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Mineralocorticoid Receptor Antagonism by Finerenone Attenuates Established Pulmonary Hypertension in Rats

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ABSTRACT

Aims: We studied the ability of the non-steroidal Mineralocorticoid Receptor (MR) antagonist finerenone to attenuate vascular remodeling and pulmonary hypertension (PH) using two complementary preclinical models [the monocrotaline (MCT) and Sugen/Hypoxia (SuHx) rat models] of severe PH.

Methods and Results: We first demonstrated that MR is overexpressed in experimental and human pulmonary arterial hypertension (PAH) and that its inhibition following siRNA-mediated MR silencing or finerenone treatment attenuates proliferation of pulmonary artery smooth muscle cells (PA-SMCs) derived from idiopathic PAH patients. In addition, we obtained evidence that MR-overexpressing (*hMR*⁺) mice display increased right ventricular systolic pressures (RVSP), right ventricular hypertrophy and remodeling of small pulmonary vessels. Consistent with these observations, finerenone (1 mg/kg/day by gavage), started 2 weeks after MCT injection or 5 weeks after Sugen (SU5416) injection, partially reversed established PH, reducing total pulmonary vascular resistance and vascular remodeling. Finally, we also found that continued finerenone treatment decreases inflammatory cell infiltration and vascular cell proliferation in lungs of MCT and SuHx rats.

Conclusion: Finerenone treatment appears to be a potential therapy for PAH worthy of investigation and evaluation for clinical use in conjunction with current PAH treatments.

Keywords: *Pulmonary hypertension, Pulmonary vascular remodeling, Mineralocorticoid receptor, Aldosterone, Finerenone,*

1 **Introduction**

2 Pulmonary arterial hypertension (PAH) is a rare and severe cardiopulmonary condition for which there
3 is no cure at this time, resulting in progressive right heart failure and functional decline^{1,2}. A
4 dependable treatment that could directly interfere with specific mechanisms involved in the
5 progression of obstructive pulmonary vascular remodeling to limit the chronic elevation in pulmonary
6 vascular resistance (PVR) is therefore urgently needed. Accumulation of pulmonary artery smooth
7 muscle cells (PA-SMCs) and perivascular inflammatory cell infiltrate are two central mechanisms in
8 this process²⁻⁴, but the exact underlying mechanisms remain unknown.

9 Evidence suggests that Mineralocorticoid Receptor (MR), a steroid hormone receptor belonging
10 to the nuclear receptor superfamily of transcription factors, could be targeted in PAH to limit
11 pulmonary vascular cell accumulation⁵⁻⁹. The dysfunctional pulmonary endothelium is indeed a local
12 source of the classical MR ligand aldosterone in remodeled vessels of PAH patients¹⁰, which through
13 MR activation, may facilitate SMC proliferation, inflammation and oxidative stress^{5,6,10-19}. In addition,
14 MR can be activated by a direct and rapid action of oxidative stress and/or angiotensin (Ang)II,
15 through AT1 receptor binding in a non-classical pathway¹⁵⁻¹⁷. Since, in PAH, there is also a local
16 overproduction of endothelial-derived AngII associated with the AT1 overexpression in PA-SMCs²⁰,
17 we hypothesized that a local over-activation of MR signaling in resident vascular cells or/and in
18 perivascular immune cells may play an important role in the pathogenesis of PAH. Consistent with
19 this notion, both spironolactone, which is a non-selective steroidal MR antagonist (MRA), and
20 eplerenone, a selective steroidal MRA, have been reported to reduce vascular and cardiac remodeling
21 in monocrotaline (MCT) and chronic hypoxia (CHx) models of pulmonary hypertension (PH)^{10-14,21}.

22 Finerenone is a first-in-class nonsteroidal MRA that has a higher selectivity and stronger affinity
23 for MR than steroidal MRAs in preclinical models^{22,23}. Although chronic treatments with finerenone
24 have anti-inflammatory, anti-fibrotic, and anti-proliferative properties in several *in vivo* experimental
25 models²⁴⁻²⁷, its efficacy in rat models of severe PH has not been studied yet. The purpose of this study
26 was to determine the potential benefit of chronic treatments with finerenone in two complementary
27 preclinical models [the monocrotaline (MCT) and Sugen/Hypoxia (SuHx) rat models] of severe PH.

28 **Methods**

29 **Animal models of severe PH and *in vivo* treatment**

30 Four-week-old male Wistar rats weighing 100 g (Janvier Labs, Saint Berthevin, France) were studied
31 3 weeks after a single subcutaneous injection of MCT (60 mg/kg; Sigma-Aldrich, Saint-Quentin-
32 Fallavier, France) or vehicle²⁸. Male rats were used to minimize hormonal effects. At day-14, MCT-
33 injected rats were randomly divided into two groups and treated for 2 weeks with daily *per os*
34 treatment with either vehicle [ethanol:PEG400:water, 10:40:50%] or finerenone in vehicle (1
35 mg/kg/day, Bayer AG, Wuppertal, Germany). Two additional groups of age-matched control rats were
36 constituted and treated for 2 weeks with daily *per os* treatment with vehicle or finerenone. To further
37 strengthen the findings obtained in the MCT rat model, a second rat model of severe PH was used.
38 Briefly, 16 male Wistar rats (100 g, Janvier Labs) received a single subcutaneous injection of SU5416
39 (20 mg/kg) and were exposed to normobaric hypoxia for 3 weeks before to return to room air for 5
40 weeks. At 5-weeks post-SU5416 injection, pulsed-wave doppler during transthoracic
41 echocardiography was used to validate the presence of established PH by assessing pulmonary artery
42 acceleration time (AT)/ ejection time (ET) ratio, using Vivid E9 (GE Healthcare, Velizy-Villacoublay,
43 France). Then, SuHx rats were randomized to receive vehicle or finerenone (1 mg/kg/day).

44

45 **Echocardiography and right ventricle (RV) hemodynamic measurements**

46 Rats were anesthetized *via* inhaled isoflurane at 2.0% in room air and body temperature was
47 maintained at 37°C. Transthoracic echocardiography was used to blindly determine AT/ET. Then,
48 pulmonary pressures were measured blindly by closed chest right heart catheterization, as previously
49 described. Briefly, a polyvinyl catheter was introduced into the right jugular vein and pushed through
50 the RV into the pulmonary artery. In parallel, a carotid artery was cannulated for the measurement of
51 systemic arterial pressure. Cardiac output (CO) in rats was measured using the thermodilution method.
52 Hemodynamic values were automatically calculated by the physiological data acquisition system
53 (LabChart 7 Software; ADInstruments Co., Shanghai, China). After measurement of hemodynamic
54 parameters, the thorax was opened and the left lung immediately removed and frozen. The right lung

55 was fixed in the distended state with formalin buffer. The heart was then removed, and the RV wall,
56 LV wall, and interventricular septum (S) were dissected and weighed, and the ratio of RV to LV plus
57 S weight [RV/(LV+S)] was calculated as the Fulton index to assess RV hypertrophy. The percentage
58 of wall thickness [$(2 \times \text{medial wall thickness} / \text{external diameter}) \times 100$] and of muscularized vessels
59 were performed as previously described²⁸.

60

61 **Transgenic mice overexpressing MR**

62 To generate MR-overexpressing mice (*hMR*⁺ mice), the human *MR* gene was placed under the control
63 of the proximal P1 promoter into the B6D2F1 mouse strain as previously described²⁹. The offspring
64 genotypes were determined by PCR and we confirmed that the mice are viable and fertile, and have a
65 normal life span and growth rate.

66 Mice were studied in room air at approximately 12 weeks of age. For echocardiography, mice were
67 anesthetized under gaseous anaesthesia (Isoflurane-Vetflurane, Virbac 1.8-2% in a 1:1 mixture of
68 oxygen:air). Hairs of the thoracic area were removed (hair-removing cream for sensitive skin), and the
69 animal was positioned on a heating platform linked to the echography system (Vevo[®] 3100 LT,
70 Fujifilm VisualSonics Inc., Toronto, Canada) allowing the registration of ECG and respiratory rate.
71 MS-550D (55 MHz) transducer was used for image acquisition and determination of AT:ET ratio and
72 CO; this transducer is specifically dedicated to mouse cardiac imaging (VisualSonics). As previously
73 described³⁰, hemodynamic parameters were measured in unventilated anesthetized mice *via* inhaled
74 isoflurane at 2.0% in room air using a closed chest technique by introducing a 1.4-F Millar catheter
75 (ADInstruments, Paris, France) into the jugular vein and directing it to the RV to assess the right
76 ventricular systolic pressure (RVSP). After the hemodynamic assessments were completed, the heart
77 and lungs were then removed *en bloc* to assess RV and pulmonary vascular changes.

78

79 **Isolation, culture, and functional analyses of PA-SMCs from idiopathic PAH patients**

80 Primary cultures of pulmonary artery smooth muscle cells (PA-SMCs) from idiopathic PAH patients
81 were isolated using an explant-outgrowth method and cultured as previously described³¹⁻³⁴. Briefly,
82 small pieces of freshly micro-dissected pulmonary arteries were cultured in Dulbecco modified Eagle

83 medium supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (100
84 unit/ml penicillin and 100 µg/ml streptomycin). The isolated pulmonary PA-SMCs were strongly
85 positive for α -smooth muscle actin, smooth muscle-specific SM22 protein, and calponin, and
86 negative for von Willebrand factor and CD31. PA-SMCs were routinely tested for mycoplasma and
87 used at early passages < 7. To knockdown MR expression, PA-SMCs were transfected with 100 nM of
88 MR siRNA or with scrambled sequence (Horizon Discovery, Cambridge, UK) using lipofectamine
89 RNAiMAX according to the manufacturer's recommendations. To assess cell proliferation, PA-SMCs
90 were seeded in 96-well plates at a density of 3,000 cells/well and allowed to adhere. After being
91 subjected to growth arrest for 48 h in medium lacking FCS, the PA-SMCs were treated with vehicle or
92 finerenone in presence of 5% FCS. Then, PA-SMC proliferation was assessed by measuring 5-bromo-
93 2-deoxyuridine incorporation using a DELFIA kit (Perkin Elmer, Villebon-Sur-Yvette) as
94 recommended by the manufacturer. BrdU incorporation was determined by measuring Eu-
95 fluorescence in a time-resolved EnVision Multilabel Reader (PerkinElmer, Waltham, MA, USA).

96

97 **RNA purification and RNA sequencing data analysis**

98 Total RNA quality was assessed on an Agilent Bioanalyzer 2100, using RNA 6000 pico kit (Agilent
99 Technologies). Directional RNA-Seq Libraries were constructed using the TruSeq Stranded Total
100 RNA library prep kit (Illumina), following the manufacturer's instructions, 1 µg of total RNA was
101 used. After Ribo-Zero depletion, the samples were checked on the Agilent Bioanalyzer for proper
102 rRNA depletion. Final library quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent
103 High Sensitivity DNA Kit. Libraries were pooled in equimolar proportions and sequenced on three
104 paired-end 42bp runs, on an Illumina NextSeq500 instrument, using NextSeq 500 High Output 75
105 cycles kits. Demultiplexing has been done (bcl2fastq2 V2.15.0) and adapters removed (Cutadapt1.3),
106 only reads longer than 10pb were kept for analysis. All data analysis were performed using R and
107 RStudio software. Reads were independently mapped to the human genome GRCh38 using Rsubread
108 package³⁵. Read count was generated with Rsubread using ensembl GTF file release-105 at gene level.
109 Genes with read count mean lower than 1 were discarded for downstream analyses. TMM
110 normalization was done using edgeR package³⁶ and normalized data were linearized with voom

111 function from Limma R package³⁷. To find differentially expressed genes, we applied a two-way
112 analysis of variance for treatment and batch effect for each gene and made pairwise Tukey's post hoc
113 tests between groups. We then considered as significant genes with p-value < 0.01 and fold-change >
114 1.5 for upregulation and fold-change < -1.5 for downregulation. For functional enrichment analysis we
115 used MSigDB v7.5 as geneset database and applied Fisher exact test with FDR correction p-value for
116 multiple testing. All raw and processed data have been submitted on GEO NCBI database with the
117 accession number GSE202698.

118

119 **Western blot and immunostaining**

120 Tissues were homogenized and sonicated in RIPA buffer containing protease and phosphatase
121 inhibitors (Sigma Aldrich). From 20 to 50 µg of protein was used to detect MR and β-actin as
122 previously described³⁸. For *in situ* studies, we used lung specimens obtained after lung transplantation
123 of patients with idiopathic PAH and lobectomy or pneumonectomy for localized lung cancer of control
124 subjects. Preoperative echocardiological evaluations, including echocardiography, were performed on
125 the control subjects to rule out PAH and the lung specimens from the control subjects were collected
126 distant from the tumor foci. The absence of tumoral infiltration was retrospectively established for all
127 tissue sections by histopathological analysis. Immunohistochemistry or immunocytofluorescent
128 staining for MR (39N antibody^{39,40}), alpha-smooth muscle actin (α-SMA, Santa Cruz Biotechnology),
129 CD68 (Santa Cruz Biotechnology, Heidelberg, Germany), and proliferating cell nuclear antigen
130 (PCNA, Dako, Les Ulis, France) and KI67 (Zytomed Systems, Berlin, Germany) were performed in
131 human or rodent lung paraffin sections. Briefly, 5-µm thick lung sections were deparaffinized and
132 stained with Hematoxylin and Eosin, Sirius red (Sigma-Aldrich), or incubated with retrieval buffer.
133 Then, sections were saturated with blocking buffer and incubated overnight with specific antibodies,
134 followed by incubation with the appropriate dilution of the corresponding secondary fluorescently
135 labeled antibodies (ThermoFisher Scientific, Illkirch, France). Nuclei were labeled using DAPI
136 (ThermoFisher Scientific). Mounting was performed using ProLong Gold antifade reagent
137 (ThermoFisher Scientific). The images were captured using an LSM700 confocal microscope (Zeiss,
138 Marly Le Roi, France) with ZEN software. Other lung sections were used for immunochemistry using

139 vectastain ABC kit according to the manufacturer's instructions (Abcys, Courtaboeuf, France) and
140 counterstained with Hematoxylin (Sigma-Aldrich). Images were taken using Eclipse 80i microscope
141 (Nikon Instruments, Champigny-sur-Marne, France).

142

143 **Statistical Analyses**

144 The data are expressed as the means±SEM. Mann-Whitney *U*-tests were used to assess the statistical
145 significance of differences between two groups. Comparisons concerning more than two groups were
146 calculated by ANOVA, followed by the Tukey test for data with a normal distribution. Differences
147 were considered significant for $P<0.05$. Analyses were performed using PRISM software (GraphPad,
148 la Jolla, CA, USA, version 7).

149

150 **Ethics statement**

151 Animal experiments were approved by the Ethics Committee of the Université Paris-Saclay and
152 carried out in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the
153 National Institute of Health and Medical Research (Inserm). The use of human tissue was approved by
154 the local ethics committee (CPP EST-III n°18.06.06, Le Kremlin-Bicêtre, France) and all patients gave
155 informed consent before the study.

156

157

158 **Results**

159

160 ***MR is overexpressed in remodeled PAs in lungs of idiopathic PAH patients, MCT and SuHx rats***

161 Confocal microscopic analyses and double labeling for MR and α -SMA were used to determine
162 and compare MR protein expression patterns in lung specimens from control subjects and patients
163 with idiopathic PAH as well as in lungs of control, MCT and SuHx rats. In control human lung tissues,
164 we found that MR is expressed at low levels in different cell types of the pulmonary arterial wall of
165 remodeled pulmonary arteries including in PA-SMCs, endothelial cells and different cells present in
166 the adventitia. However, PAH lung specimens displayed a more pronounced staining for MR relative

167 to control lung tissues. This MR overexpression was independent of vessel diameter (**Figure 1A**). Our
168 data indicate a 3-fold increase in the relative fluorescence mean intensity (FMI) of the MR signals in
169 the smooth muscle (FMI=44±19 vs 13±5; $P=0.01$, respectively) and in PA-SMC nuclei (FMI=66±17
170 vs. 22±6; $P=0.04$, respectively) between idiopathic PAH and controls. In sharp contrast, no difference
171 was found in the relative FMI of the MR signals in the endothelial layer (FMI=36±14 vs 22±9;
172 $P=0.41$, respectively) and EC nuclei (FMI=68±19 vs 40±12; $P=0.41$, respectively) between lungs
173 from idiopathic PAH and controls.

174 These observations were replicated in two different animal models of severe PH, with a more
175 pronounced MR staining in PA-SMCs of remodeled vessels in MCT- and SuHx-rat lungs
176 (FMI=15.4±7.1 vs 9.9±3.5; $P=0.05$, and 37.4±4.1 vs 13.1±0.6; $P=0.001$ between MCT or SuHx vs
177 control rat lungs, respectively) (**Figure 1B**). Consistent with the *in situ* data, the mean mRNA and
178 protein levels of MR were increased in cultured PA-SMCs derived from idiopathic PAH patients,
179 relative to controls (**Supplemental Fig. S1**). In contrast, no difference in MR protein levels were
180 found between cultured pulmonary microvascular endothelial cells derived from idiopathic PAH
181 patients and controls (0.94±0.16 vs 0.59±0.94; *NS*, respectively).

182

183 ***MR inhibition following siRNA-mediated MR silencing or finerenone treatment attenuates*** 184 ***proliferation of PAH PA-SMCs***

185 To obtain a global view of the role of MR in human PA-SMCs, comparative transcriptome
186 analyses between PA-SMCs transfected with either a MR siRNA or a scrambled sequence were
187 performed in our study (**Figure 2A-C and Supplemental Fig. S2**). Transfecting PAH PA-SMCs with
188 MR siRNA decreased the MR protein level by more than 50% compared with scrambled sequence. A
189 total of 908 differentially expressed genes (DEGs) including 536 upregulated genes and 372
190 downregulated genes were detected in PAH PA-SMCs transfected with MR-siRNA as compared with
191 PAH PA-SMCs transfected with Src Seq (**Figure 2A**). The gene ontology (GO) analysis of DEGs with
192 significant differences revealed that they are involved in critical biological processes and molecular
193 pathways, such as mitotic cell cycle, microtubule-based process, macromolecule catabolic process,

194 organonitrogen compound catabolic process, intracellular transport, and cell cycle (**Figure 2B and**
195 **Supplemental Fig. S3**). The analysis of enriched KEGG and Reactome pathways also confirmed the
196 association of the DEGs in cell cycle mitotic, cell cycle, and DNA replication (**Figure 2B and**
197 **Supplemental Fig. S3**). The volcano plot (**Figure 2C**) showed global expression changes in MR
198 knockdown PA-SMCs. Taken together, these RNAseq data suggest that knocking out MR expression
199 slowed down PA-SMC proliferation *in vitro*.

200 Subsequent studies were carried out to confirm the association between MR activation and PCNA
201 staining in lung specimens from control subjects and patients with idiopathic PAH using confocal
202 microscopic analyses and double labeling for MR and PCNA. Positive staining of both MR and PCNA
203 were noted in vascular cells in remodeled PAs in lungs of idiopathic PAH patients (**Figure 2D**). Since
204 our data indicate that cultured PA-SMCs derived from PAH patients at early passages (< 7)
205 maintained *in vitro* their MR overexpression (**Supplemental Figure S1**), we next explored the effect
206 of MR inhibition on the abnormal proliferation of PA-SMCs derived from idiopathic PAH patients.
207 Compared with scrambled sequence, MR siRNA decreased the proliferation of PAH PA-SMCs
208 induced by 5% FCS (**Figure 2E**) and reduced expression of several genes found to be regulated by
209 MR and involved in cell proliferation and cell cycle control, such as *CCND1*, *CDC45*, *ESPL1*, *FZRI*,
210 *MCM5*, and *PLK1* (**Supplemental Fig. S2 and Figure 2C**). Consistent with these findings, finerenone
211 (10^{-6} M) also reduced PAH PA-SMCs proliferation compared to vehicle (**Figure 2F**). Taken together,
212 these results support the notion that MR contributes to the PH-associated pulmonary vascular
213 remodeling.

214

215 ***Increased pulmonary vascular resistance and remodeling of pulmonary vessels in transgenic mice*** 216 ***overexpressing MR (hMR^+)***

217 Next, to better understand the functional importance of MR activation in the pulmonary vascular
218 remodeling characteristic of PH, we used hMR^+ mice in which the *hMR* gene is ubiquitously
219 overexpressed (**Supplemental Fig S4**). Doppler-echocardiographic assessment of the left ventricular
220 (LV) structure and function was first performed in anesthetized adult WT and hMR^+ mice and no
221 significant changes in the cardiac output (CO) and AT/ET were found (**Figure 3A**). Invasive

222 hemodynamic using right heart catheterization confirmed the presence of mild PH in hMR^+ mice as
223 reflected by a significant elevation in the values of right ventricular systolic pressure (RVSP) ($P<0.05$)
224 and Fulton index [$RV/(LV+S)$] ($P<0.05$), indicators of right ventricular hypertrophy (**Figure 3B**), and
225 the percentage of medial thickness and muscularized pulmonary arteries (**Figure 3C**). Consistent with
226 our previous study indicating that hMR^+ mice do not display changes in mean arterial blood pressure²⁹,
227 no difference in left ventricle (LV)+ Septum (S) mass was observed between hMR^+ and WT mice
228 (101.8 ± 5.2 vs 104.2 ± 4.7 ; NS, respectively). Finally, we found that in association with the increased
229 expression of cyclin D1 in hMR^+ mouse lungs (**Supplemental Fig S4**), the number of positive cells for
230 the cell proliferation antigen KI67 in remodeled pulmonary vessels from hMR^+ mice and the number
231 of perivascular CD68-positive cells (macrophages) were significantly higher than in WT mouse lungs
232 (**Figure 3D**), supporting the notion that MR activation confers PH and increased pulmonary vascular
233 remodeling in mice.

234

235 *Chronic treatment with finerenone partially reverses PH in the monocrotaline (MCT) and*
236 *sugen+hypoxia (SuHx) rats*

237 Based upon these findings, we next tested the effect of chronic finerenone treatment against the
238 progression of PH induced in rats by MCT or SuHx. No rats died during these studies. Compared with
239 control rats, a substantial decrease in the ratios of AT/ET were found in vehicle-treated MCT rats 4
240 weeks post-MCT injection, without changes of systemic blood pressure. However, this reduction was
241 less pronounced in rats chronically treated with finerenone (**Figure 4A-C**). Consistent with these
242 echocardiographic data, invasive hemodynamic measurements using right heart catheterization
243 confirmed the presence of PH in vehicle-treated MCT rats as reflected by the increase in mean
244 pulmonary artery pressure (mPAP), total pulmonary vascular resistance (TPVR) and $RV/(RV+LV)$
245 ratio, together with a decrease in CO (**Figure 4D**). Importantly, finerenone treatment partially reversed
246 established PH in MCT rats, reducing total pulmonary vascular resistance and right ventricular
247 hypertrophy. Consistent with our observations in the MCT rat model, we confirmed that curative
248 finerenone treatment also partially reverses PH in a second experimental model of severe PH, namely
249 the SuHx rat model (**Figure 4E**). Before treatment (week-5), transthoracic echocardiography validated

250 that SuHx rats exhibit established PH, as reflected by lower values of AT/ET ratio (**Figure 4F**).
251 Compared with control rats, vehicle- and finerenone-treated rats exhibited lower AT/ET ratios 8 weeks
252 post-SU5416 injection, but this reduction was less pronounced in finerenone-treated SuHx rats
253 compared to vehicle-treated rats, suggesting protective effect of finerenone treatment against the
254 pulmonary vascular remodeling induced by SuHx (**Figure 4F**). There were no significant differences
255 in mean blood pressure between groups (**Figure 4G**). In addition, we found that values of mPAP,
256 TPVR and RV/(RV+LV) ratio were reduced in finerenone-treated SuHx rats than in vehicle-treated
257 SuHx rats. Furthermore, we observed that finerenone-treated SuHx rats exhibit an improved cardiac
258 output (**Figure 4H**).

259 Consistent with these results, vehicle-treated MCT and SuHx rats exhibited an increase in the
260 accumulation of collagen (stained with picosirius red) in the RV (**Figure 5A**) as well as in the
261 percentages of medial wall thickness and of muscularized distal pulmonary arteries (**Figure 5B**). In
262 contrast, our results indicated that finerenone-treated MCT and SuHx rats display a reduced
263 accumulation of collagen in the RV and lower percentages of medial wall thickness and of
264 muscularized distal pulmonary arteries relative to vehicle-treated MCT and SuHx rats (**Figure 5**). We
265 next showed that chronic treatment with finerenone decreases inflammatory cell infiltration (CD68
266 staining) and vascular cell proliferation (KI67 staining) in lung of MCT and SuHx rats (**Figure 6**).

267 Taken together, our results indicate that chronic treatment with finerenone partially attenuates
268 PA-SMCs proliferation and decreases inflammatory cell infiltration in these two different animal
269 models of severe PH.

270

271

272 **Discussion**

273 Although finerenone has anti-inflammatory, anti-fibrotic, and anti-proliferative properties in several *in*
274 *vivo* experimental models²⁴⁻²⁷, its efficacy in rat models of severe PH has never been studied. Herein,
275 we obtained evidence indicating that MR contributes to the aberrant cell accumulation within the
276 pulmonary vasculature in human and experimental PAH. We first showed that MR is overexpressed in

277 PAH remodeled vessels relative to control subjects and that its inhibition following siRNA-mediated
278 MR silencing or finerenone treatment attenuates the proliferation of PA-SMCs derived from patients
279 with idiopathic PAH. We also demonstrated that transgenic mice that overexpress MR (*hMR*⁺ mice)
280 present increased right ventricular systolic pressures, right ventricular hypertrophy, and remodeling of
281 small pulmonary vessels and a 2-fold increase in the percentage of PCNA-positive pulmonary vascular
282 cells compared with their wild-type littermates. Finally, we also demonstrated that chronic treatment
283 with finerenone at a dose which does not modify systemic blood pressure, partially attenuates PA-
284 SMC proliferation and inflammatory cell infiltration in lungs of MCT and SuHx rats with established
285 PH.

286 Elevated plasma and lung tissue levels of the two MR activators aldosterone and Ang-II are found
287 in patients with idiopathic PAH^{8,20}, suggesting that MR may be overactivated in lungs of PAH
288 patients. Our study provides direct experimental evidence that MR is overexpressed in PA-SMCs of
289 remodeled pulmonary arteries in patients with idiopathic PAH as well as in MCT and SuHx rats with
290 established PH. Since MR is known to promote vascular oxidative stress, inflammation, proliferation,
291 migration, vasoconstriction, vascular remodeling, and fibrosis and because circulating aldosterone and
292 Ang-II levels have been reported to correlate with the disease severity^{7,10,20}, MR may play a pivotal
293 role in PAH pathogenesis. To support this hypothesis, we performed Doppler-echocardiographic
294 assessment of the left ventricular (LV) structure and function in anesthetized adult WT and *hMR*⁺ mice
295 and showed that *hMR*⁺ mice exhibit mild pulmonary vascular remodeling and right ventricular
296 hypertrophy in room air.

297 Several preclinical studies have demonstrated that MR antagonism with either spironolactone
298 (first generation) or eplerenone (second generation) have beneficial effects in the chronic hypoxia-
299 induced PH with or without administration of the VEGFR2 inhibitor SU5416^{11,12} as well as in the
300 MCT and SuHx rat models of severe PH^{10,13}. However, it is well known that clinical use of these two
301 steroidal MRAs presents several disadvantages. Unselective binding of spironolactone to the androgen
302 receptor as an antagonist and to the progesterone receptor as an agonist is responsible for sexual
303 adverse effects including gynecomastia and impotence⁴¹. Eplerenone is much more selective than
304 spironolactone but has a relatively low *in vitro* affinity for the MR. Importantly, the use of both

305 steroidal antagonists is associated with the risk of developing life-threatening hyperkalemia and
306 worsening of renal function⁴¹. Recently, the non-steroidal third generation of MRA finerenone, a
307 potent and highly selective MRA has been developed²²⁻²⁶. Furthermore, addition of finerenone to
308 optimal renin-angiotensin system (RAS) blockade reduced cardiovascular (CV) and kidney outcomes
309 in two large phase III trials in patients with chronic kidney disease (CKD) and type 2 diabetes
310 (T2D)^{42,43}. The incidence of hyperkalemia-related discontinuation (2.3% vs. 0.9% in placebo) was
311 markedly lower than with spironolactone on top of RAS blockade in CKD patients⁴³. In contrast to the
312 other MR antagonists, finerenone has been reported to act as an inverse MR agonist preventing the
313 recruitment of key transcriptional cofactors on the promoter of the different MR target genes^{25,44}.
314 Herein, we obtained evidence that MR inhibition following siRNA-mediated MR silencing or
315 finerenone treatment attenuates the proliferation of PA-SMCs derived from patients with idiopathic
316 PAH and partially reverses experimental PH in the MCT and SuHx rat models of severe PH.

317 The molecular mechanism underlying the protective effects of finerenone against the pulmonary
318 vascular remodeling in the MCT and SuHx rats remains unknown, however our findings indicate that
319 it may partly be mediated by a decrease in PA-SMC proliferation and a reduction of inflammatory cell
320 infiltration in lungs of finerenone-treated MCT and SuHx rats. These data are consistent with the
321 recent study from Menon and colleagues²¹, which showed that the degree of perivascular lung
322 inflammation is higher in mice with a smooth muscle-specific deletion of MR when they are subjected
323 to SU5416 in combination with chronic hypoxia. Consistent with these results, we also found a
324 significant increase in the percentage of KI67-positive pulmonary vascular cells and a perivascular
325 accumulation of CD68-positive cells in lungs of *hMR*⁺ mice. As underlined by other studies, MR is a
326 pleiotropic factor with central roles in endothelial or immune cell responses, therefore we cannot
327 exclude that finerenone exerts also protective effects through the modulation of the endothelial-^{12,13}
328 and/or macrophage-MR signaling^{45,46}. Using genetically modified mouse models with tissue-specific
329 MR deletion, Kowalski *et al.* have indeed recently demonstrated that only mice with deletion of the
330 *MR* gene in endothelial cells, but not in SMCs, fibroblasts, or in myeloid cells, are less prone to
331 remodel pulmonary vessels under chronic hypoxia than wild-type mice¹². However, Menon *et al.*²¹
332 recently reported that endothelial MR deletion was not sufficient to protect mice exposed to chronic

333 hypoxia combined with SU5416 injection. However, these two different studies found beneficial
334 effects of MR inhibition by eplerenone¹² and spironolactone²¹ in these mouse models of experimental
335 PH, respectively. Taken altogether, these studies reveal cell-type specific roles of MR in the context of
336 PAH that is consistent with the decrease in PA-SMC proliferation and the reduction of inflammatory
337 cell infiltration observed in lungs of finerenone-treated MCT and SuHx rats. Therefore, further studies
338 are needed to study whether finerenone could attenuate abnormal phenotypic features of pulmonary
339 endothelial cells found in idiopathic PAH.

340

341 In summary, these findings underline that MR contributes to the aberrant cell accumulation
342 within the pulmonary vasculature in human and experimental PAH. Our data also demonstrate that
343 finerenone treatment attenuates pulmonary vascular remodeling and decreases pulmonary arterial
344 pressure in two different experimental models of severe PH. Our data indicate that this beneficial
345 effect is mediated not only by a decrease in PA-SMCs proliferation, but also by reduction of
346 inflammatory cell infiltration in lungs of finerenone-treated MCT and SuHx rats. Our findings should
347 encourage clinical investigations and especially the evaluation of the use of finerenone in conjunction
348 with current PAH therapies.

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Authors Contributions: Conception and design: LT, SV, ML and CG; Analysis and interpretation: all; Drafting manuscript: LT, SV, ML and CG. All authors read and corrected the manuscript and approved the final manuscript.

Abbreviations: *α-smooth muscle actin: α-SMA; acceleration time: AT; arbitrary unit: AU; blood pressure: BP; bone morphogenetic protein receptor type II: BMPRII; 5-bromo-2'-deoxyuridine: BrdU; cardiac output: CO; ejection time: ET; fetal calf serum: FCS; left ventricle: LV; mean pulmonary arterial pressure: mPAP; monocrotaline: MCT; mineralocorticoid receptor : MR; pulmonary arteries: PAs; pulmonary arterial hypertension: PAH; pulmonary artery-smooth muscle cell: PA-SMC; proliferating cell nuclear antigen PCNA; right ventricle: RV; right ventricular systolic pressure: RVSP; septum: S; SU5416 combined with hypoxia: SuHx; total pulmonary vascular resistance: TPVR; wild-type: WT.*

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FIGURE LEGENDS:

Figure 1. Increased expression of mineralocorticoid receptor (MR) in vascular cells of remodeled pulmonary arteries in lungs of idiopathic PAH patients, monocrotaline (MCT) and sugen+hypoxia (SuHx) rats. (A) Representative photomicrographs of distal pulmonary arteries in lung sections from controls and PAH patients showing overexpression of MR (red color) within pulmonary arterial wall in controls and idiopathic PAH patients and (B) in control, MCT, and SuHx rats. Pulmonary artery smooth muscle cells (PA-SMCs) were visualized with an anti-smooth muscle α -actin (α -SMA) antibody (green color). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; blue color). L: lumen.

Figure 2. Mineralocorticoid receptor (MR) inhibition attenuates proliferation of human pulmonary artery smooth muscle cells (PA-SMCs) derived from PAH patients. (A) Hierarchical clustering analysis reveals the differentially expressed genes (DEGs) between transcripts in human PA-SMCs treated with scrambled or MR siRNA with a cutoff of P -value < 0.01 and a fold change > 1.5 ($n = 4$ each group). (B) Graph representation of the top 10 enriched terms derived from GO Biological Process, KEGG and Reactome. The blue line indicates with a $pFDR < 0.01$. (C) Volcano plot highlighting significant genes differentially expressed between human PA-SMCs transfected with either a scrambled sequence (src seq) or MR siRNA. Red spots indicate upregulated genes in PA-SMCs transfected with MR siRNA. Blue spots indicate genes downregulated in PA-SMCs transfected with MR siRNA. Genes of the Reactome pathway related to the mitotic cell cycle shown in B are highlighted by boxes. (D) Representative photomicrographs of distal pulmonary arteries in lung sections from controls and PAH patients showing the colocalization of MR (red color) with proliferating cell nuclear antigen (PCNA; white color). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; blue color). (E) Proliferative potential of human PA-SMCs cultured with 5% fetal calf serum (FCS) with or without MR inhibition by RNA-interference or (F) in presence of finerenone. Horizontal lines display the mean \pm SEM. ** P -value < 0.01 , *** P -value < 0.001 versus 0% FCS; # P -value < 0.05 versus 5% FCS.

Figure 3. Transgenic mice overexpressing mineralocorticoid receptor (MR) exhibit remodeling of pulmonary vessels with increased wall thickness in room air: (A) Echocardiographic analysis of the cardiac output (CO) and ratio of acceleration time (AT) to ejection time (ET) in hMR^+ and wild-type mice. (B) Values of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy expressed by the Fulton Index in hMR^+ and wild-type mice. (C) Representative images of hematoxylin-eosin (HE) staining and α -smooth muscle (SM)-actin and quantification of the percentage of wall thickness and muscularized distal pulmonary arteries in lungs from hMR^+ and wild-type mice. (D) Representative images of KI67, PCNA and quantification of the percentage of KI67-positive cells in the muscularized wall of distal pulmonary arteries and of CD68-positive cells in lungs from hMR^+ and wild-type mice. Scale bar= 50 μ m in all sections. Horizontal lines display the mean \pm SEM. * P -value < 0.05 , ** P -value < 0.01 versus wild-type (WT) littermates. AT= acceleration time; AU= arbitrary unit; ET= ejection time; LV= left ventricle; ns= non-significant; RV= right ventricle; S= septum.

Figure 4. Chronic treatment with finerenone partially reverses PH in the monocrotaline (MCT) and sugen+hypoxia (SuHx) rat models: (A) Experimental strategies used to test the curative effects of finerenone in the MCT rat model of PH. (B) Ratios of acceleration time (AT) to ejection time (ET) obtained by transthoracic echocardiography. (C) Values of mean systemic blood pressure (BP), (D) mean pulmonary artery pressure (mPAP), cardiac output (CO), total pulmonary vascular resistance

(TPVR) and right ventricular hypertrophy by the Fulton Index. **(E)** Experimental strategies used to test the curative effects of finerenone in the SuHx rat model of PH. **(F)** Ratios of AT/ET obtained by transthoracic echocardiography. **(G)** Values of mean systemic BP, **(H)** mPAP, CO, TPVR and Fulton Index. * *P*-value < 0.05, *** *P*-value < 0.001, **** *P*-value < 0.0001 vehicle-treated control rats; # *P*-value < 0.05; ## *P*-value < 0.01; #### *P*-value < 0.0001 *versus* MCT or SuHx rats treated with vehicle. AT= acceleration time; AU= arbitrary unit; ET= ejection time; LV= left ventricle; ns= non-significant; RV= right ventricle; S= septum.

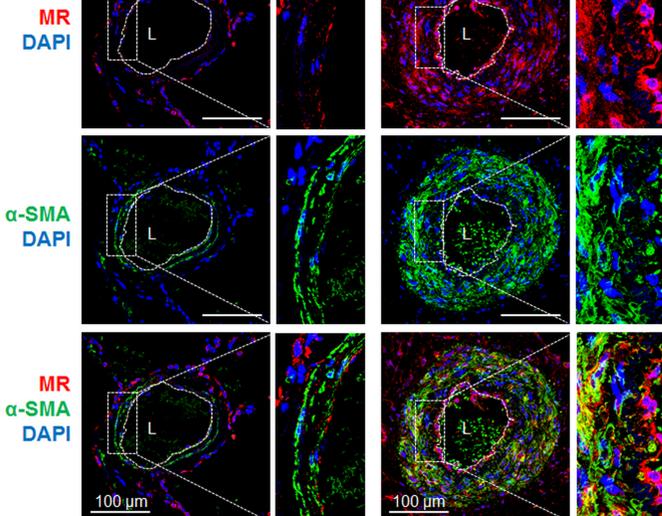
Figure 5. Chronic treatment with finerenone attenuates remodeling of both the right ventricle (RV) and pulmonary arteries (PAs) in monocrotaline (MCT) and sugen+hypoxia (SuHx) rats: **(A)** Representative images and quantifications of picrosirius red staining in the RV myocardium tissues. **(B)** Representative images of hematoxylin-eosin (HE) and α -smooth muscle actin (α -SMA) immunostaining and quantification of the percentage of wall thickness and of muscularized distal PAs in lungs of control, MCT and SuHx rats treated or not with finerenone. Scale bar= 50 μ m in all sections. Horizontal lines display the mean \pm SEM. *** *P*-value < 0.001; **** *P*-value < 0.0001 *versus* control rats; # *P*-value < 0.05, ## *P*-value < 0.01; ### *P*-value < 0.001; #### *P*-value < 0.0001 *versus* MCT or SuHx rats treated with vehicle. AU=arbitrary unit.

Figure 6. Chronic treatment with finerenone attenuates inflammatory cell infiltration and vascular cell proliferation in lungs of monocrotaline (MCT) and sugen+hypoxia (SuHx) rats: Representative images and quantifications of the cell proliferation antigen KI67 and CD68 positive cells per pulmonary vessels. Scale bar= 50 μ m in all sections. Horizontal lines display the mean \pm SEM. ** *P*-value < 0.01; *** *P*-value < 0.001; **** *P*-value < 0.0001 *versus* control rats; # *P*-value < 0.05; ## *P*-value < 0.01; ### *P*-value < 0.001; #### *P*-value < 0.0001 *versus* MCT or SuHx rats treated with vehicle. AU=arbitrary unit.

A**Human pulmonary arteries (< 200 μm)**

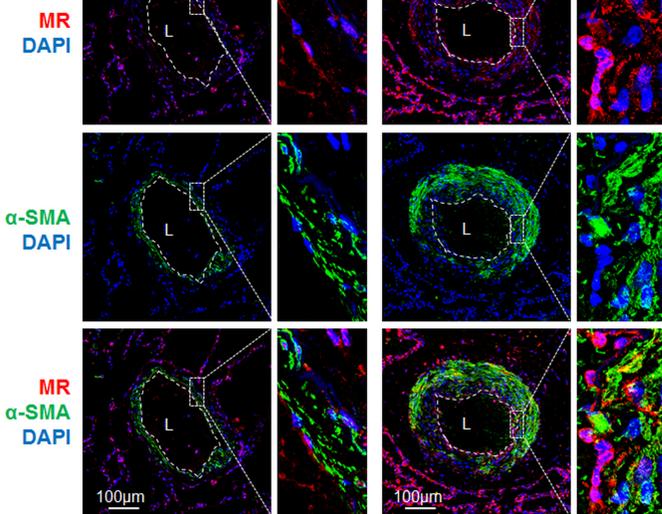
Control

Idiopathic PAH

**Human pulmonary arteries (> 200 μm)**

Control

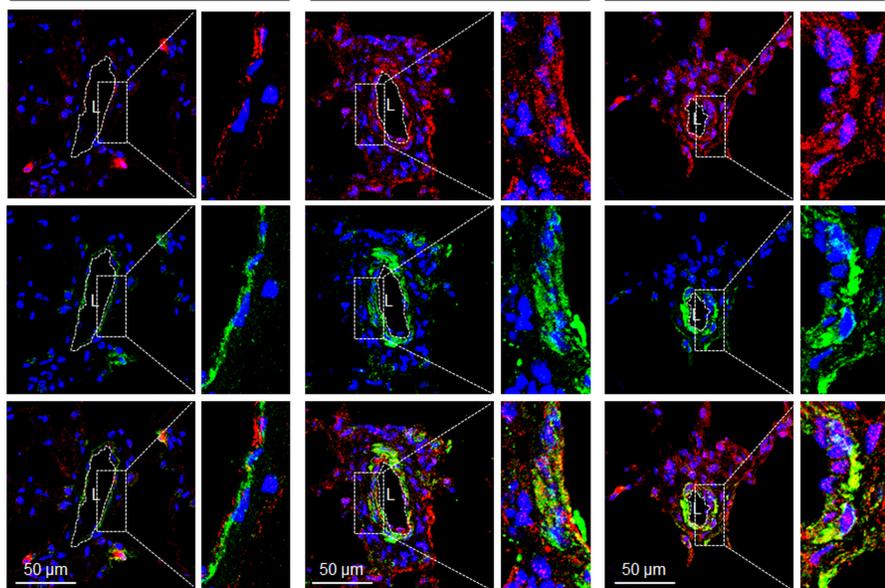
Idiopathic PAH

**B****Rat pulmonary arteries (< 100 μm)**

Control

MCT

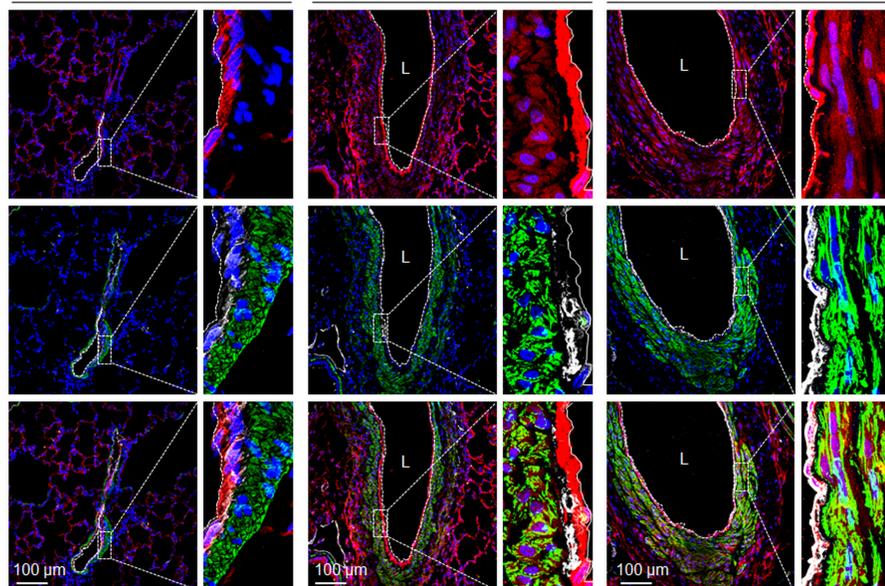
SuHx

**Rat pulmonary arteries (> 100 μm)**

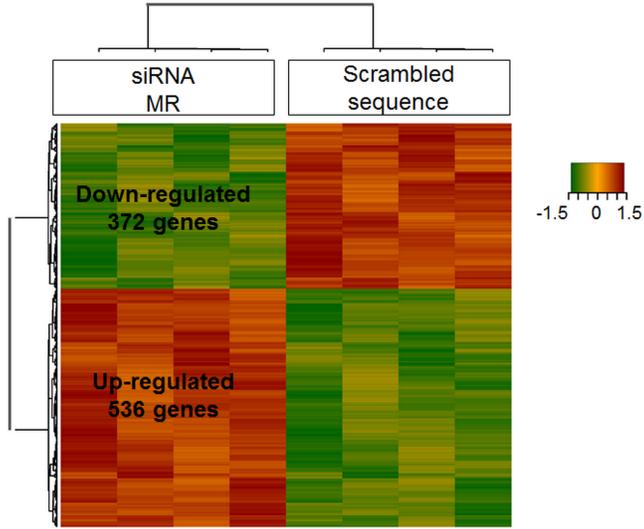
Control

MCT

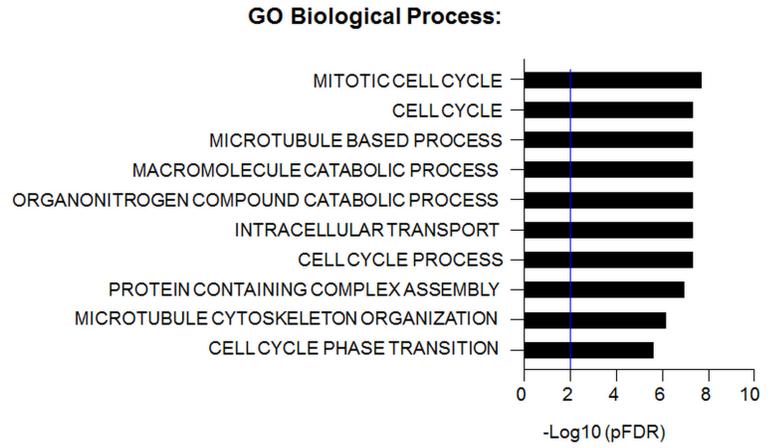
SuHx



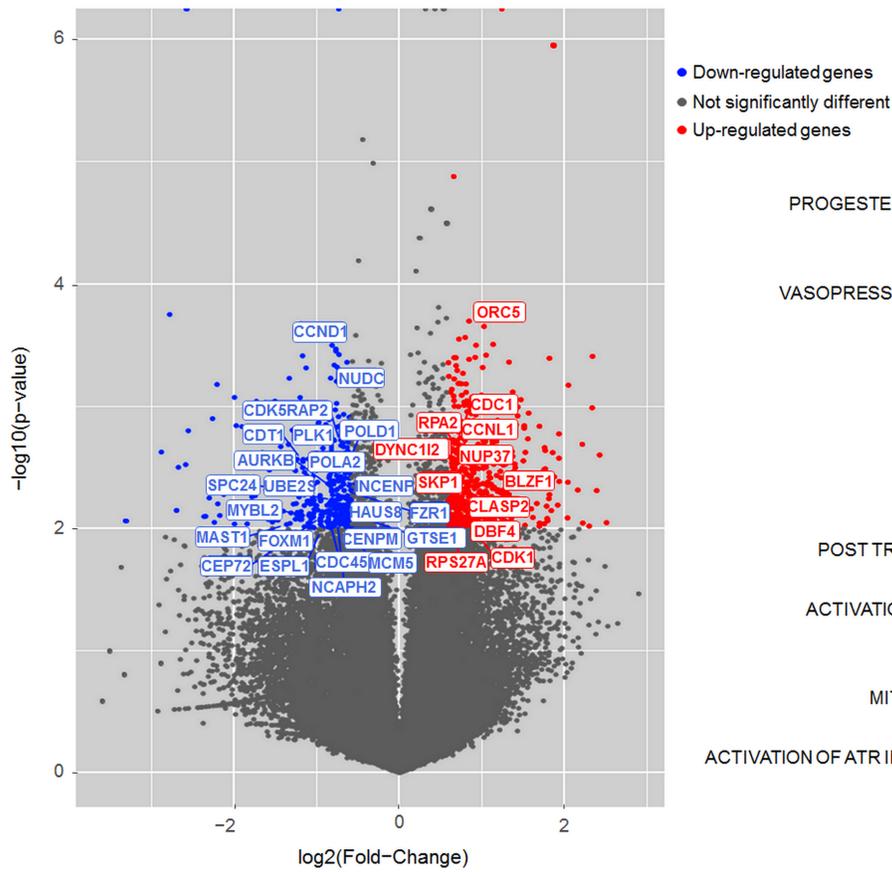
A Human PA-SMCs:



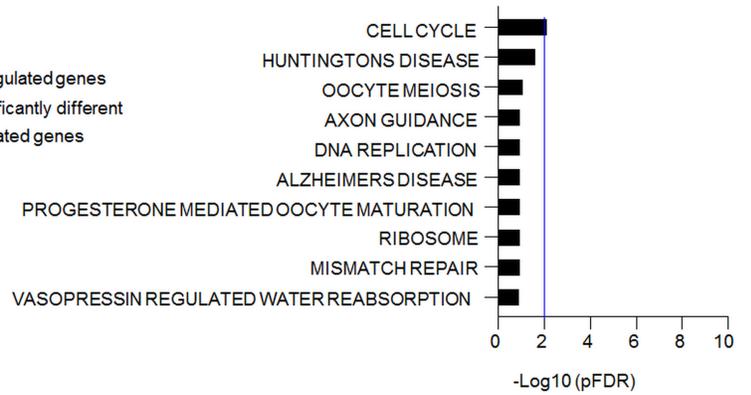
B Human PA-SMCs:



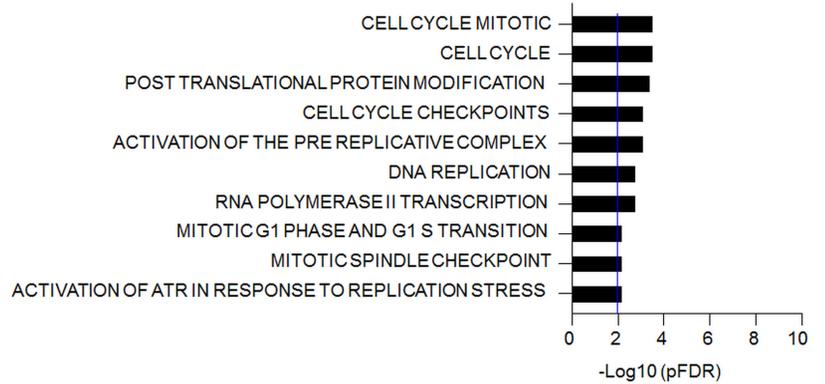
C Human PA-SMCs:



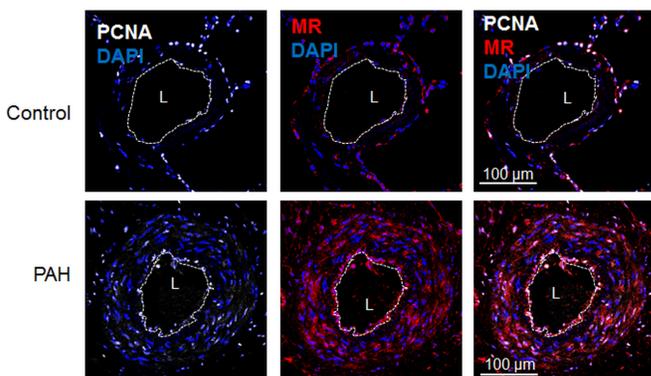
KEGG pathways:



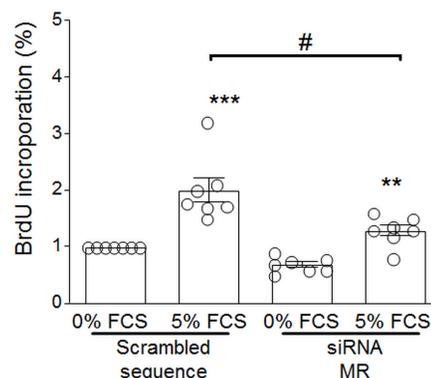
Reactome:



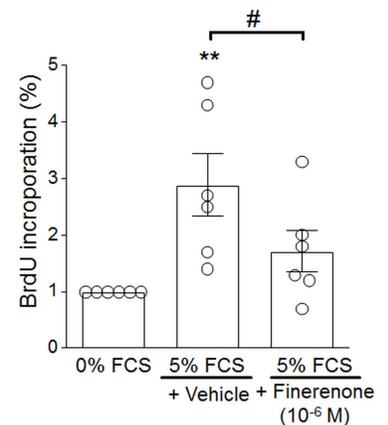
D Human Lungs:



E Human PA-SMCs:



F Human PA-SMCs:



A

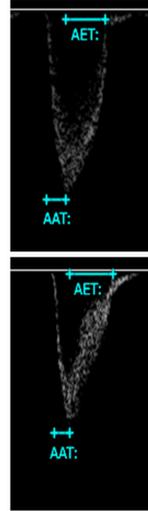
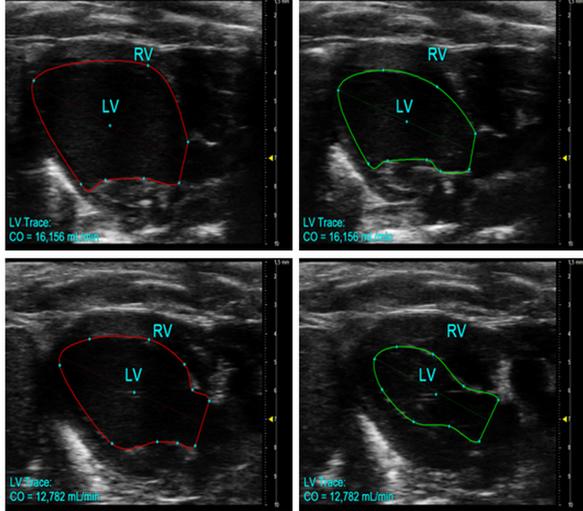
Cardiac Output

Diastole

Systole

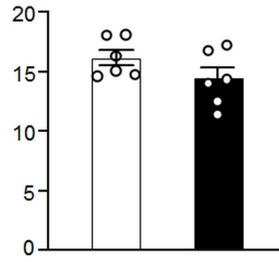
AT/ET

WT

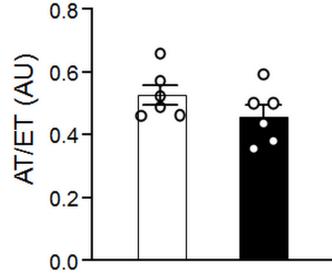
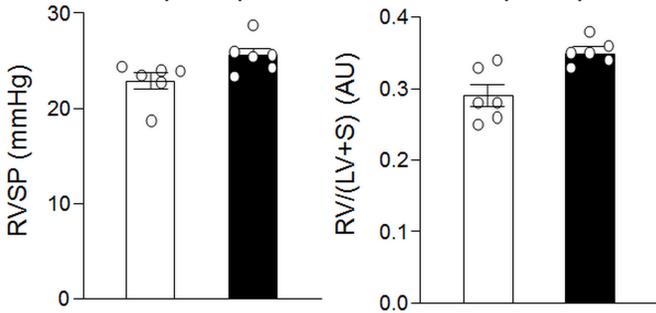
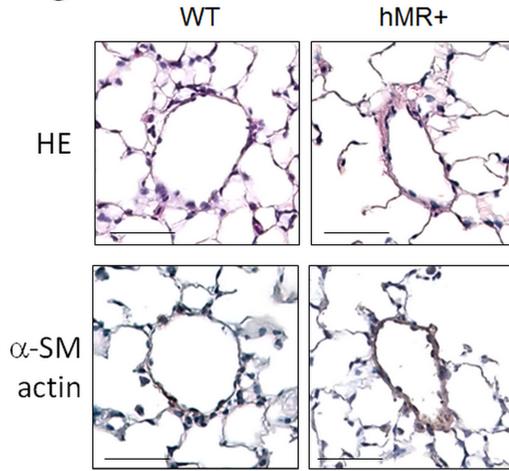


□ Wild-type mice
■ hMR+ mice

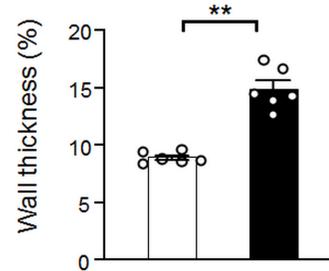
CO (mL/min)



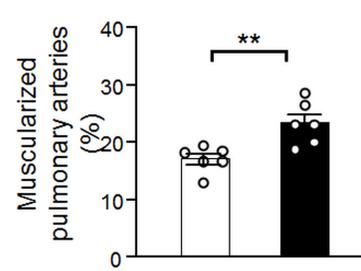
AT/ET (AU)

**B****C**

Wall thickness (%)

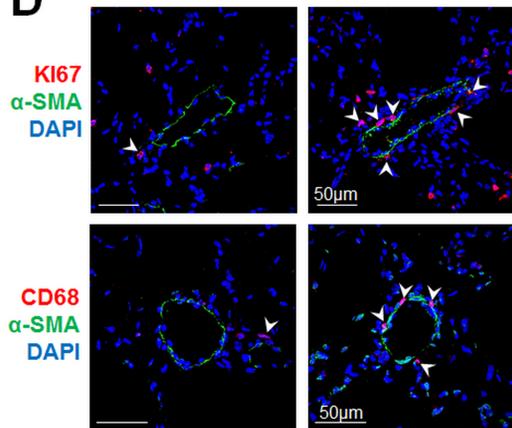


Muscularized pulmonary arteries (%)

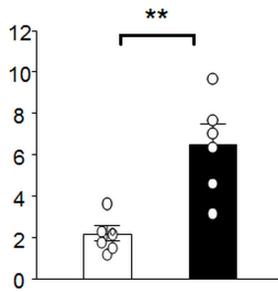
**D**

WT

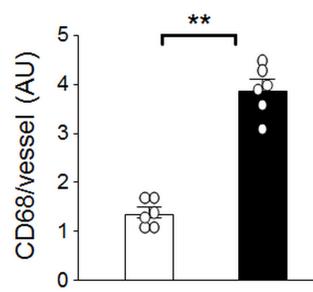
hMR+

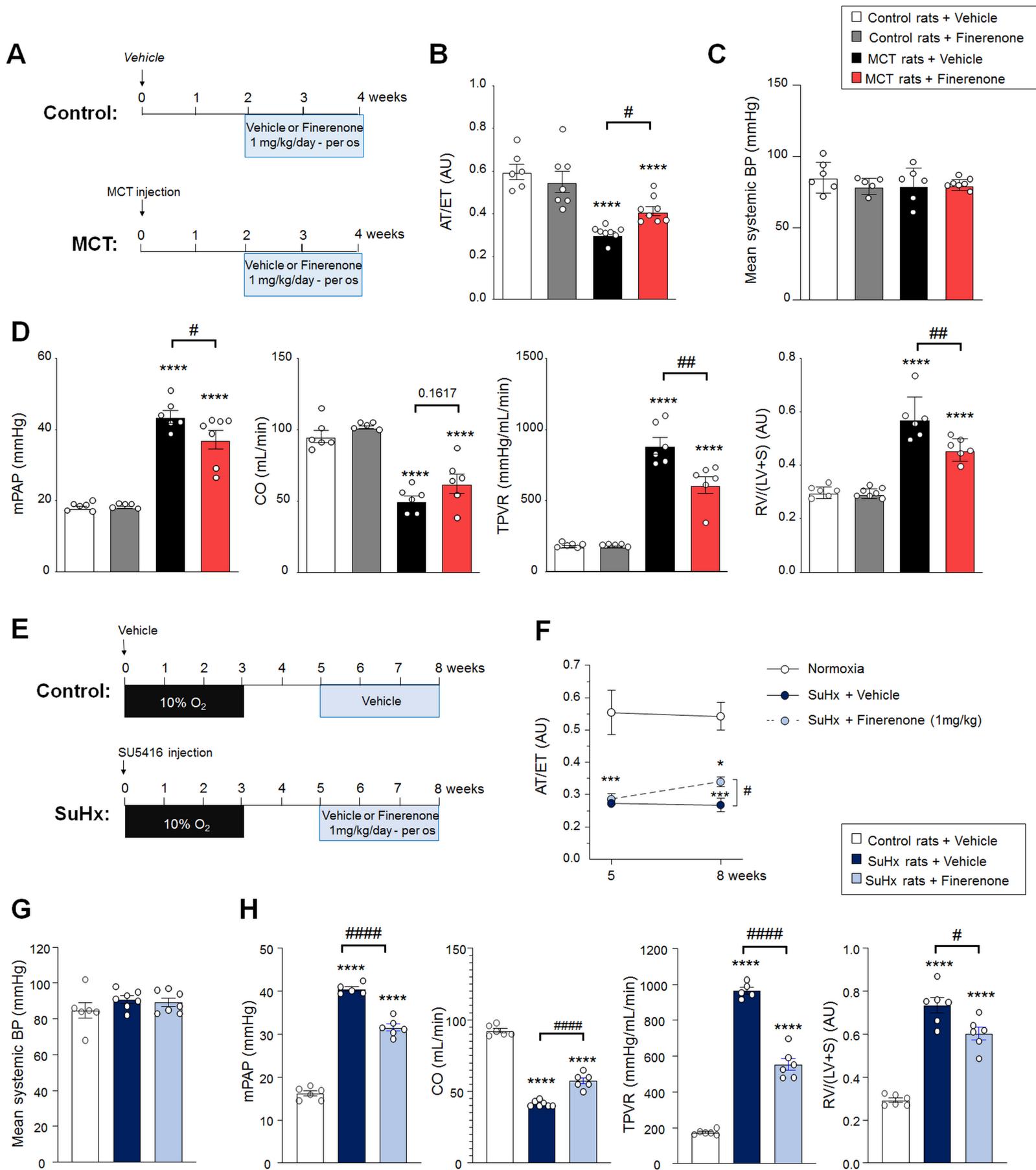


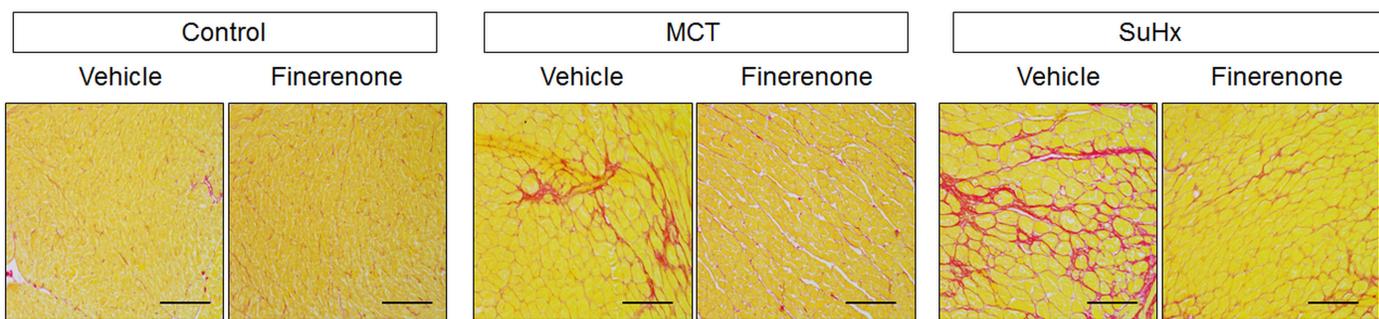
Ki67+/vessel (AU)



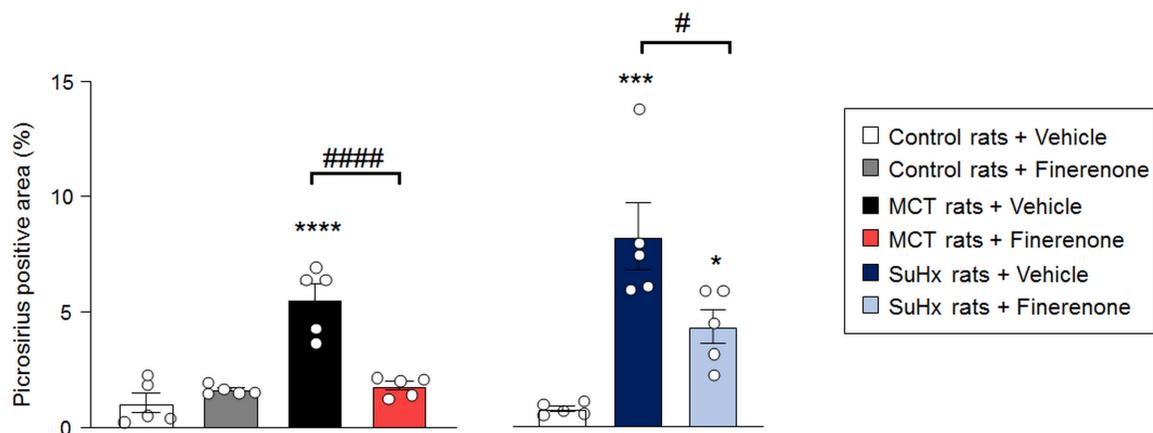
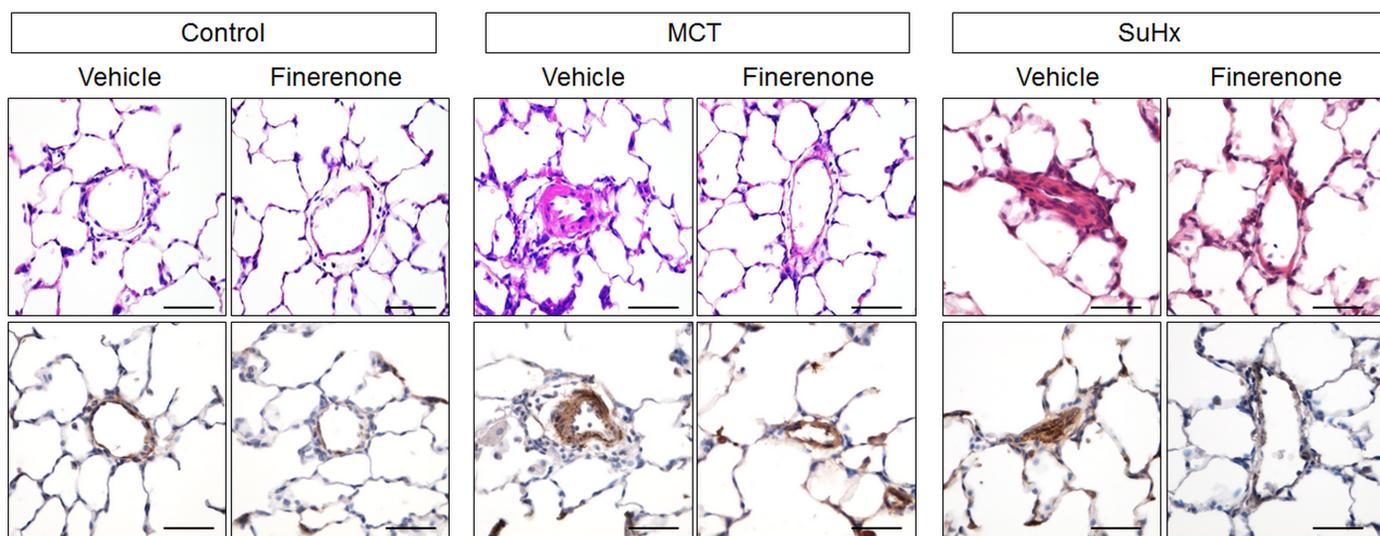
CD68/vessel (AU)





A

Picrosirius red

**B**

HE

α-SM actin

