ejection time (PAAT and PAET, respectively), PA velocity-time integral (PAVTI), RV diastolic dimension (RVDD), right atrium area (RAA), and interventricular septum diastolic dimension (IVSDD).

Invasive hemodynamic assessment

As previously described[21], rats were sedated (100 μg/kg and 5 mg/kg i.p., fentanyl and midazolam, respectively) and anesthetized (inhalation of 8% sevofluorane for induction and 2-3.5 % for maintenance) and intubated. Using an open chest approach pressure-volume catheters were introduced in the RV and LV (SPR-869 and SPR-847, respectively, Millar Instruments). A flow probe was implanted around the ascending aorta (MA2.5PSB, Transonic Systems). Baseline and inferior vena cava occlusion recordings were obtained with ventilation suspended at end-expiration. Pressure and volume signals were continuously acquired (MPVS Ultra, Millar Instruments), digitally recorded (PowerLab 16/30, ADInstruments) and analyzed off-line (LabChart 7 Pro, ADInstruments). Parallel conductance was computed after hypertonic saline bolus.

Sample collection and morphometric analysis

Following anaesthetic overdose, and immediately after exsanguination, heart and lungs were excised. RV free wall, LV + septum (LV+S), and lungs were dissected and weighed separately. Tibial length (TL) was used for normalization. RV samples were collected, snap

frozen in liquid nitrogen and stored at -80 ºC. For mRNA quantification, samples were submerged in RNA stabilization reagent (RNAlater, Qiagen) and for histological analysis samples were stored in buffered 10% formaldehyde.

Evaluation of RV and Lung remodelling

After fixation, histological samples were embedded in paraffin and sections were obtained from RV, lung and isolated arterial rings. Haematoxylin and eosin (HE) staining was used to quantify cardiomyocyte and pulmonary artery morphology, Picro Sirius Red staining was used to quantify RV fibrosis, and Verhoeff–Van Gieson staining was used to measure isolated arterial rings remodelling. Sections were digitally photographed (Olympus XC30, Olympus) and measured using imaging software (Cell^B, Olympus). Pulmonary artery medial wall thickness was expressed as follows: %WT = [(Medial wall thickness×2)/Arterial external diameter] x 100.

Assessment of isolated pulmonary artery endothelial function Second generation pulmonary arteries (200-400 µm diameter) were dissected from the left upper lobe of rats. Arterial rings were isolated, and mounted in a bath myograph system (720MO, DMT). Maximum tension development was assessed with 80 mM KCl solution, and a dose-response curve to acetylcholine was attained (10^{-9} to 10^{-5} M, in 0.5 logarithmic units intervals), after pre-contraction with phenylephrine (10^{-5} M). At the end of the protocol, the arterial rings were collected for histological evaluation of pulmonary arterial remodelling of large diameter vessels. Maximal relaxation to acetylcholine (Emax), and the concentration of acetylcholine required for 50% of the maximal response (EC50) were calculated.

Quantitative RT-PCR, immunoblot and cytokine ELISA

RV mRNA expression of NRG-1, B-type natriuretic peptide (BNP), caspase-3, endothelin-1 (ET-1), hypoxia inducible factor 1 alpha subunit (HIF-1α), interleukin 6 (IL-6), (NRG-1) and tumor necrosis factor alpha (TNF-α) was quantified (primer sequences in Table S1). Lung mRNA expression of NRG-1 was also quantified. Total mRNA was extracted using the RNeasy kit according to manufacturer's instructions (Qiagen). Two-step RT-PCR was used for relative mRNA quantification (Step-One^{TM}, Applied Biosystems). Results are expressed in arbitrary units (AU), normalized to GAPDH, which did not differ between groups.

Blood was collected and centrifuged in EDTA-containing tubes for plasmatic quantification of IL-6 and TNF-α concentrations, using solid phase sandwich Enzyme-Linked-Immuno-Sorbent Assay (ELISA) according to manufacturer's instructions (Rat IL-6 ELISA Kit and Rat TNF-α ELISA Kit, Invitrogen).

In order to determine rhNRG-1 activity, RV total protein was extracted from acutely treated animals and separated in a 10% SDS-PAGE gel, electro-blotted into nitrocellulose membrane and probed for ErbB4 (ErbB4 (C-18): sc-283, SantaCruz Biotechnology) and phospho-ErbB4 (Phospho-HER4/ErbB4 (Tyr1284)(21A9) #4757, Cell Signaling Technologies).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). 2-way ANOVA was used to statically analyze all the presented parameters (which followed a normal distribution).Endothelial function was analysed with a 2-way repeated measures ANOVA test, and the comparison of control and MCT animals 14 days after MCT administration was analysed with the t-student. Holm-Sidak's method was performed for post hoc comparisons between groups. Group data are presented as means ± SEM and differences with $p<0.05$ were considered statistically significant.

RESULTS

RhNRG-1 improves pulmonary arterial flow and attenuates cardiac and pulmonary arterial remodelling in MCT-induced PAH

MCT-induced PAH results in a decrease in PAAT and PAVTI with a midsystolic decrease in PA flow, RV dilation, and IVS hypertrophy and flattening[22]. Accordingly, MCT animals presented altered PA flow, with faster acceleration and a consequent decrease in the PAAT/PAET ratio (Figure 1 – A), a midsystolic notch (Figure 1 – F, white arrowhead) and decreased PAVTI (Figure 1 – B), representative of decreased stroke volume. Treatment with rhNRG-1 was able to normalize these changes, restoring pulmonary circulation. M group also presented RV dilation (Figure 1 – C), as measured by the dimension of the tricuspid annulus (Figure 1 – F), RA enlargement (Figure 1- D), IVS thickening (Figure 1 – E) and flattening, as showed by the rectilinear position of the IVS (Figure 1 – F). The aforementioned pathological heart remodelling (RV and RA increase and IVS thickening), observed in MCT animals through echocardiography, was restored by rhNRG-1 treatment.

Treatment with rhNRG-1 was able to attenuate body weight loss, in MCT treated animals (Figure 2 – A). In addition, the RV/LV+S ratio, a surrogate of RV hypertrophy, was greatly increased in the PAH group (Figure 2 – B), and was significantly attenuated by rhNRG-1 treatment. Together with this finding, pulmonary oedema, as quantified by the Lung/TL ratio was also reduced by rhNRG-1 treatment when compared to the MCT group (Figure 2 – C). This shows a decrease of fluid build-up in the lungs, potentially as a result of improved cardiac function and cardiac output (CO) in treated animals.

Animals with PAH and without pharmacological intervention presented increased cardiomyocyte cross-sectional area, as well as fibrosis deposition (Figure 2 – D and E). RhNRG-1 treatment normalized both cardiomyocyte size and fibrotic tissue deposition. Pulmonary small artery remodelling, measured by medial layer thickness was also attenuated by rhNRG-1 treatment (Figure 2 – F).

RhNRG-1 amends reduced cardiac function in MCT-induced PAH

Monocrotaline-induced PAH results in RV dysfunction 3 weeks after MCT administration (Figure 3 – A). MCT animals also present an increase in pulmonary vascular resistance (PVR, Figure 3 – B)and this results in higher right ventricular end-systolic pressure (ESP, Figure 3 – C), consistent with increased RV hypertrophy, RV dilation (increased end-diastolic volume, EDV, Figure 3 – D), and RV dysfunction as shown by the decrease of ejection fraction (EF, Figure 3 – E) and CO (Figure 3 – F), despite intrinsic myocardial contractility increase (higher load-independent contractility index, end-systolic elastance, Ees, Figure 3 – I). As mentioned above, by reducing pulmonary vascular remodeling, chronic treatment with rhNRG-1 was able to attenuate PVR, therefore reducing RV afterload and improving RV function. Consistently with increased fibrosis, MCT animals had diastolic dysfunction, with higher filling pressures, impaired relaxation and increased diastolic stiffness, quantified by higher end-diastolic pressure (EDP, Figure 3 – G), increased isovolumic relaxation time constant (tau, Figure 3 – H) and increased end-diastolic elastance (Eed, Figure 3 – J), respectively. PAH animals treated with rhNRG-1 showed improved RV diastolic function, with a more compliant chamber, and restored relaxation, as shown by normalized Eed, EDP and tau. Overall, chronic treatment with rhNRG-1 starting 2 weeks after MCT administration, when signs of PAH are already present (Figure S2), was able to noticeably improve RV function 3 weeks after MCT administration.

Pressure-volume analysis of the LV (Figure 4 – A) showed decreased contractile LV performance in MCT treated animals as shown by the decrease in ESP (Figure 4 – B), and was paralleled with decreased EDV (Figure 4 – C), and diastolic impairment (increased tau

and Eed, Figure 4 – D and E, respectively). This might result from LV unloading subsequent to decreased RV ejection, and septal bulging (shown by echocardiography). Similarly to the RV, rhNRG-1 treatment improved global LV function, recovering both systolic and diastolic function.

RhNRG-1 attenuates pulmonary endothelial dysfunction in MCT-induced PAH

We found a lack of relaxation in a dose-response test to acetylcholine (Figure 5 – A and B) in pulmonary arteries isolated from MCT animals. Treating animals with rhNRG-1 did not change phenylephrine-induced maximal tension, but significantly enhanced endothelial function, by increasing the maximal response to acetylcholine by 12% (Figure 5 – C). Furthermore, rhNRG-1 decreased the EC50 (Figure 5 –D), increasing receptor sensitivity to acetylcholine. Pulmonary arterial remodelling was also reversed in arterial rings (large diameter vessels, Figure 5 – E and F) isolated from rhNRG-1 treated animals, in conformity with its effects on small diameter arteries (Figure 2 – F and G).

Besides decreasing pulmonary arterial remodelling, rhNRG-1 treatment improved endothelial function, contributing to the improvement of PVR in the treated group.

RhNRG-1 abrogates molecular changes in the RV and attenuates systemic inflammation in MCT-induced PAH

MCT-induced PAH resulted in an increase in NRG-1 gene expression in the RV (Figure 6 – A), which is associated with impaired RV function, as observed by a negative significant correlation between NRG-1 and EF (Figure 6 – B). No changes were observed in NRG-1 expression in the lung of the different experimental groups (Figure 6 – C). Animals treated with rhNRG-1 showed a reversal of RV NRG-1 expression to control levels when compared to the MCT group without treatment. As expected, administration of rhNRG-1 resulted in ErbB4 receptor phosphorylation (Figure 6 – D), demonstrating the binding of the peptide to the receptor and its activation.

MCT animals presented increased RV expression of markers of hypertrophy and overload, namely, ET-1 (Figure 6 – E) and BNP (Figure 6 – F). We also found increased RV expression of caspase-3, as a surrogate for apoptosis (Figure 6 – G) and of HIF-1α as a tissue hypoxia marker (Figure 6 – H). RhNRG-1 treatment was able to restore the RV expression levels of all the mentioned cardiac damage markers.

Although myocarditis has been reported as a "side effect" of MCT administration[23], we did not find changed RV pro-inflammatory cytokine expression (Figure 6 – I and Figure 6 – J). We did find increased IL-6 (Figure 6 – L) expression in the lung of both MCT groups, demonstrating that pulmonary inflammation, secondary to MCT administration, was not attenuated by rhNRG-1.

However, plasmatic levels of TNF-α (Figure 6 – M) and IL-6 (Figure 6 – N), which were increased in animals from the MCT group, pointing to systemic inflammation, were attenuated by rhNRG-1 treatment.

RhNRG-1 improves RV structure in animals with RV hypertrophy induced by pulmonary artery banding

Using the PAB model, we sought to distinguish rhNRG-1's effect on the RV, independent from its effect on the pulmonary vasculature. PAB surgery resulted in compensated RV hypertrophy, as measured by the RV/LVS ratio (Figure 7 – A), increased cardiomyocyte CSA (Figure 7 – B) and fibrosis (Figure 7 – C), and preservation of RV function, as seen by an unchanged CO (Figure 7 – D). RhNRG-1 treatment attenuated RV structural changes, by decreasing RV hypertrophy and fibrosis in the PAB model, demonstrating that it has also

direct myocardial effects that are independent from its effects on pulmonary vasculature seen in MCT animals.

DISCUSSION

In this work, we tested the effect of rhNRG-1 treatment in a rat model of MCT-induced PAH and RV overload. Consistent with our hypothesis, rhNRG-1attenuated the severity of this disease, as evident from the salutary effects of rhNRG-1 on pulmonary and RV remodelling and overall cardiac function. Beneficial effects of rhNRG-1 were evident both at the functional and at the histological/structural level. Furthermore, using a model of pressure loading of the RV without PAH, we also demonstrated that rhNRG-1 treatment has direct beneficial effects on RV structure, by reducing hypertrophy and fibrosis.

The cardiac NRG-1/ErbB system has been intensely studied, and there is compelling evidence that this system is activated during compensated LV failure[8]. Treatment of various animal models with LV dysfunction has resulted in improved cardiac function, LV remodelling and reduced heart failure mortality [10-15, 24, 25], and has instigated ongoing clinical trials with NRG-1 in heart failure[16, 17]. Although it is generally believed that beneficial effects of NRG-1 in heart failure mainly result from direct effects on cardiomyocytes[25, 26] and, perhaps, on cardiac fibroblasts[10], the physiological effects of NRG-1 may be more pleiotropic, including effects on vascular endothelial cells[27, 28], vascular smooth muscle cells[19] and inflammatory cells[29].

In line with these observations, the favourable effects of NRG-1 observed in the present study seem to result from effects on both the pulmonary vasculature (MCT model) and directly on the RV myocardium (PAB model). Both pulmonary arterial medial hypertrophy and pulmonary arterial endothelial dysfunction were markedly attenuated by NRG-1. This led to reduction of pulmonary arterial resistance, RV afterload and consequently of RV hypertrophy and RV contractility. Although the precise mechanisms of these beneficial effects of NRG-1 on the pulmonary endothelium and vasculature remain to be deciphered, inhibitory effects of rhNRG-1 on platelet-derived growth factor induced smooth muscle cell proliferation[19], and stimulatory actions on nitric oxide synthesis may participate[26, 30].

Lung inflammation is associated with the development of PAH[31], and inflammatory markers as TNF-α and IL-6 are increased in MCT-induced PAH[32]. Our finding that rhNRG-1 treatment did not attenuate lung inflammation shows that the improvement of pulmonary and RV function and structure was not achieved through attenuation of an acute inflammatory response in MCT-induced PAH[23]. In the same perspective and regardless of previous evidence associating inflammatory cardiomyopathy to MCT-induced PAH[23], we did not observed TNF-α and IL-6 altered expression in the RV of MCT animals, which suggests that RV myocardial inflammation does not seem to play a role in our experimental setting. Despite this observation, PAH-associated systemic inflammation[33, 34], was attenuated by rhNRG-1 treatment, possibly as a result of overall improved function, revealing NRG-1's potential function as an anti-inflammatory agent in PAH. Also, control animals treated with rhNRG-1 did not show increased proinflammatory cytokine levels showing that intraperitoneal administration of this peptide does not elicit an inflammatory response by itself.

Besides decreasing vascular remodelling and dysfunction, NRG-1 seems to also act on the myocardium in MCT-induced PAH. The anti-hypertrophic effects of NRG-1 in the RV

observed in this study are consistent with previous observations in which rhNRG-1 inhibits LV cardiomyocyte hypertrophy during post-infarct remodelling[25]. Strikingly, in the MCT-induced PAH model, rhNRG-1 also prevented LV dysfunction. LV contractile dysfunction[35, 36] and impaired relaxation[37] are generally present in PAH, and were both attenuated by rhNRG-1 treatment. LVEDV, which was restored with treatment, was lower in rats with PAH. In PAH patients[38] these LV functional parameters are associated with increased mortality, underscoring the putative translational implication of this NRG-1 effect.

In the PAB model, a model without vascular disease, but with an identical degree of RV overload and hypertrophy to the MCT-induced PAH model used, rhNRG-1 was also able to mitigate hypertrophy and fibrosis, demonstrating that in fact, a direct effect on the RV myocardium is present, and importantly contributes to the improved RV function and structure observed in MCT animals treated with rhNRG-1.

Myocardial remodelling, increased wall stress, hypoxic damage and apoptosis, are associated with MCT-induced PAH increased RV expression of ET-1[39], BNP[40], HIF-1α[41] and caspase-3[42]. Accordingly, all these markers were upregulated in the RV of MCT animals, agreeing with the functional and structural changes observed. Either by directly acting on these signalling pathways, potentially regulating its expression, or by decreasing RV remodelling and improving its function, rhNRG-1 treatment was able to restore the expression of all the above mentioned RV damage markers. In the present study, we also observed that NRG-1 was endogenously upregulated during PAH. RV NRG-1 mRNA expression was increased in MCT animals, and was associated with poorer RV function, possibly as a result of increased afterload and myocardial stress[8].

The beneficial effects of NRG-1 on both heart and vessels, by acting on cardiomyocytes[25, 26], cardiac fibroblasts[10], endothelial cells[27, 28], vascular smooth muscle cells[19] and inflammatory cells[29], might be an advantage in the treatment of PAH, when compared with current therapeutic agents that are more focused on arterial pulmonary vasoconstriction. Clinical translation of these observations is ongoing, especially with regard to the treatment of heart failure[16, 17].

Previous studies have shown that two weeks after MCT administration, rats already present increased RV and pulmonary dysfunction and remodeling[43]. Our data (Figure S2) shows that, 14 days after MCT administration, animals develop RV hypertrophy with maintained function, lung oedema, possibly as a result of an early inflammatory response[23, 44], and compromised pulmonary flow, where PAVTI and PAAT/PAET are already as decreased as 21 days after MCT administration (data not shown). This confirms that 2 weeks after MCT administration pulmonary dysfunction is established. This finding suggests that treatment with rhNRG-1 recovers already established pulmonary flow dysfunction, attenuating overload of the RV and improving its function and structure. Therefore, by beginning rhNRG-1treatment at day 14 we showed that rhNRG-1 has a role in treating already established PAH, thus facilitating its transition to clinical practice.

Limitations of our work include the lack of subcellular mechanisms for the beneficial role of the NRG-1, and although potential mechanisms were suggested, this will be the object of another line of research. Additionally, the plexiform lesions that are found in the lungs of PAH patients, as well as in angioproliferative models of PH, are not usually seen in the MCT model. Still, the MCT model shares several main characteristics with both primary and secondary pulmonary hypertension in humans, such as pulmonary vascular remodelling, as

well as RV and endothelial dysfunction[32]. As rhNRG-1 ameliorates most of these parameters, we propose that NRG-1 could potentially serve as a treatment option for both forms of pulmonary hypertension in humans.

In conclusion, this study shows, for the first time, that NRG-1/ErbB signalling may have an important role in PAH and RV dysfunction and that rhNRG-1 treatment improves both cardiopulmonary function and structure. NRG-1 decreases pulmonary arteries remodelling, improves endothelial function, and restores RV function. These beneficial effects may improve outcome in PAH. These data should encourage further studies to elucidate the underlying mechanisms through which NRG-1 attenuates the pathophysiology of PAH.

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Conflict of interest

No conflict of interest to declare

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Figure Legends

Figure 1 – RhNRG-1 improves pulmonary flow and RV structural changes in MCT-induced PAH. (A) Pulmonary acceleration time normalized to pulmonary ejection time (PAAT/PAET) is decreased in MCT animals, and recovered with rhNRG-1 treatment. (B) Pulmonary artery velocity time integral (PAVTI) is also decreased in PAH, and restored with treatment. (C) RV end-diastolic diameter (RVEDD) is increased in MCT animals, while MCT animals treated with rhNRG-1 show no differences from control animals. (D) Right atria area (RAA) is higher in MCT animals, and normalized in MCT animals treated with rhNRG-1. (E) Interventricular septum (IVS) thickness is higher in MCT animals and is normalized by rhNRG-1 treatment. (F) Representative images of pulmonary flow, and parasternal long axis (Apical 4 chamber view) and parasternal short axis of the heart of the different experimental groups. White arrowhead points to midsystolic notch, and white arrows delineate the tricuspid valve (Apical 4 chamber view) and the IVS (short axis view). Bars represent mean ± SEM of 8-11 rats per group. *P<0.05 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT; $^{\#\#\#}$P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 2 – MCT-induced RV and Lung remodelling is attenuated by rhNRG-1 treatment. (A) Weight loss was evident in MCT animals, while treated animals showed a significantly higher body weight (BW). (B) RV hypertrophy, as measured by RV/LV+S ratio, was increased in MCT animals, while the RV of treated animals was significantly less hypertrophied. (C) Lung oedema, as measured by the Lung/TL ratio, was present in both MCT groups, and treatment with rhNRG-1 was able to attenuate this change. (D) RV cardiomyocyte cross sectional area (CSA) was increased in MCT-induced PAH, while rhNRG-1 treatment reversed cardiomyocyte hypertrophy. (E) RV fibrosis was also increased in MCT-induced PAH, and treatment with rhNRG-1 was able to normalize RV fibrosis. (F) Pulmonary arterial remodelling was increased in MCT animals, as shown by an increase in medial wall thickness, and was attenuated with rhNRG-1 treatment. (G) Representative photomicrographs of haematoxylin-eosin (H&E) and Red Sirius staining of RV sections, and H&E lung sections. Black scale lines represent 20 µm (400X magnification) and 20 µm (200X magnification) and 100 µm (200X magnification) for RV H&E and Red Sirius, and lung H&E photomicrographs, respectively. Bars represent mean ± SEM of 14-16 rats per control group and 24 rats per MCT group for the morphometric data, and 6-12 rats per group in the histological data. *P<0.05 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT; $^{\#\#\#}$P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 3 – RhNRG-1 treatment improves RV function in MCT-induced PAH. (A) Representative pressure-volume loops of the different experimental groups. (B) Pulmonary vascular resistance (PVR) is increased in MCT animals and is attenuated with rhNRG-1 treatment. (C) MCT animals show higher RV end-systolic pressure (ESP), while MCT animals treated with rhNRG-1 show a significant reduction of ESP. (D) RV dilation, as measured by the end-diastolic volume (EDV) occurs in MCT-induced PAH, while treatment restores RV volume. (E) RV ejection fraction (EF) is compromised in MCT animals, and normalized in MCT animals treated with rhNRG-1. (F) Cardiac output (CO) is severely decreased in MCT-induced PAH and significantly improved with treatment. (H) Relaxation, as measured by the isovolumic relaxation time constant (tau) is compromised (increased tau) in MCT animals and is normalized in MCT animals treated with rhNRG-1. (I) End-systolic elastance (Ees) is increased in MCT animals and significantly attenuated in MCT animals

treated with rhNRG-1. (J) End-diastolic elastance (Eed) is increased in MCT-induced PAH and is restored by rhNRG-1 treatment. Bars represent mean ± SEM of 14-16 rats per control group and 24 rats per MCT group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT; $^{\#\#\#}$P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 4 – RhNRG-1 treatment improves left ventricular function in MCT-induced PAH. (A) Representative pressure-volume loops of the different experimental groups. (B) LV end-systolic pressures were decreased in MCT animals, and treatment with rhNRG-1 reversed this change. (B) End-diastolic volume was decreased in MCT-induced PAH and normalized with rhNRG-1 treatment. (D) Isovolumic relaxation time constant (tau) was increased in MCT animals and normalized with rhNRG-1 treatment. (E) End-diastolic elastance (Eed) was increased in MCT-induced PAH and normalized with rhNRG-1 treatment. Bars represent mean ± SEM of 14-16 rats per control group and 24 rats per MCT group. **P<0.01 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT; $^{\#\#\#}$P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 5 – Treatment with rhNRG-1 attenuates endothelial dysfunction and attenuates large diameter pulmonary arteries remodelling. (A) MCT-induced PAH resulted in decreased vasorelaxation induced by acetylcholine, while chronic treatment with rhNRG-1 improved vasorelaxation. (B) Representative tracings of the different acetylcholine dose-response curves. (C) Acetylcholine-induced relaxation maximal response (Emax) was decreased in MCT animals, and significantly improved in MCT animals treated with rhNRG-1. (D) Dose-response curve to acetylcholine EC50 was increased in MCT-induced PAH, and attenuated by rhNRG-1 treatment. (E) Isolated large diameter pulmonary arteries wall thickness was increased in MCT animals and normalized in MCT animals treated with rhNRG-1. (F) Representative photomicrographs of isolated pulmonary arteries stained with Verhoeff–Van Gieson stain. Black scale line represents 200 μm (200X magnification). Bars represent mean ± SEM of 6-8 rats per group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT. Two-way repeated measures ANOVA was used for the dose-response curve to acetylcholine, while two-way ANOVA was used for all the other parameters presented.

Figure 6 – Expression of cardiac disease markers is reversed by rhNRG-1 treatment. (A) RV NRG-1 mRNA levels are increased in MCT-induced PAH and normalised with rhNRG-1 treatment. (B) Increased levels of RV NRG-1 are negatively correlated with EF (Pearson r = -0.8782; P<0.0001). Data used for this correlation analysis was obtained from animals that had both PV-Loop analysis and mRNA quantification of RV NRG-1. (C) Lung NRG-1 mRNA expression does not change in MCT-induced PAH. (D) Administration of rhNRG-1 results in ErbB4 receptor phosphorylation. ET-1 (E), BNP (F), Caspase-3 (G) and HIF-1α (H) upregulation is normalized by rhNRG-1 treatment. RV proinflammatory cytokine - TNF-α (I) and IL-6 (J) - expression is unchanged in all the experimental groups. Lung proinflammatory cytokine TNF-α (K) expression is not changed, while IL-6 (L) expression is increased in MCT-induced PAH and is not affected by rhNRG-1 treatment. Systemic inflammation, as measured by plasmatic levels of TNF-α (M) and IL-6 (N) is increased in MCT-induced PAH and attenuated by rhNRG-1 treatment. Bars represent mean ± SEM of 6-12 rats per group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; $^{\#}$P<0.05 vs MCT; $^{\#\#}$P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 7 – PAB-induced RV hypertrophy and fibrosis are attenuated by rhNRG-1 treatment. Treatment with rhNRG-1 attenuated the (A) PAB increased RV/LV+S ratio (B) PAB induced cardiomyocyte hypertrophy, quantified as increased cardiomyocyte cross sectional area (CSA), (C) PAB increased fibrosis. . (D) Cardiac output (CO) was similar in all experimental groups. (E) Representative photomicrographs of haematoxylin-eosin (H&E) and Red Sirius staining of RV sections. Black scale lines represent 20 µm (40X magnification) and 50 µm (25X magnification) for H&E and Red Sirius, respectively. Bars represent mean ± SEM of 6-8 rats per group. *P<0.05 vs Sham; **P<0.01 vs Sham; ***P<0.001 vs Sham; $^{\#}$P<0.05 vs Banding. Two-way ANOVA was used for all the parameters presented.

Figure 1
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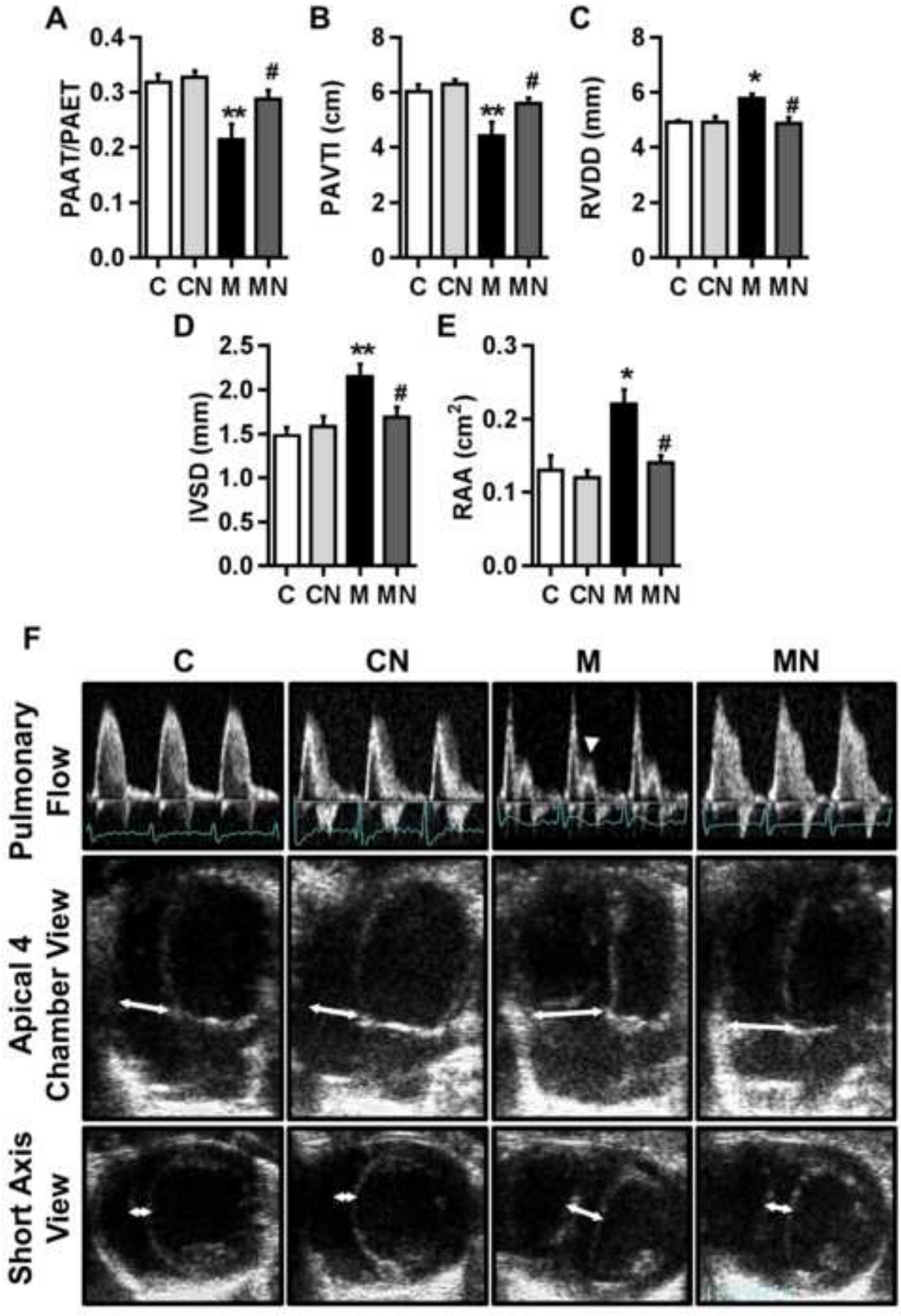

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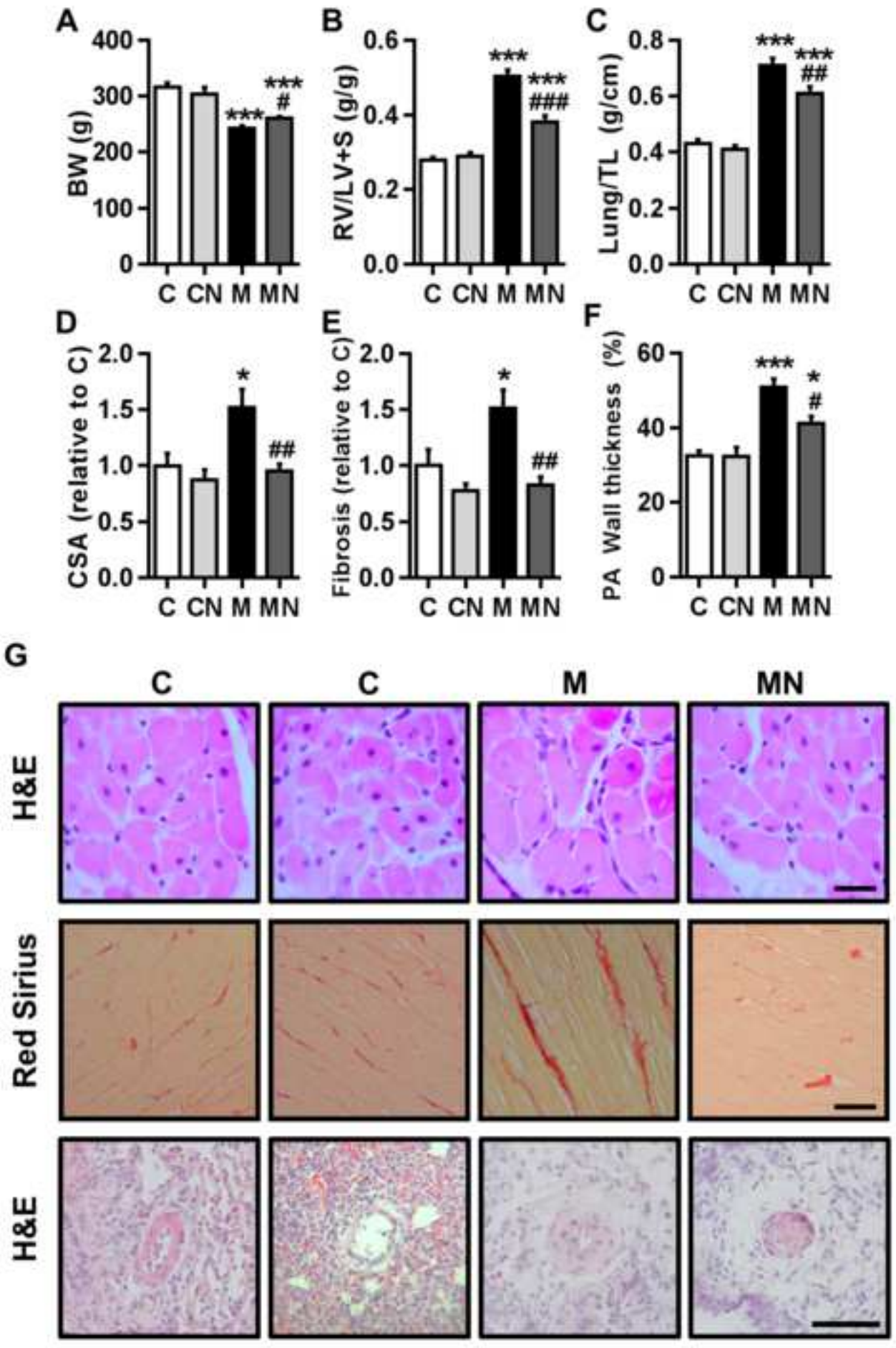

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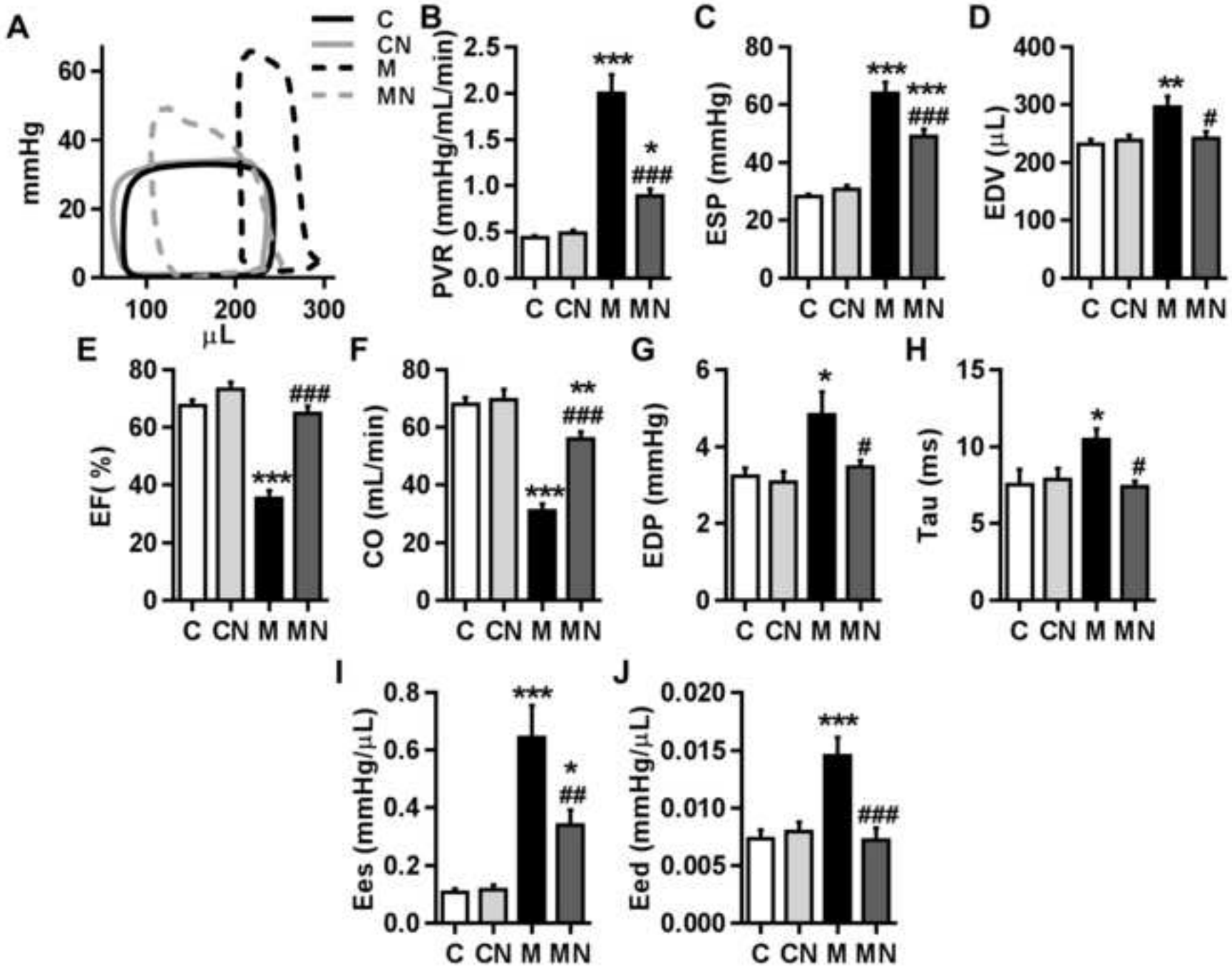

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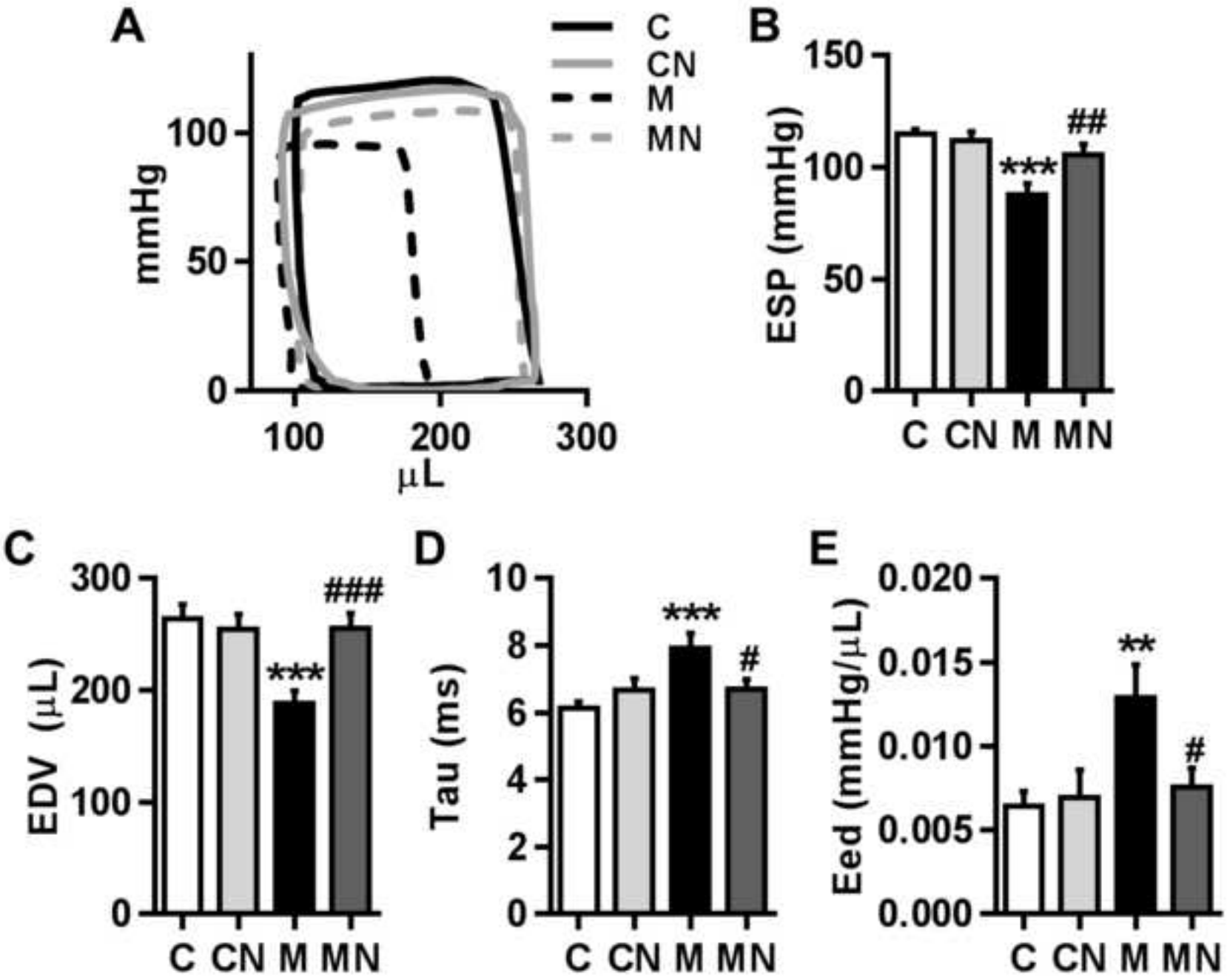

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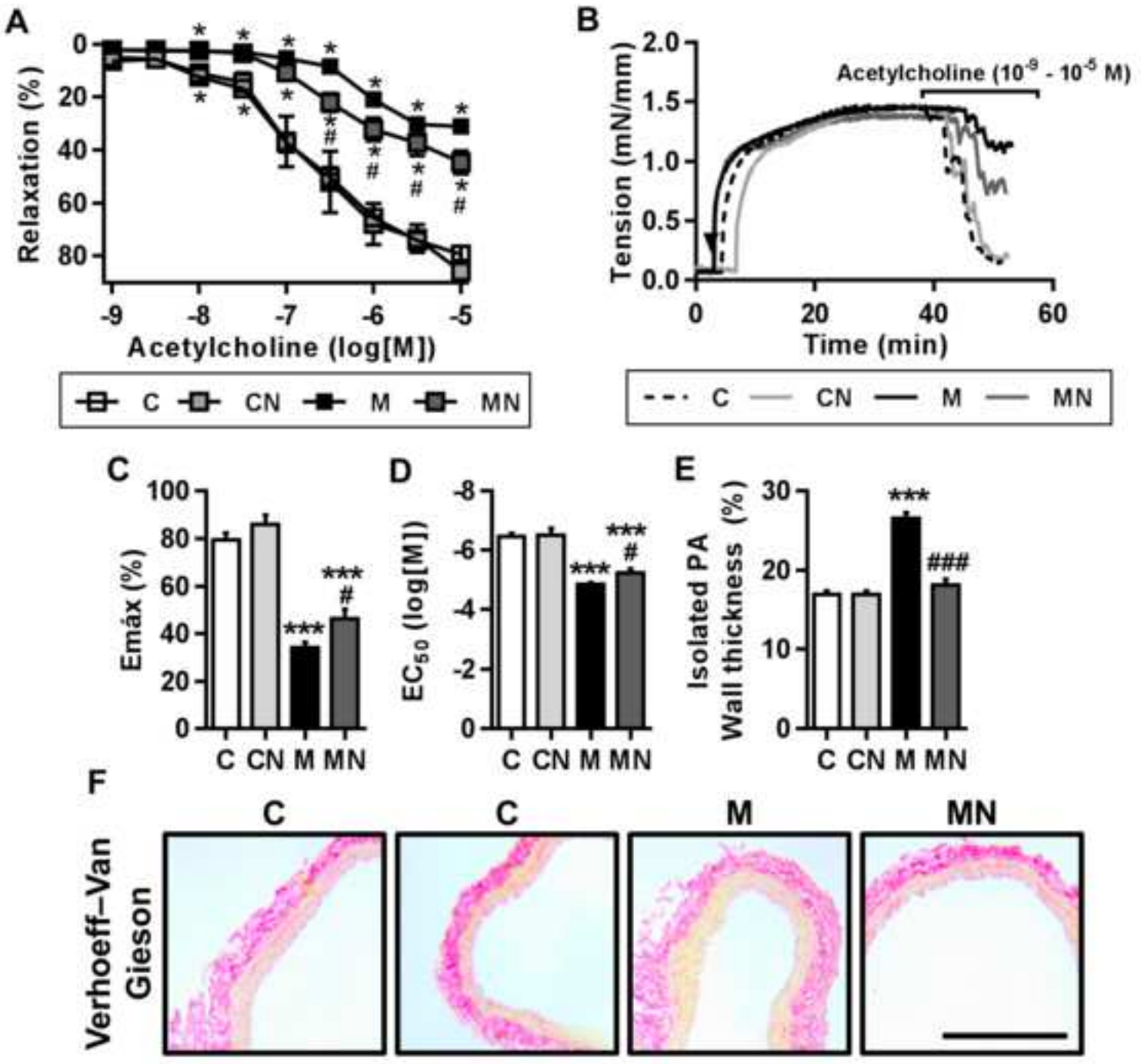

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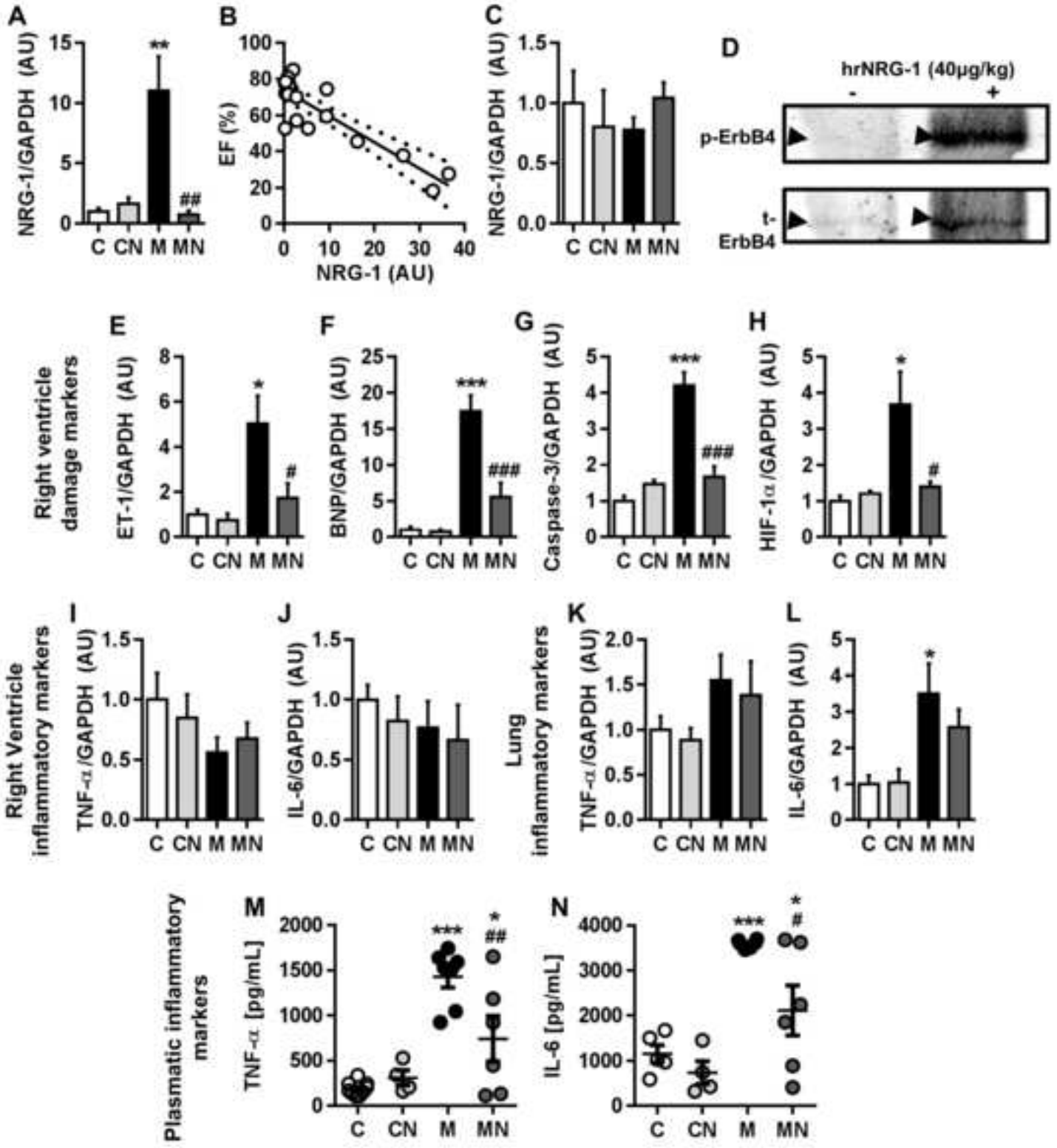

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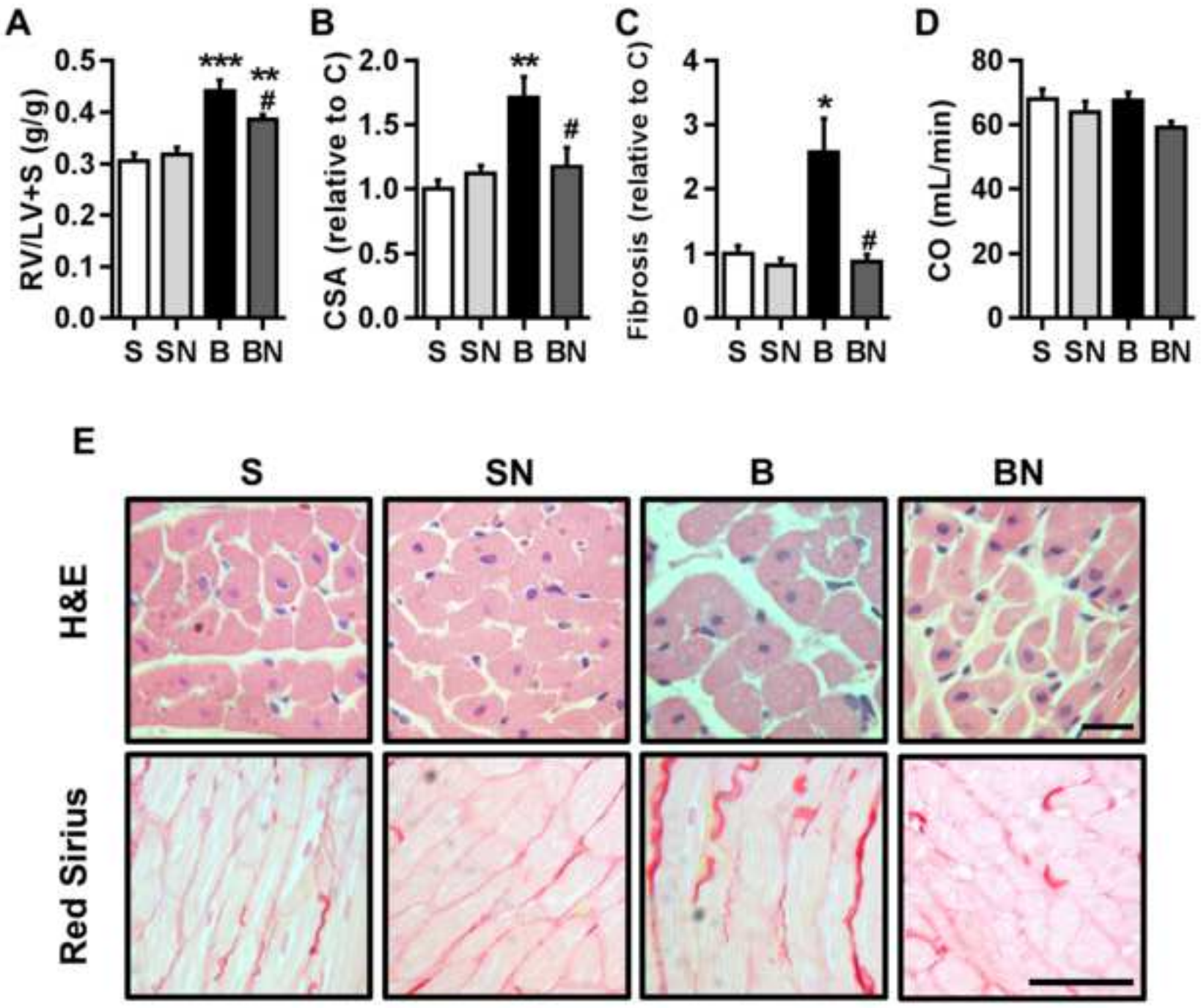

SUPPLEMENTARY MATERIAL

Neuregulin-1 improves right ventricular function and attenuates monocrotaline-induced pulmonary arterial hypertension

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SUPPLEMENTARY METHODS

Animal models and experimental design

Seven-to-eight week-old male Wistar rats (Charles River Laboratories) weighing 180–200 g, were randomly assigned to receive a subcutaneous injection of 60 mg/kg monocrotaline (MCT, Sigma-Aldrich) or an equal volume of vehicle (saline). Two weeks (14 days) after MCT/vehicle administration, rats were randomly assigned to receive 40 μg/kg rhNRG-1 i.p. (Peprotech) or vehicle (0.1 % BSA) daily during 1 week, resulting in 4 groups: Ctrl+vehicle (Group C, n=16); Ctrl+rhNRG-1 (Group CN, n=14); MCT+vehicle (Group M, n=24); MCT+rhNRG-1 (Group MN, n=24). Rats were grouped 5 per box, in a controlled environment, with a light-darkness cycle of 12:12h, controlled temperature, and water and food *ad libitum*. In order to determine if MCT-induced PAH was already present prior to treatment an additional group of animals underwent the same experimental protocol (MCT administration) and were evaluated at an earlier time point (14 days).

Another group of animals was submitted to pulmonary artery banding (PAB), and submitted to the same randomization, time points, and chronic treatment protocol.

Seven week old (180-200 g) male Wistar rats (Charles River Laboratories) were sedated, anesthetized and monitored as above mentioned (for the hemodynamic evaluation). The animal was placed in right lateral decubitus, the left thorax was shaved, depilated and wiped clean with iodine solution. Under a surgical microscope (Wilde M651, Leica microsystems), a small incision was created at the axillary level, the pectoral muscles were carefully dissected and retracted, and the thorax was opened at the second intercostal space. The thymus was moved and the pulmonary artery visualized. Using blunt forceps, the pulmonary artery was dissected free from both sides and a perforated spinal cord hook (Fine Science Tools) was used to pass a 6-0 prolene suture around the pulmonary artery. A 16 gauge needle with a blunt tip was placed parallel to the pulmonary artery, and the suture was quickly tied leaving a 1.65 mm constriction. The thorax was closed with 5-0 prolene, taking care not to cause pneumothorax, the pectoral muscles were put back in place and the skin was closed using 7-0 prolene. The suture site was infiltrated with lidocaine (1 %) and a subcutaneous injection of saline was administered to compensate for fluid losses, as well as analgesia (morphine, 4 mg/kg every 4 hours for the first 48 hours). Anaesthesia was interrupted and the animals were allowed to recover until full motion was achieved, and placed in a clean cage on top of a heating pad with easy access to food and water.

Suture integrity was checked for the next 3-4 days to ensure proper wound closure. Sham animals underwent the exact same protocol, except that the suture around the PA was loosely tied, and were not failed PAB attempts. No mortality was observed 3 weeks after PAB.

This protocol resulted in 4 groups: Sham+vehicle (Group S, n=8); Sham+rhNRG-1 (Group SN, n=7); PAB+vehicle (Group B, n=8); PAB+rhNRG-1 (Group BN, n=10). Applying a 1.65 mm constriction resulted in a degree of hypertrophy and RV overload identical to the MCT-induced PAH model used in this study (see Figure S1).

Three weeks (21 days) after MCT and PAB, rats were submitted to echocardiographic (MCT protocol) and hemodynamic evaluation, with subsequent sample collection for *in vitro* functional studies, morphological, histological and molecular analysis.

Echocardiography

Fourteen, or 21 days after a single subcutaneous injection of saline or MCT, or PAB/Sham surgery, all surviving rats were anesthetized with an i.p. injection of

ketamine/xylazine (75mg/kg and 10 mg/kg, respectively), placed in left lateral decubitus and the skin was shaved and depilated. After applying warm echocardiography gel, a 12MHz probe (GE Healthcare) was gently placed on the thorax.
Acquisitions (General Electrics Vivid 7 echocardiograph, GE Healthcare) were averaged from three consecutive heartbeats. Bi-dimensional and M-mode images were obtained, in parasternal short-axis, at the LV papillary muscle level, interventricular septum dimension was evaluated. In this projection pulmonary artery flow was also evaluated, and pulmonary artery acceleration (PAAT) and ejection time (PAET) were quantified, as well as pulmonary artery velocity-time integral (PAVTI). In apical projection of 4 cavities right ventricular end-diastolic diameter (RVEDD), and right atria area (RAA). After the echocardiographic analysis, animals were allowed to recover in a clean cage placed on top a heating pad and were monitored until full recovery.

Invasive hemodynamic assessment

Fourteen, or 21 days after MCT administration, anaesthesia was induced, after sedation (100 μg/kg and 5 mg/kg i.p., fentanyl and midazolam, respectively), by inhalation of 8% sevoflurane (Penlon Sigma Delta). Endotracheal intubation was performed using a 14 gauge catheter and mechanical ventilation controlled by a rodent ventilator (MouseVent G500, Kent Scientific) with an animal weight-defined tidal volume. Animal temperature was monitored and regulated with a warming pad and rectal temperature sensor, and digitally controlled, together with pulse oxymetry measured through a paw sensor, measurement of exhaled CO_2 (PhysioSuite, Kent Scientific), and ECG (Animal Bio Amp, ADinstruments). Anaesthesia was maintained with sevofluorane (2.5-3.0% vol/vol), and anaesthetic depth was determined through the toe-pinch reflex. The internal femoral vein was catheterized (24G catheter) under surgical microdissection (Wilde M651, Leica microsystems) for infusion (Multi-Phaser, NE-100, New Era Pump Systems) of warm lactate Ringer's solution at a rate of 32 mL/Kg/h. Alternatively if the animal was not too vasoconstricted, catheterization of the dorsal pedal vein was performed. Animals were placed in right-lateral decubitus and after tricotomy, a left thoracotomy was performed. Pericardium and pleura were carefully dissected, and the phrenic nerve was severed. A 3-0 surgical silk was passed around the inferior vena cava (IVC) for transient occlusion during the protocol, and pressure-volume catheters were inserted through the apex of the RV and LV, and positioned along the long axis (SPR-869 and SPR-847, respectively, Millar Instruments). A flow probe was implanted around the ascending aorta (MA2.5PSB, 2.5mm, Precision S-Series, Transonic Systems), and connected to an ultrasonic transit time volume flowmeter (TS420, transit-time perivascular flowmeter, Transonic systems). The experimental preparation was allowed to stabilize for 15 minutes, and during the procedure blood loss was accounted for with saline bolus. For pulmonary artery gradient measurement, another catheter (PVR-1045, Millar Instruments) was placed in the pulmonary artery through the RV outflow tract, and advanced through the constriction.
Baseline and IVC occlusions recordings were obtained with ventilation suspended at end-expiration. Pressure and volume signals were continuously acquired (MVPS 300, Millar instruments), digitally recorded at a sampling rate of 1000Hz (ML880 PowerLab 16/30, ADInstruments, Oxford, UK), and analyzed off-line (LabChart 7 Pro, ADInstruments, and PVAN 3.5, Millar Instruments).
Parallel conductance for the volume catheter was computed after bolus injection of 50μL hypertonic saline (10% sodium chloride), calibration for factor alpha (field inhomogeneity) was determined through the cardiac output measured by the aortic flow probe and the ultrasonic transit time volume flowmeter.

Following anaesthetic overdose, blood was retrieved for storage and further analysis, animals were exsanguinated and heparinized blood was collected and used for volume calibration with standard cuvettes (P/N 910-1048, Millar Instruments, Texas, USA).

Histological analysis

RV, lung and isolated pulmonary artery samples for histology were submersed in a fixative solution of 10% formaldehyde. After the initial fixation step, samples underwent dehydration (using ethanol in decreasing percentages), diaphanisation (using xylene) and impregnation in liquid paraffin (54°C). Later they were placed and properly oriented in metal molds, and serial sections of 4 μm were obtained, in a Minot type microtome.

Haematoxylin and eosin (HE) stained sections of the RV and lung tissue were used to measure cardiomyocyte cross-sectional area, and pulmonary artery muscularization. Sections were digitally photographed (Olympus XC30 Digital colour Camera, Olympus) and measured using imaging software (cell^B, Olympus). Fifty muscle fibres per animal were analyzed and only nuclei-centred cardiomyocytes were considered for analysis of cardiomyocyte dimensions, and ten pulmonary arteries per animal (50 - 200 μm) were analyzed. Measurements and quantifications were performed by two blinded observers. Picro Sirius Red staining was used to quantify RV fibrosis. Ten sections per animal were obtained using imaging software (cell^B, Olympus) and analyzed with image analysis software (Image J). Alternatively, 3 μm sections were obtained from isolated pulmonary arterial rings (after the endothelial function evaluation protocol). Verhoeff–Van Gieson staining, which distinguishes smooth muscle from collagen fibres, was used to determine the large diameter pulmonary artery medial wall thickness. Sections were digitally photographed and analyzed as mentioned above for the lung sections.

Pulmonary artery endothelial function

In a separate group of animals, after euthanasia (100 mg/kg sodium pentobarbital, Eutasil, CEVA), lungs and heart were excised en bloc and submerged in modified Krebs Ringer solution (130 mM NaCl, 4.7 KCl, 1.18 mM KH2PO4, 1.17 mM MgSO4, 14.9 mM NaHCO3, 5.5 mM glucose, 0.026 mM EDTA and 1.6 mM CaCl2). Under a stereo microscope (Stemi 2000C Stereo Microscope, Zeiss), second generation pulmonary arteries (200 - 400 μm diameter) were carefully dissected from the left upper lobe, and arterial rings (2 ± 0.2 mm length) were isolated. Preparations were mounted between two metallic pins in a horizontal bath myograph system (Tissue Bath System – 720MO, DMT), submerged in modified Krebs-Ringer solution, at a constant temperature of 37ºC, 95% O_2/5% CO_2 and pH 7.4. After a stabilization period, a tension-length curve (DMT normalization) was obtained through progressive stretching of the arterial rings (20% increase intervals) until the target transmural pressure was achieved (target transmural pressure was obtained through right heart catheterization as previously described). The preparations were allowed to stabilize at target transmural pressure and stimulated with 80 mM KCl solution to determine maximum tension development. Three to four solution changes followed in order to relax the arterial rings and endothelial function was evaluated through a dose-response curve to acetylcholine (10-9 to 10-5 M, in 0.5 logarithmic units intervals) performed on pre-contracted rings (10-5 M phenylephrine). Analysis was performed offline using appropriate software (LabChart 7 Pro, ADInstruments).

Quantitative RT-PCR, immunoblot and cytokine ELISA

Samples collected in RNAlater (Qiagen), snap frozen in liquid nitrogen and kept at -80ºC were used for total mRNA isolation. RV mRNA was completely extracted through the guanidinum thiocyanate silica-gel membrane-binding method, according to the manufacturer's instructions (RNeasy, Qiagen). Concentration and purity of RNA were measured with a spectrophotometer (NanoDrop® ND-1000 spectrophotometer, Thermo Fisher Scientific), assuming as ideal A260/A280 ratio values of 1.8 – 2.1. Relative quantification of mRNA expression was performed by two-step Real-Time Polymerase Chain Reaction (RT-PCR). Using RV samples from the control group, standard curves were created to all the studied genes, correlating the initial total mRNA quantity and the threshold cycle. Reverse transcription was performed in a conventional thermocycler (TPersonal Thermocycler, Biometra), and consisted of 10 minutes at 22ºC, 50 minutes at 50ºC and 10 minutes at 95ºC. Ten percent of the obtained cDNA was amplified and detected by RT-PCR (Step-One™, Applied Biosystems), using SYBR Green as a marker (PerfeCta® SYBR Green FastMix, Rox, Kit, Quanta Biosciences), according to the manufacturer's instructions. Amplification curves were analyzed with the equipment-provided software (Step-One™ Software v2.2.2, Applied Biosystems). Melting curves of each PCR reaction were used in order to exclude the formation of primer-dimmers and unspecific products, confirming the purity of the amplified product. GAPDH was chosen as reference gene, since no significant changes were observed in the different experimental groups. Final gene expression results are presented in arbitrary units (AU), assuming as 1 AU the control group mean value, after normalization to GAPDH. All assays were performed twice. Primers used were designed in-house (Table S1) with the appropriate software (DNAstar™, DNAstar Inc).

Blood samples were collected and transferred into 1 mL disposable tubes containing 2-natrium-ethylenediamine tetra-acetic acid (EDTA) for plasma determinations. After sampling, tubes were placed on ice immediately, and plasma was separated within 30 min of collection by centrifugation at 4°C (5000 g for 15min), divided into aliquots and stored at −80 °C until quantification. All blood samples were collected at the same time points, and were stored under the same conditions. Plasma concentrations of IL6 and TNF-α were measured using a solid phase sandwich Enzyme-Linked-Immuno-Sorbent Assay (ELISA) according to manufacturer's instructions. Rat IL6 Elisa Kit (Invitrogen) and Rat TNF-α Elisa Kit (Invitrogen) were used for quantitative detection.

Thirty minutes after intraperitoneal administration of 40 µg/kg of rhNRG-1, animals were euthanized (100 mg/kg sodium pentobarbital, Eutasil, CEVA), and RV tissue was immediately homogenized on ice in 1 mL lysis buffer (RIPA, Cell Signaling Technologies) containing protease and phosphatase inhibitors (Sigma-Aldrich). After 1 hour incubation at 4ºC, samples were centrifuged for 20 minutes at 12000 rpm, and the supernatant collected. Bradford protein assay (Bio-Rad) was used to determine protein concentration, and the equivalent to 30 µg of protein was treated with sample buffer (Cell Signaling Technologies) according to manufacturer's instruction, and separated in a 10% SDS-PAGE gel. After proper separation, the gel was electro-blotted into nitrocellulose membrane overnight, blocked using 5% BSA (Sigma-Aldrich) and probed for ErbB4 (ErbB4 (C-18): sc-283, SantaCruz Biotechnology) and phospho-ErbB4 (Phospho-HER4/ErbB4 (Tyr1284) (21A9) #4757, Cell Signaling Technologies). After washing, membranes were incubated with secondary antibodies (IRDye 800CW and 680LT, Li-COR Biosciences) for 1 hour at room temperature, and a 185 kDa band (corresponding to ErbB4 molecular weight) was visualized using an appropriate imaging system (Odyssey, Li-COR Biosciences).

Supplementary Table

Table S1. RT-PCR primer sequences.

Gene	Forward	Reverse
BNP	5'CAG AGC TGG GGA AAG AAG AG3'	5'GGA CCA AGG CCC TAC AAA AGA3'
Caspase-3	5'CGG GTG CGG TAG AGT AAG3'	5'CTG GAC TGC GGT ATT GAG ACA3'
ET-1	5'CGG GGC TCT GTA GTC AAT GTG3'	5'CCA TGC AGA AAG GCG AAT GTG3'
GAPDH	5'TGG CCT TCC GTG TTC CTA CCC3'	5'CCG CCT GCT TCA CCA CCT TCT3'
HIF-1α	5'TCA TAG GCG GTT TCT TGT AGC3'	5'CTA ACA AGC CGG AGG AC3'
IL-6	5'GAA GTT GGG GTA GGA AGG AC3'	5'CCG TTT CTA CCT GGA GTT TG3'
NRG-1	5'AAG CTG GCC ATT ACG TAG TTT TG3'	5'TGT GCG GAG AAG GAG AAA ACT TTC3'
TNF- α	5'TGG GCT ACG GGC TTG TCA CTC3'	5'GGG GGC CAC GCT CTT C3'

BNP – B-type natriuretic peptide; ET-1 – endothelin-1; GAPDH - Glyceraldehyde-3-phosphate dehydrogenase; HIF-1α – hypoxia-inducible factor 1 alpha subunit; IL-6 – interleukin 6; NRG-1 – neuregulin-1; TNF- α – tumor necrosis factor alpha.

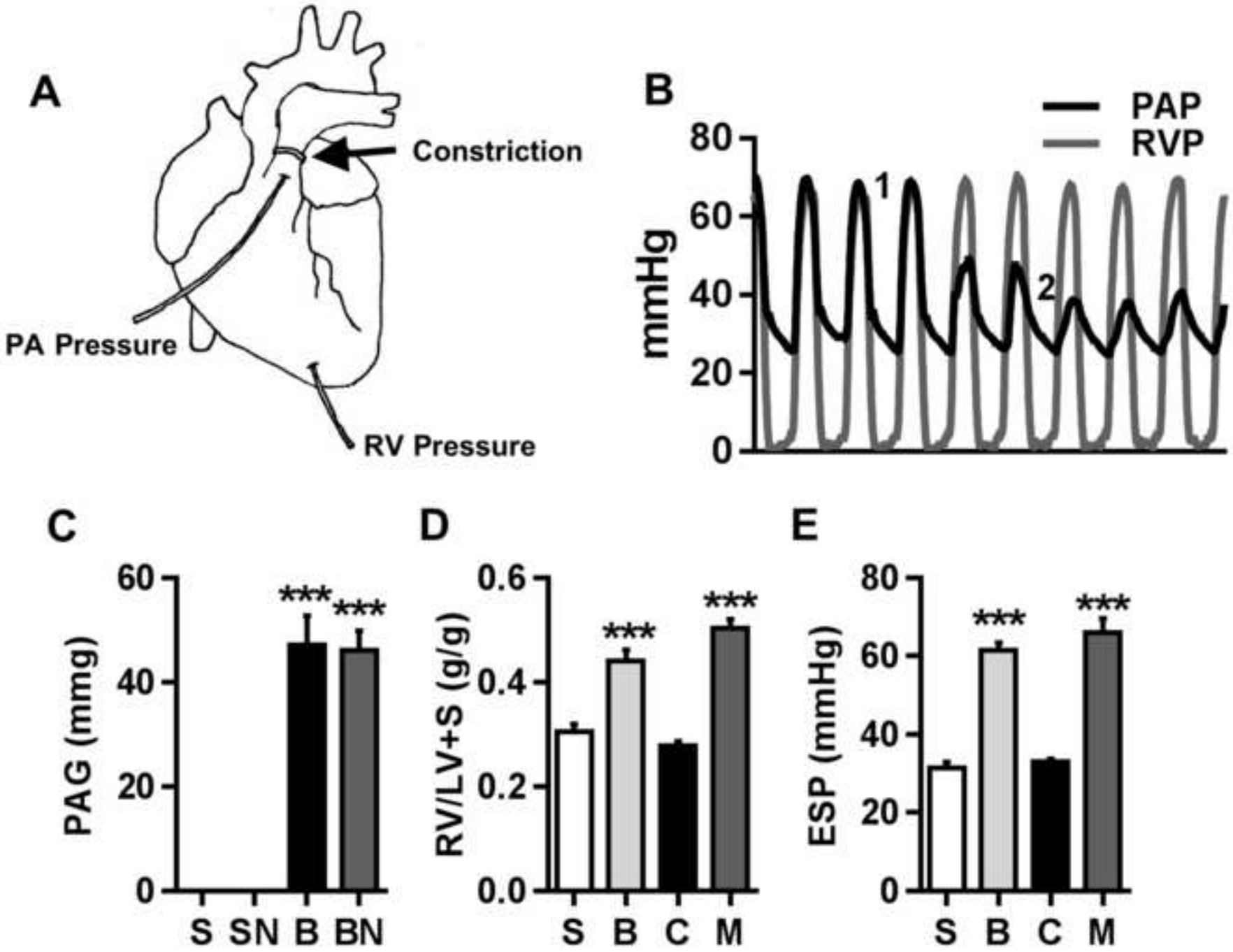
Supplementary Figure Legends

Figure S1 - Pulmonary artery banding (PAB) results in similar degrees of RV hypertrophy and overload to the MCT-induced PAH model. (A) Illustration of the placement of the catheters for the measurement of the pressure gradient (PAG) across the constriction. One of the catheters (PVR-1045) was introduced in the pulmonary artery and the other one in the RV. (B) Representative tracing of pressures at the RV and at the pulmonary artery before the constriction (1) and after the constriction (2). (C) PAB resulted in a pressure gradient of ~ 47 mmHg in both PAB groups. (D) PAB animals present values of RV/LV+S ratio identical to animals with MCT-induced PAH, pointing to the same degree of hypertrophy in both animal models. (E) PAB animals present similar values of end-systolic pressure (ESP), pointing to similar degrees of RV pressure overload in both animal models. C – Control; M – Monocrotaline; B – Pulmonary artery banding; BN – Pulmonary artery banding + rhNRG-1; S – Sham; SN – Sham + rhNRG-1. Bars represent mean ± SEM of 7-10 rats per group in the PAB protocol, and 16-24 in the MCT protocol. ***P<0.001 vs respective control. Two-way ANOVA was used for PAG comparison. One-way ANOVA was used for comparison of the MCT and PAB protocol.

Figure S2 – Signs of PAH 14 days after MCT administration. (A) Representative pulmonary flow from age-matched controls and MCT animals 14 days after MCT administration. Pulmonary flow is already compromised at this stage, with decreased pulmonary artery velocity-time integral (VTI) (B) and the pulmonary acceleration/ejection time (PAAT/PAET) ratio (C). (D) Right ventricular hypertrophy is already present 14 days after MCT administration, as well as Lung oedema (E). (F) Representative PV-Loops of control and MCT animals 14 days after MCT administration. RV increased afterload, as measured by pulmonary vascular resistance (PVR) (G) is present 14 days after MCT, as well as RV end-systolic pressure (ESP) (H). RV dilation (I) and function (J) is not compromised 14 days after MCT administration. C – Control; M14D – Monocrotaline 14 days after administration. Bars represent mean ± SEM of 5-7 rats per group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control. T-student test was used for all the parameters presented.

Supplementary Figure 1

Click here to download Supplementary Material - Other: Figure S1.tif



Supplementary Figure 2

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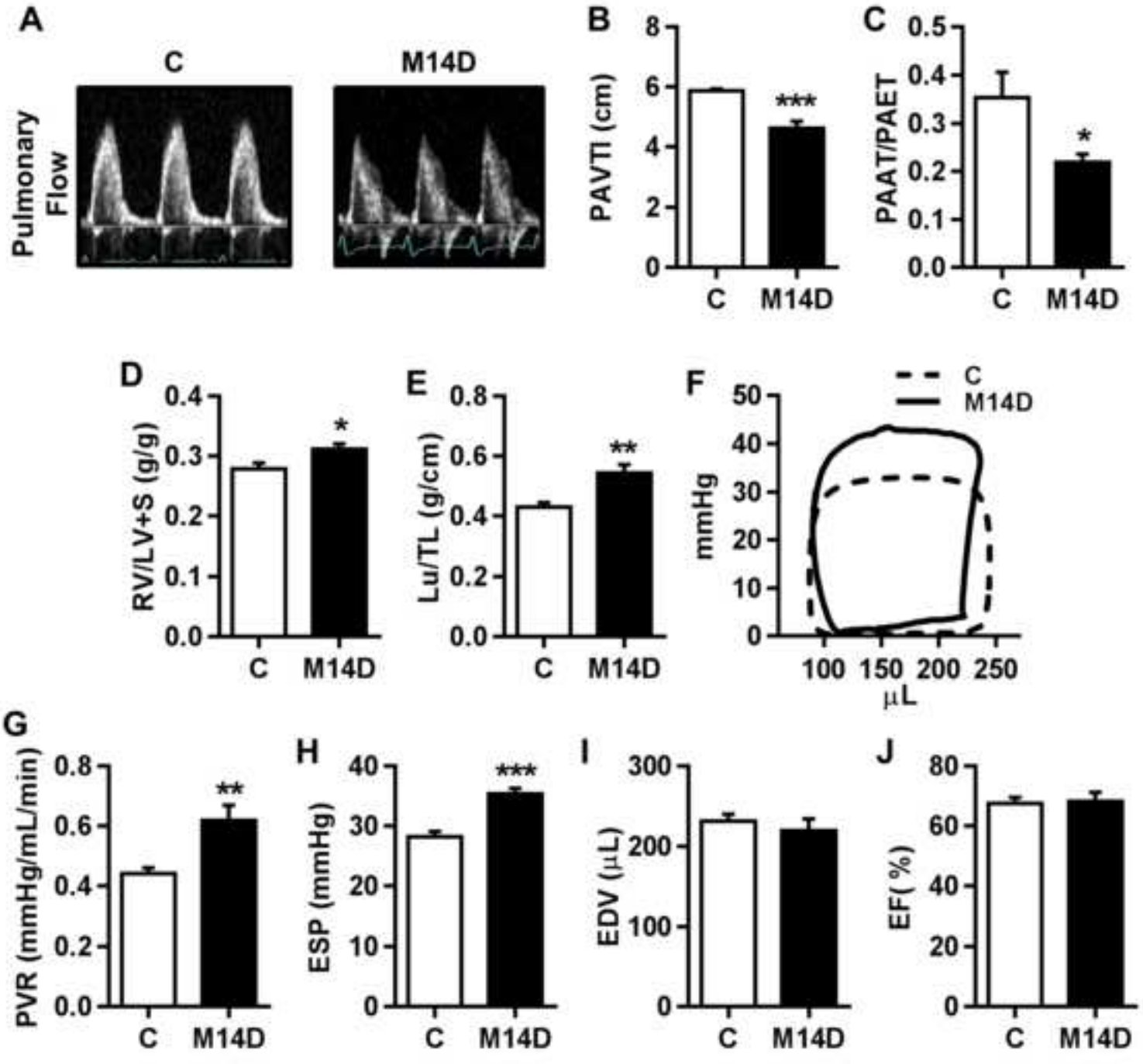

Aprovado pelo Senado da Universidade do Porto, a 16 de novembro de 2005
Publicado no Diário da República, II Série, nº 242, a 20 de dezembro de 2005

REGULAMENTO DE PROPRIEDADE INTELECTUAL DA UNIVERSIDADE DO PORTO

Preâmbulo

A Universidade do Porto, doravante designada UP, considera que a protecção e valorização dos resultados de I&D e de outras actividades realizadas no seu seio constituem um incentivo à produtividade e inovação, em especial para todos os que na Universidade realizam actividades com forte ligação ao tecido empresarial.

Por outro lado, o sucesso alcançado na protecção desses resultados é também um forte contributo para o reforço da imagem interna e externa da UP e para o seu reconhecimento como uma universidade inovadora e empreendedora.

A protecção e valorização dos referidos resultados pode ainda ser uma fonte de rendimentos e de constituição de património próprio para a UP. Pode também constituir-se como um reconhecimento da actividade exercida por alguns dos membros da comunidade académica da UP, através dos incentivos financeiros de que poderão ser beneficiários na sequência da protecção e valorização de resultados da sua actividade na UP.

Tendo em vista a importância que a protecção e valorização de tais resultados tem para a UP, é necessário que exista um normativo que permita assegurar a referida protecção e valorização sempre que sejam gerados, bem como salvaguardar os legítimos interesses da UP, no seu todo e das entidades que a constituem, e dos membros da sua comunidade académica.

É assim elaborado o presente Regulamento de Propriedade Intelectual da UP, que se rege pelas cláusulas seguintes:

TÍTULO I
DOS DIREITOS DE PROPRIEDADE INDUSTRIAL

PARTE I
OBJECTO E ÂMBITO DE APLICAÇÃO

Artigo 1º
Objecto e âmbito de aplicação

1- A propriedade industrial visa a protecção legal das criações do domínio da indústria, comércio e serviços, bem como marcas e outros sinais distintivos do comércio.
2- Para efeitos de interpretação e aplicação do presente Regulamento entendem-se por Direitos de Propriedade Industrial, nos termos da lei geral, as patentes de invenção, modelos de utilidade, desenhos ou modelos, obtenções vegetais e topografias dos produtos semicondutores, doravante designados como invenções ou criações.
3- Os princípios consagrados no presente Regulamento serão igualmente aplicáveis às invenções que contenham programas de computadores com conteúdo técnico implícito e aplicabilidade industrial, ou seja, que contribuam ou venham a contribuir para a resolução de problemas técnicos.
4- O presente regulamento será ainda aplicável a novos objectos de Direitos de Propriedade Industrial que venham a ser juridicamente tutelados.

PARTE II
TITULARIDADE DOS DIREITOS

Artigo 2º
Regra geral

1. Salvo o disposto no artigo 5º, a Universidade do Porto consagra, como princípio geral, o seu direito à titularidade dos Direitos de Propriedade Industrial que incidam ou venham a incidir sobre as invenções ou outras criações concebidas e realizadas pelos seus docentes, investigadores e demais funcionários ou agentes que exerçam funções na Universidade do Porto.

2. Idêntico princípio se aplica às invenções ou criações concebidas e realizadas pelo demais pessoal contratado sempre que as mesmas resultem de actividades realizadas em virtude do vínculo contratual estabelecido.
3. A aplicação dos princípios enunciados nos números 1 e 2 do presente artigo estende-se até ao final do ano civil seguinte ao termo do vínculo contratual com a UP, no que concerne às invenções ou criações divulgadas durante esse período e derivadas de trabalho realizado ainda enquanto vigorava o vínculo contratual com a UP.
4. No caso de a actividade que deu origem à invenção ou criação decorrer no âmbito de um contrato ou protocolo celebrado entre a Universidade do Porto e uma terceira entidade, aplicar-se-ão as disposições constantes do artigo 5º do presente regulamento.

Artigo 3º
Utilização de meios e recursos da Universidade

1. Sem prejuízo das disposições legais que impõem ou venham a impor regime diverso, a Universidade do Porto será titular dos Direitos de Propriedade Industrial relativos às invenções ou outras criações concebidas e realizadas no todo ou em parte com a utilização dos seus meios e recursos por pessoas com ou sem vínculo contratual à Universidade, incluindo discentes de qualquer ciclo, independentemente da entidade que financia.
2. A participação de toda e qualquer pessoa, não vinculada à Universidade do Porto por contrato que preveja a realização de actividades inventivas ou de investigação, em projectos ou outras actividades que impliquem a utilização de meios e/ou recursos da Universidade obriga à assinatura prévia de uma declaração, conforme modelo 1 anexo ao presente regulamento e que dele faz parte integrante, nos termos da qual o inventor ou criador reconheça a sujeição da sua participação à aplicação do presente regulamento.

Artigo 4º
Investigadores de carreira

1. O regime geral da titularidade de direitos de propriedade industrial apresentados nos artigos 2.º e 3.º deste regulamento aplica-se também aos investigadores contratados pela UP e abrangidos pelo Estatuto da Carreira de Investigação Científica.
2. Os investigadores referidos no número 1 do presente artigo poderão optar, através de requerimento dirigido ao Reitor da UP, pelo regime de co-propriedade e em partes iguais, à Universidade e ao Investigador, segundo disposição legal do Decreto-Lei nº 124/99 de 20 de Abril. Neste regime de co-propriedade os custos inerentes ao processo e gestão da protecção jurídica dos resultados de investigação, assim como os benefícios financeiros líquidos obtidos pela exploração económica desses resultados serão repartidos entre a Universidade e o inventor em partes iguais.

Artigo 5º
Contratos com terceiras entidades

1. Os contratos e protocolos celebrados entre a Universidade e outras entidades, de qualquer natureza, independentemente da sua forma de financiamento, deverão prever, obrigatoriamente a regulamentação sobre os Direitos de Propriedade Industrial.
2. Na celebração do contrato ou protocolo poderão as partes estipular outro titular dos direitos inerentes aos resultados obtidos que não a Universidade do Porto, por negociação ou entendimento entre as partes.
3. A participação de qualquer elemento, nomeadamente docentes, investigadores, outro pessoal contratado, bolseiros e discentes, na execução dos contratos, deverá ser precedida da celebração de um acordo escrito com a Universidade, no qual se reconhece que a titularidade dos Direitos de Propriedade Industrial sobre os resultados é da Universidade ou da entidade por esta designada no contrato.
4. O contrato poderá determinar que os elementos participantes assinem um documento no qual assumem um dever de confidencialidade quanto às informações e conhecimentos a que tiverem acesso durante a execução do contrato.

Artigo 6º
Direito moral do inventor

Sem prejuízo do estabelecido nos artigos anteriores relativamente à titularidade dos Direitos de Propriedade Industrial, o inventor ou criador tem o direito a ser mencionado como tal no requerimento e título do direito, salvo quando solicite por escrito o contrário.

PARTE III
PROTECÇÃO LEGAL

Artigo 7º
Protecção Legal

1. Nas situações previstas nos artigos 2º e 3.º, a Universidade do Porto decidirá do âmbito de protecção legal da invenção ou criação e da sua manutenção, ficando obrigada ao pagamento dos custos inerentes ao processo de protecção jurídica e manutenção dos direitos outorgados.
2. Caso a Universidade do Porto, no âmbito dos poderes de gestão e administração dos seus Direitos de Propriedade Industrial, decida desistir da manutenção e consequente protecção legal de um Direito de Propriedade Industrial deverá, previamente a tal desistência, comunicar tal facto ao(s) inventor(es) oferecendo-lhe(s) a oportunidade de assumir(em) a titularidade do direito em questão.
3. A comunicação referida no n.º 2 anterior deve ser efectivada com uma antecedência mínima de 90 dias relativamente a qualquer prazo limite para conservação de direitos que estejam em vigor.
4. Caso o(s) inventor(es) pretenda(m) assumir(em) a titularidade do direito em questão, deverá ser celebrado um contrato de transferência da titularidade do direito para o(s) inventor(es).

PARTE IV
EXPLORAÇÃO DOS DIREITOS

Artigo 8º
Competência

1. Nas situações previstas nos artigos 2º e 3º do presente Regulamento, competirá à Universidade a prática de todos os actos que conduzam à exploração adequada dos Direitos de Propriedade Industrial.
2. O inventor e a unidade orgânica a que pertence, serão informados de todas as diligências referentes ao processo de exploração dos Direitos de Propriedade Industrial, bem como sobre os termos precisos das propostas contratuais dirigidas à Universidade.
3. O inventor fica obrigado a colaborar com a Universidade no processo de valorização dos resultados de investigação.

Artigo 9º
Repartição de Benefícios

1. Os benefícios financeiros líquidos obtidos pela exploração económica dos resultados de investigação serão objecto de repartição nas seguintes proporções:
   a) 10% para a Universidade do Porto;
   b) 30% para a Unidade Orgânica ou outra entidade do universo da UP em que se realizou a actividade que conduziu a uma invenção ou criação;
   c) 60% para o Inventor;
2. Os benefícios referidos reportam-se aos montantes obtidos depois de serem deduzidos os custos inerentes à protecção legal dos resultados e outros custos, eventualmente incorridos no processo de comercialização dos mesmos resultados protegidos.

Artigo 10º
Pluralidade de beneficiários

1. Sempre que existam vários inventores ou criadores, os benefícios que lhes caibam, de acordo com a forma utilizada no artigo anterior, deverão ser objecto de repartição igualitária, salvo se entre eles

existir acordo que estipule de forma diversa e desde que os próprios levem ao conhecimento da Universidade do Porto esse mesmo acordo.

2. Caso existam várias Unidades Orgânicas e/ou outras entidades do universo da UP envolvidas no projecto de investigação que originou os proveitos, estes serão objecto de repartição igualitária, salvo se existir acordo que estipule de forma diversa.

PARTE V
ORGANIZAÇÃO

Artigo 11º
Competências da Universidade do Porto

Compete à Universidade do Porto, designadamente:

1. Implementar o presente Regulamento e os demais procedimentos necessários à sua correcta aplicação;
2. Decidir e efectuar a protecção jurídica dos resultados da investigação, nomeadamente o pedido de patente;
3. Administrar e explorar os Direitos de Propriedade Industrial que lhe pertençam, em exclusividade ou não;
4. Celebrar contratos relativos à exploração dos Direitos de Propriedade Industrial que lhe pertençam.

PARTE VI
PROCEDIMENTOS

Artigo 12º
Dever de Informação e Confidencialidade

1. Como regra geral, o inventor ou criador deverá informar a UPIN – Universidade do Porto Inovação da realização da invenção ou criação no prazo máximo de três meses a partir da data em que esta é considerada concluída.
2. Nos casos em que exista na unidade orgânica, a que pertence o inventor ou criador, um serviço responsável pela gestão das questões de Propriedade Intelectual relativas a essa unidade orgânica, o inventor ou criador deverá informar esse serviço da realização da invenção ou criação no prazo máximo de três meses a partir da data em que esta é considerada concluída. O serviço em questão, por sua vez, tem o dever de informar a UPIN no prazo de 10 dias úteis a contar da data da recepção da comunicação do inventor ou criador, para que possa ser dado início ao processo de eventual protecção dos direitos existentes.
3. Em qualquer caso, a UPIN deverá informar, num prazo máximo de 10 dias úteis, o órgão de gestão competente da unidade orgânica a que pertence o inventor ou criador da recepção da comunicação da invenção ou criação.
4. Sem prejuízo do disposto nos números 1 e 2 deste artigo, no decorrer da sua actividade, o inventor ou criador deverá dar conhecimento, às entidades referidas nos mesmos números 1 e 2 conforme aplicável, dos resultados já obtidos e dos potenciais resultados finais do projecto, de forma a permitir a esta uma avaliação atempada das suas possibilidades de protecção e valorização.
5. O inventor ou criador deverá disponibilizar todas as informações referentes à invenção ou criação que se considerem necessárias ou relevantes para os processos de decisão relativos à sua protecção jurídica e exploração económica.
6. A informação referida nos números anteriores deverá ser elaborada por escrito, assinada pelo inventor ou criador, precisando os elementos técnicos relativos ao objecto e âmbito de aplicação da invenção.
7. As informações serão enviadas às entidades referidas nos números 1 e 2 deste artigo, conforme aplicável, em envelope fechado contendo a menção *"confidencial"* e serão tratadas no decorrer de todo o processo de forma sigilosa, de modo a não prejudicar a possibilidade de protecção jurídica da invenção, obrigando assim todos os intervenientes do processo, nomeadamente quem represente a Universidade do Porto, o inventor e terceiros que, por qualquer forma, estejam envolvidos no procedimento.
8. O inventor ou criador deverá abster-se de publicar ou divulgar qualquer tipo de dados ou informações acerca da invenção ou criação antes de cumprir o dever de informação referido nos números anteriores e da consequente notificação pela Universidade da decisão prevista no artigo seguinte.

9. Em caso de pluralidade de inventores deverá ser designado um Responsável pela invenção ou criação ao qual caberá zelar pelo cumprimento dos deveres estabelecidos nos números anteriores.

Artigo 13º Processo de decisão

1. No prazo máximo de 30 dias úteis a contar da recepção da informação completa referida no n.º 6 do artigo anterior, a UPIN elaborará um parecer fundamentado acerca da solicitação de patente ou de outro título jurídico, que entregará ao Reitor ou a outrem por este designado.
2. O Reitor ou a pessoa por ele designada, contando com as assessorias que considere oportunas, decidirá sobre o interesse ou não de solicitar a patente ou outro título jurídico e disso mesmo informará por escrito o inventor ou criador no prazo máximo de 30 dias úteis contados a partir da data da recepção do parecer, referido no n.º1 deste artigo.
3. O prazo referido no n.º 2 deste artigo poderá ser, excepcionalmente, prorrogado por 30 dias úteis, sempre que as circunstâncias o justifiquem.
4. A solicitação da protecção jurídica para a invenção por parte da Universidade nos prazos previstos nos n.º 1 a 3 do presente artigo constitui presunção *inilidível* da manifestação do interesse da Universidade em assumir a titularidade da invenção.
5. No caso previsto no número anterior, a UPIN deverá no prazo de 5 dias úteis dar conhecimento ao inventor do pedido de protecção legal efectuado, informando igualmente do facto a unidade orgânica a que pertence o inventor.
6. Caso a Universidade do Porto opte por ceder os direitos ao inventor, ou na ausência de uma manifestação da intenção da Universidade em assumir a titularidade da invenção formulada nos termos previstos nos números anteriores, o inventor adquirirá de imediato os direitos sobre a invenção, incluindo os de exploração, podendo requerer em seu nome e a seu encargo a respectiva protecção.
7. No caso referido no número anterior, a actividade de investigação ou desenvolvimento no domínio técnico da invenção poderá realizar-se na Universidade desde que esta o autorize previamente.
8. Caso exista alguma actividade de investigação ou desenvolvimento a realizar-se na Universidade do Porto, esta ficará com o direito a receber 20% dos benefícios financeiros líquidos obtidos pela exploração económica dos resultados.

TÍTULO II

DIREITOS DE AUTOR E DIREITOS CONEXOS

PARTE I
OBJECTO E ÂMBITO DE APLICAÇÃO

Artigo 14º Objecto e âmbito de aplicação

1. Para efeitos de aplicação do presente regulamento e nos termos da lei geral, consideram-se como criações susceptíveis de protecção pelo direito de autor ou direitos conexos as criações intelectuais do domínio literário, científico e artístico, qualquer que seja o género ou forma de expressão, nomeadamente, obras literárias, obras de arte, obras audio-visuais, obras de multimédia, programas de computador que não se enquadrem no n.º 3 do artigo 1.º, ou qualquer outra criação que possa ser considerada como obra.
2. As disposições do presente regulamento serão igualmente aplicáveis a novos objectos de direito de autor ou direitos conexos que eventualmente venham a ser juridicamente tutelados.

PARTE II
TITULARIDADE

Artigo 15º
Regra geral

A Universidade reconhece e consagra como princípio básico que pertence ao respectivo criador ou autor a titularidade dos direitos relativos às obras concebidas e realizadas por docentes, investigadores, outros funcionários e discentes de qualquer ciclo resultantes do desempenho das suas actividades desenvolvidas ou decorrentes de serviços realizados na Universidade, salvo acordo escrito em contrário nos termos previstos e admitidos na Lei Geral.

Artigo 16º
Casos especiais

1. A Universidade do Porto poderá assumir a titularidade dos direitos de autor e direitos conexos, mediante acordo escrito prévio, com o autor ou criador sempre que ocorra uma das seguintes situações:
   a) A obra realizada decorra da execução de um contrato celebrado entre a Universidade e outra entidade, no qual se estipula expressamente que a titularidade dos Direitos de Autor pertencem à Universidade.
   b) A realização ou conclusão da obra implica uma utilização significativa de meios ou de dotações da Universidade.
2. Em qualquer circunstância o criador da obra manterá os direitos morais, previstos na legislação aplicável sendo sempre designado nessa qualidade.

Artigo 17º
Utilização significativa de meios da Universidade

1. No caso previsto na alínea b) do n.º 1 do artigo anterior, sempre que se preveja a utilização significativa dos meios e dotações da Universidade na elaboração de uma obra ou criação intelectual susceptível de protecção pelos Direitos de Autor e Direitos Conexos, deverá ser antecipadamente requerida a autorização da Universidade.
2. A autorização da Universidade ficará dependente da celebração de um acordo escrito entre a Universidade e o(s) autor(es), seguindo os requisitos formais impostos pela Lei Geral, no qual se estabeleçam as regras relativas à titularidade e exploração dos respectivos direitos de autor.

Artigo 18º
Contratos

1. Os contratos celebrados entre a Universidade do Porto e outras entidades, cujo objecto principal ou acessório contemple directa ou indirectamente a criação de obras, deverão prever obrigatoriamente a regulamentação sobre a titularidade e exploração dos respectivos direitos de autor ou direitos conexos.
2. Os contratos referidos no número anterior poderão estipular outro titular dos direitos inerentes que não a Universidade do Porto, por negociação ou entendimento entre as partes.
3. Os contratos referidos no número 1 incluem os que visam o financiamento do trabalho a ser realizado pela Universidade.

Artigo 19º
Benefícios

1. Os benefícios financeiros líquidos obtidos pela Universidade referentes à exploração dos direitos cuja titularidade lhe pertença serão objecto da seguinte repartição:
   a) 10% para a Universidade do Porto;
   b) 30% para a Unidade Orgânica ou outra entidade do universo da UP em que se desenvolveu a Investigação;
   c) 60% para o criador.
2. No caso de existirem vários criadores será atribuída uma repartição igualitária, excepto se existir acordo escrito celebrado entre estes que estabeleça outra forma de repartição e desde que os próprios levem ao conhecimento da Universidade esse mesmo convénio.

PARTE III
ORGANIZAÇÃO

Artigo 20º
Competências da Universidade do Porto

Compete à Universidade do Porto, designadamente:

1. Implementar o presente Regulamento e os demais procedimentos necessários à sua correcta aplicação;
2. Decidir sobre a protecção jurídica dos resultados da criação cuja titularidade lhe pertença;
3. Administrar e explorar os direitos de autor e direitos conexos que lhe pertençam em exclusividade ou não.

TÍTULO III

DISPOSIÇÕES FINAIS E TRANSITÓRIAS

Artigo 21º
Interpretação e Casos omissos

A interpretação e integração do presente Regulamento, nomeadamente dos casos omissos, far-se-á de acordo com a Lei Geral e com os princípios gerais de Direito.

Artigo 22º
Entrada em Vigor

O presente Regulamento entrará em vigor imediatamente após a sua aprovação pelo Senado da Universidade do Porto e publicação em Diário da República.

Artigo 23º
Norma revogatória

1. O presente Regulamento revoga o Regulamento de Propriedade Industrial aprovado na reunião plenária do Senado da Universidade do Porto de 10 de Julho de 2002, sem prejuízo dos Direitos de Propriedade Intelectual originados antes da entrada em vigor deste presente Regulamento.
2. O presente regulamento derroga e sobrepõe-se a todo e qualquer diploma normativo existente e em vigor na Universidade do Porto e suas Unidades Orgânicas respeitante à regulamentação dos Direitos de Propriedade Intelectual.

Artigo 24º
Revisão

Este regulamento poderá ser revisto pelo Senado sempre que seja considerado necessário.

U.PORTO

Intellectual Property Regulation of the University of Porto

Preamble

The University of Porto, hereinafter also referred to as UP, believes that the protection and valorisation of the R&D results and of other activities carried out within UP are an incentive to productivity and innovation, particularly to all those whose activities within the University are strongly connected to the business sector.

On the other hand, the success achieved in the protection of these results is also a strong contribution to strengthening UP's internal and external image and to its recognition as an innovative and entrepreneurial university.

The protection and valorisation of these results can also be a source of income and a way to constitute assets for UP. They can also be the recognition of the activity carried out by some of UP's academic community members, through the financial incentives from which they may benefit following the protection and valorisation of the results of their activity within UP.

In view of the importance that the protection and valorisation of such results have to UP, it is essential that there are rules ensuring this protection and valorisation whenever results are generated and that the interests of UP, as a whole and also of its entities, and of the members of its academic community are safeguarded.

The Intellectual Property Regulation of UP is therefore drawn up, governed by the following clauses:

TITLE I
Industrial property rights

PART I
Object and extent of application

Article 1
Object and extent of application

1. Industrial property seeks to legally protect the creations in the industrial, commercial and services areas, as well as brands and other distinctive commercial signs.

2. For the purposes of interpreting and applying this regulation, Industrial Property Rights are, as provided for in the general law, patents for invention, utility models, drawings or models, new varieties of plants and topographies of semiconductor products, hereinafter referred to as inventions or creations.

3. The principles drawn up in this Regulation are also applicable to inventions that contain computer programmes with implied technical content and industrial applicability, i.e. which contribute or could contribute in the future to solve technical problems.

4. This regulation shall also be applicable to new objects of Industrial Property Rights that may become object of juridical tutelage in the future.

PART II
Ownership of rights

Article 2
General rule

1. Except as otherwise provided in Article 5, the University of Porto establishes, as a general principle, its right to the ownership of the Industrial Property Rights regarding inventions or other creations designed and made by its faculty, researchers and other employees or agents who work at the University of Porto.

2. The same principle applies to the inventions or creations designed and made by all other non-permanent staff, whenever these result from activities carried out under the work contract.

3. The principles laid out in paragraphs 1 and 2 of this article are applicable until the end of the calendar year that follows the resolution of the contract with UP with regard to the inventions or creations disclosed during that period, and which result from work carried out while the work contract with UP was still in force.

4. Should the activity that originated the invention or creation be carried out under a contract or protocol signed between the University of Porto and a third party, the provisions contained in Article 5 of this regulation shall apply.

Article 3

Use of University means and resources

1. Notwithstanding the provisions that impose a different regime, the University of Porto shall own the industrial property rights regarding the inventions or other creations designed and made, wholly or in part, using its means and resources by people with or without a work contract with the University, including any students, regardless of the funding institution.

2. The participation of any person that does not have a contract with the University of Porto which foresees the realisation of inventive or research activities in projects or other activities that imply the use of University means and/or resources requires a previously signed statement, in accordance with Model 1 attached herein, and which is an integral part of this regulation, under the terms of which the inventor or creator recognises that his/her participation is subject to the application of this regulation.

Article 4

Career researchers

1. The general regime of industrial property rights ownership presented in articles 2 and 3 of this regulation also applies to the researchers hired by UP and covered by the Scientific Research Career Statute.

2. The researchers referred to in paragraph 1 of this article may opt, by means of a petition addressed to the Rector of UP, for the regime of joint ownership in equal parts, for the University and the researcher, according to the provision of Decree-law No. 124/99, of 20 April. In this regime of joint ownership, the costs related to the process and management of the legal protection of the research results, as well as the net financial benefits obtained through the economic exploitation of these results, shall be split between the University and the inventor in equal parts.

Article 5

Contract with third parties

1. The contracts signed between the University of Porto and other institutions, of any nature and regardless of their financial agreements, shall include the rules on the industrial property ownership.

2. During the establishment of the contract all parties may stipulate another entity for the rights inherent to the profits, other than the University of Porto, by negotiation or understanding between the parties involved.

3. The participation of any person, namely staff members, researchers and other contracted staff, researchers with grants or scholarships and students, in the activities of the contract should be preceded by a written contract with the University in which it must be recognised that the ownership of the industrial property rights belongs to the University or to an institution designated by the University in the contract.

4. The contract may include an item according to which the participants must sign a confidentiality agreement related to the information and knowledge shared during the execution of the project.

Article 6

Moral rights of the inventor

Notwithstanding the provisions in the previous articles on the ownership of Industrial Property Rights, the inventor or creator has the right to be mentioned as the creator or inventor in the Title Form of the Patent (or other title document), unless otherwise requested in writing.

PART III

Legal Protection

Article 7

Legal protection

1. In the situations included in articles paragraphs 2 and 3, the University of Porto shall decide on the extent of legal protection of the invention or creation and of its maintenance, being responsible for the payment of costs inherent to the legal protection process and maintenance of the rights granted.

2. In the case where the University of Porto, within the power of administration and management of its Industrial Property Rights, decides to give up the maintenance and subsequent legal protection of a property industrial right previously to such action, it shall inform the inventors of this fact and provide them the opportunity of assuming the ownership of those rights.

3. The information referred to in paragraph 2 should be given no later than 90 days prior to any deadline of ownership maintenance.

4. If the inventors wish to assume the ownership of those rights, a right ownership transfer contract should be established transmitting the right to the inventors.

PART IV
Exploitation of the rights

Article 8
Competence

1. In the situations previously foreseen in articles 2 and 3 of this Regulation, the University of Porto shall conduct all the procedures leading to the adequate exploitation of the Industrial Property Rights.

2. The inventor and the Organic Unit (School) to which he belongs shall be informed of all steps related to the exploitation process of Industrial Property Rights, as well as on the exact conditions/terms of the contractual proposals made to the University.

3. The inventor must collaborate with the University in the process of valorising the research results.

Article 9
Sharing of profits

1. The net financial profits resulting from the economic exploitation of the research results shall be split according to the following percentages:

   a) 10% for the University of Porto (UP);
   b) 30% for the Organic Unit (FEUP or other) or another institution of the U.P. in which the inventor has carried out the activity leading to an invention;
   c) 60% for the Inventor.

2. These benefits refer to the actual amounts less the legal protection costs of results and any other costs incurred during the commercialisation process of those protected results.

Article 10

Joint Beneficiaries

1. Whenever there are several inventors or creators, the benefits to which they may qualify shall, according to article 9, be split in equal parts, except when there is an agreement between the inventors splitting them otherwise and if this agreement is known by the University.

2. If there are several Organics Units (Schools) and/or other institutions of the U.P. involved in the research project that gave rise to the profits, these shall be split into equal parts, except as otherwise stipulated in an agreement.

Part V

Organization

Article 11

UP responsibilities

It is the specific responsibility of UP:

1. To implement this Regulation and other procedures necessary to its correct implementation.

2. To decide on and legally protect the results of the research, namely the application for a patent.

3. To administrate and exploit the Industrial Property Rights that it owns exclusively or not.

4. To celebrate contracts related to the exploitation of Industrial Property Rights that it owns.

PART VI

Procedures

Article 12

Duty of information and confidentiality

1. As a general rule, the inventor or creator shall disclose an invention to UPIN – University of Porto, within no more than three months from the date on which the invention is considered to be concluded.

2. In the case of FEUP (our School), where there is a structure responsible for the management of intellectual property issues, the inventor shall disclose the invention to FEUP within no more than three months from the date on which the invention is considered to be finished. FEUP must inform UPIN within 10 working days from the date of the reception of disclosure so that UPIN may start the process for an eventual protection of existing rights.

3. In any case, UPIN shall inform FEUP's Cooperation Office, within no more than10 working days, of the reception of disclosure of any invention, when the inventor belongs to FEUP.

4. Notwithstanding the provisions laid out in paragraphs 1 and 2, during its activity, the inventor shall inform FEUP or UPIN, as applicable, of the results obtained and of potential final results of the project, so as to enable the timely assessment of protection and valorisation opportunities.

5. The inventor shall provide all information related to the invention that is necessary or relevant to the decision processes related to its legal protection and economic exploitation.

6. The information referred to in the preceding paragraphs shall be in writing, signed by the inventor or creator, specifying the technical elements related to the object and field of application of the invention.

7. The information shall be sent to FEUP (Organic Unit from the University) or UPIN, as applicable, in a closed envelope containing the word "Confidential" and shall be treated during the process in a confidential manner so as not to hinder the possibility of legal protection, thus binding all stakeholders in the process, especially those representing UP, the inventor and third parties who, by any means, shall be involved in the process.

8. The inventor shall refrain from publishing or disseminating any kind of data or information about the invention before he fulfils the duty of providing the information referred to in the preceding paragraphs and of subsequent notification by UP of the decision referred to in the subsequent article.

9. Should there be more than one inventor, a responsible entity for the invention shall be appointed, having the obligation of ensuring compliance with the duties established in preceding paragraphs.

Article 13

Decision process

1. UPIN shall give its justified opinion on the patent request or other legal title within no more than 30 working days after receiving the information referred to in paragraph 6 of the preceding article. This information shall be given to the Rector or to a person appointed by the Rector.

2. The Rector, or the person appointed by the Rector, relying on the consultants which it may consider appropriate, shall decide on the interest or not to apply for a patent or other legal title and shall inform the inventor in writing within no more than 30 working days after receiving the statement referred to in paragraph 1 of this article.

3. The period referred to in paragraph 2 of this article may be exceptionally extended by 30 working days whenever the circumstances justify it.

4. The request for legal protection for the invention on behalf of UP, within the periods referred to in paragraphs 1 and 3 of this article, constitutes an irrebuttable presumption of the expression of interest by UP in assuming the ownership of the invention.

5. In the case defined in the preceding paragraph, UPIN shall inform the inventor within 5 working days that the application for legal protection has been filed, also informing FEUP (Organic Unit from UP) of that fact.

6. If UP decides to transfer the rights to the inventor, or in the absence of the expression of intent by UP in assuming the ownership of the invention formulated in terms defined in preceding articles, the inventor shall immediately acquire the rights of the invention, including the right of exploitation, having the possibility to apply, in his name and at his own expense, for the protection of the invention.

7. In the case referred to in the preceding paragraph, the activity of research and development, at a technical level, of the invention may be carried out in UP if authorised previously.

8. In the case where there is some activity of research and development being carried out in UP, the latter shall have the right to receive 20% of the net financial benefits obtained from the economic exploitation of results.

TITLE II
Copyrights and related rights

PART I
Object and extent of application

Article 14
Object and extent of application

1. For the purpose of this regulation, and as provided for in the general law, the creations liable to protection through copyrights or related rights include intellectual creations in the literary, scientific and artistic fields, regardless of nature or form of expression, namely works related to literature, art, audiovisual, multimedia and computer programmes that do not conform to paragraph 3 of article 1, or any other creation that may be considered as work.

2. The provisions drawn up in this regulation are also applicable to new objects of copyright or related rights which may become object of legal tutelage in the future.

PART II
Ownership

Article 15
General rule

The University acknowledges and establishes as a basic principle the right to ownership of the work regarding inventions or creations designed and produced by its faculty, researchers, other employees and students of any cycle, as a result of their activities or of services done in the University, unless otherwise stated in writing under the terms foreseen and allowed in the General Law.

Article 16
Special cases

1. The University of Porto may assume the ownership of copyrights and related rights by means of a previous written agreement with the author or creator in one the following situations:

a) The work carried out stems from the enforcement of a contract signed between the University and another entity, stating explicitly that the ownership of Copyrights belongs to the University;
b) The realisation or conclusion of the work implies a significant use of University means or provisions.

2. In any case, the creator shall preserve the moral rights foreseen in the legislation in force, and shall always be designated as such.

Article 17
Relevant use of University means

1. In the case foreseen in paragraph 1(b), of the preceding article, whenever relevant use of University means and provisions is foreseen to take place in the preparation of intellectual work or creation liable to protection by Copyrights and Related Rights, then prior authorisation must be obtained from the University.

2. The University authorisation shall depend on a written agreement signed between the University and the author(s), according to the formal requirements enforced by the General Law, laying out the rules regarding ownership and exploitation of respective copyrights.

Article 18
Contracts

1. The contracts signed between the University of Porto and other entities, with the main or additional object involving directly or indirectly the creation of works, shall necessarily include the rules on ownership and exploitation of the respective copyrights or related rights.

2. The contracts referred to in the preceding paragraph may stipulate another holder of inherent rights other than the University of Porto, by negotiation or understanding between the parties involved.

3. The contracts referred to in paragraph 1 include those aimed at providing funding for the works to be carried out by the University.

Article 19
Benefits

1. The net financial profits obtained by the University relating to the exploitation of rights owned by the University shall be split as follows:
   a) 10% for the University of Porto (UP);
   b) 30% for the Organic Unit or another institution of the U.P. in which the work was developed;
   c) 60% for the creator.

2. Should there be more than one creator, the benefits shall be split into equal parts, except as otherwise stipulated in an agreement between the creators, and if this agreement is known by the University.

Part III
Organisation

Article 20
Responsibilities of the University of Porto

It is the specific responsibility of UP:

1. To implement this Regulation and other procedures necessary to its correct application;
2. Decide on the legal protection of the results of the creation that it owns;
3. To administrate and exploit the copyrights and related rights that it owns exclusively or not.

Title III
Final and transitional provisions

Article 21
Interpretation and omissions

The interpretation and integration of this Regulation, namely the issues beyond those foreseen in this Regulation, shall be done according to the General Law and with the general principles of law.

Article 22
Entry into effect

This Regulation shall enter into effect immediately after approval by the University of Porto Senate and publication in the *Diário da República*[1].

Article 23
Revoking rules

1. This Regulation revokes the Industrial Property Regulation approved in the plenary session held by the University of Porto Senate on 10 July 2002, notwithstanding the Intellectual Property Rights existing prior to the entry into force of this Regulation.

2. This Regulation revokes and supersedes all existing and effective regulatory diplomas in the University of Porto and its Organic Units with respect to the regulation of Intellectual Property Rights.

Article 24
Revision

Whenever deemed necessary, this regulation may be revised by the Senate.

[1] Official Journal