Mitomycin-Induced Pulmonary Veno-Occlusive Disease: Evidence From Human Disease and Animal Models

Running title: Perros et al.; Mitomycin-induced PVOD

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Abstract

Background—Pulmonary veno-occlusive disease (PVOD) is an uncommon form of pulmonary hypertension (PH) characterized by obstruction of small pulmonary veins and a dismal prognosis. PVOD may be sporadic or heritable because of biallelic mutations of the EIF2AK4 gene coding for GCN2. Isolated case reports suggest that chemotherapy may be a risk factor for PVOD.

Methods and Results—We reported on the clinical, functional, and hemodynamic characteristics and outcomes of seven cases of PVOD induced by mitomycin-C (MMC) therapy, from the French PH Registry. All patients displayed squamous anal cancer and were treated with MMC alone or MMC plus 5-fluoruracil. The estimated annual incidence of PVOD in the French population that have anal cancer is 3.9/1000 patients, which is much higher than the incidence of PVOD in the general population (0.5/million/year). In rats, intraperitoneal administration of MMC induced PVOD, as demonstrated by PH at right-heart catheterization at days 21–35 and major remodeling of small pulmonary veins associated with foci of intense microvascular endothelial-cell proliferation of the capillary bed. In rats, MMC administration was associated with dose-dependent depletion of pulmonary GCN2 content and decreased smad1/5/8 signaling. Amifostine prevented development of MMC-induced PVOD in rats.

Conclusions—MMC therapy is a potent inducer of PVOD in humans and rats. Amifostine prevents MMC-induced PVOD in rats and should be tested as a preventive therapy for MMC-induced PVOD in humans. MMC-induced PVOD in rats represents a unique model to test novel therapies in this devastating orphan disease.

Key words: antineoplastic agents, pulmonary veno-occlusive disease, adverse drug events, pulmonary hypertension, vascular complications, pulmonary hypertension, adverse drug event, vascular complications
Pulmonary veno-occlusive disease (PVOD) is a rare and devastating cause of pulmonary hypertension (PH). PVOD is characterized by widespread fibrous intimal proliferation of septal veins and pre-septal venules, and is frequently associated with pulmonary capillary dilatation and proliferation\(^1\). Although the pathology firmly confirms PVOD, a non-invasive diagnostic approach is usually favored in these fragile patients in whom a lung biopsy carries a high risk of life-threatening complications. Decreased diffusing capacity of the lung for carbon monoxide, severe hypoxemia, radiological abnormalities on high-resolution CTs of the chest, and the occurrence of pulmonary edema after starting pulmonary vasodilator therapy strongly argue for PVOD\(^1,2\). PVOD is a difficult form of PH to diagnose; it is frequently misclassified as idiopathic pulmonary arterial hypertension (PAH). Thus, the true incidence of PVOD is not known precisely, but is considered to represent 10% of cases of PAH that were initially considered as idiopathic or heritable, thus suggesting an incidence of less than 0.5/million/year\(^2,4\).

The pathophysiological mechanisms of PVOD remain poorly understood, in particular because of the lack of animal models conducted on PVOD. PVOD may also be heritable and associated with biallelic mutations in the \(EIF2AK4\) gene that codes for general control non-derepressible 2 protein (GCN2)\(^4\). Isolated case reports suggest that chemotherapeutic agents may induce the development of PVOD\(^5\). However, PVOD is usually reported in patients receiving multiple regimens of chemotherapy, making it difficult to individualize drugs or pathophysiological mechanisms.

We have recently published systematic analysis and the experience of the French PH network, which suggests that alkylating agents may represent the predominant chemotherapeutic class of drugs associated with PVOD\(^6\). The French PH network, which includes specialized
pulmonary vascular centers, has now facilitated a study on the trends of PH and has developed a pharmacovigilance program at national level\textsuperscript{7–9}. Since 2012, any cases of severe PVOD that have occurred in the setting of anal cancer and after therapy with mitomycin-C (MMC) have been identified in the French PH registry and reported to the French pharmacovigilance agency. The present report summarizes the clinical characteristics and outcomes of seven cases of MMC-associated PVOD identified in the French Registry. To confirm a causal link between MMC and human PVOD, we demonstrated that in rats, MMC induced specific remodeling of pulmonary venous and capillary compartments, representing a unique animal model of human PVOD. We have also demonstrated a preventive effect of amifostine, a cytoprotective agent, in animal models\textsuperscript{6}.

Methods

Patients

We prospectively collected the clinical, functional, and hemodynamic characteristics and outcomes of PVOD associated with MMC therapy from the French PH Registry. The Registry was established in accordance with the French bioethics laws (Commission Nationale de l'Informatique et des Libertés) and all patients gave their informed consent. Diagnosis of pre-capillary PH, defined as a mean pulmonary arterial pressure (mPAP) ≥25 mmHg with a normal pulmonary capillary wedge pressure (PCWP) of ≤15 mmHg, was confirmed in all patients by right heart catheterization. Right atrial pressure, mPAP, PCWP, and mixed venous oxygen saturation were recorded. Cardiac output (CO) was measured using the standard thermodilution technique. Pulmonary vascular resistance was calculated as (mPAP-PCWP)/CO, expressed in Woods unit. Acute vasodilator testing was performed during right heart catheterization using...
inhaled nitric oxide, as previously described\textsuperscript{10}. Routine evaluation at baseline included medical
history, physical examination, echocardiography, high-resolution CT of the chest,
ventilation/perfusion lung scan, abdominal ultrasound, auto-immunity screening, and HIV
serology. Age at diagnosis, modified New York Heart Association (NYHA) functional class, and
6-minute walk distance were also recorded.

Number of cycles of MMC therapy, associated chemotherapeutic agents, delay between
the last dose of MMC and diagnosis of PVOD, and outcomes were collected.

\textbf{In vivo study design}

Rats were housed at the Faculty of Pharmacy of Châtenay-Malabry (ANIMEX platform,
Châtenay Malabry, France). Experiments were conducted according to the European Union
regulations (Directive 86/609 EEC) for animal experiments and complied with our institution's
guidelines for animal care and handling. The animal facility is licensed by the French Ministry of
Agriculture (agreement N\textdegree{} B92-019-01). This study was approved by the Committee on the
Ethics of Animal Experiments CEEA26 CAP Sud. Animal experiments were supervised by Dr.
Frederic Perros (agreement delivered by the French Ministry of Agriculture for animal
experiment N\textdegree{} A92–392). All efforts were made to minimize animal suffering.

Male and female Wistar rats (Janvier, eight weeks old) were subjected to different
protocols to evaluate gender differences, dose–response relationships, and the kinetics of PH
development after MMC exposure.

\textit{Protocol 1:} Gender-specific susceptibility. Male and female rats were randomly divided
into saline (control, \(n=5\)) or MMC-exposed groups (Ametycin, Sanofi, 4 mg/kg, i.p., \(n=10\)). We
performed hemodynamic measurements, assessed right-ventricle hypertrophy, and tissues were
collected at 24 days after the injection.
Protocol 2: Dose–response relationships. Females, which harbored a more severe response to MMC (than males), were used to study MMC-induced pulmonary vasculopathy induced by fractioned lower doses. Rats were randomly divided into saline (control, \( n=5 \)) or MMC-exposed groups: i.e., 2 and 3 mg/kg/week/2 weeks (i.p. \( n=10 \) for each dose). Rats were sacrificed at 30 days after the first injection. Hemodynamic measurements were made, right-ventricle hypertrophy was assessed, and tissue samples were collected.

Protocol 3: Kinetics of PH development. Female rats were randomly divided into saline (control, \( n=5 \)) or MMC-exposed groups (3 mg/kg/week/2 weeks, i.p., \( n=10 \)). We evaluated the hemodynamics using echocardiography at baseline, and at 1, 2, 3, 4, and 5 weeks after the first MMC injection (\( n=5–10 \) rats for each time point).

Protocol 4: Prevention study. Amifostine was given i.p. at 200mg/kg 30 min prior to 3 mg/kg/week/2 weeks of MMC (\( n=15 \)). This group was compared to the MMC only-exposed group (MMC, 3 mg/kg/week/2 weeks, \( n=25 \)). Survival was evaluated over five weeks. Survivors were sacrificed at five weeks after the second CP injection and assessed for hemodynamic, RVH measurements, and tissue collection. For information about the choice of MMC doses used in this study, see the online data supplement.

Hemodynamic measurements, evaluation of right-ventricle hypertrophy, and collection of tissues

Rats were anesthetized with isoflurane (2 L/min \( O_2/3\% \) isoflurane; Minerve, Esternay, France). Hemodynamic measurements were recorded with a PowerLab 4/35 data-acquisition system and analyzed with LabChart software (AD-Instruments, Oxford, UK).

In anesthetized rats, blood from the ventral-tail artery was taken for serum analyses prior to the hemodynamic measurements. A 3.5-French umbilical-vessel catheter (Tyco, Plaisir,
France) was introduced into the right external jugular vein. With the angle directed anteriorly, the catheter was inserted 2.5 cm proximally into the right atrium. The catheter was then rotated 90° anticlockwise and advanced 1 cm distally into the right ventricle (RV) and finally into the pulmonary artery after an additional 1.5-cm advancement. Correct anatomical placement was confirmed by respective pressure contours. A T-type Ultra-Fast Thermocouple Probe (IT-23) was inserted into the left carotid artery to allow measurement of CO by the thermodilution technique, after injection of cold saline into the pulmonary artery. Data on mean pulmonary artery pressure (mPAP) in mmHg, CO in mL/min, and total pulmonary resistances in Woods units were subject to statistical analyses.

Following exsanguination, the hilum of the left lung was ligated and the right lung was distended by infusion of formalin via the trachea, and then embedded in paraffin. The non-inflated left lung was snap-frozen in liquid nitrogen and used for protein quantification. Fulton’s index for right-ventricle hypertrophy, i.e., the ratio of right-ventricular weight divided by left-ventricular (LV) plus septal weight (RV/LV+S), was calculated.

Quantification of pulmonary microvessel neomuscularization

Morphological assessment of pulmonary vascular remodeling is depicted in the online data supplement.

Hemodynamic evaluation by echocardiography

Rats were evaluated by transthoracic echocardiography (Vivid E9, GE Healthcare) using a high-frequency transducer (12 S-D 4-12MHz, GE Healthcare). Evaluation was performed under general anesthesia and spontaneous breathing (isoflurane induction 4% at room air plus isoflurane maintenance 2% at room air). Pulmonary-artery acceleration time11, CO, and RV dilatation assessed by the ratio between the RV and left ventricle and RV-wall thickness were
assessed according to guidelines from RV echocardiographic measurements\textsuperscript{12}. All data were averaged during five cardiac cycles.

**Histology and special stains**

Hematoxylin–eosin, hematoxylin–eosin–safran, and orcein stains, and α-smooth-muscle actin (clone 1A4, Sigma-Aldrich, Lyon, France) or proliferating cell-nuclear antigen (PCNA) (clone PC10, Dako, Les Ulis, France) immunohistochemical stains were applied to 5-μm-thick paraffin-embedded lung sections or 10-μm frozen sections using a routine procedure.

**Retrograde microbead injection from the left ventricle.**

Following exsanguination, a catheter was introduced into the left ventricle via and tied to the aorta. Then polystyrene microbeads (10 μm diameter) (Polysciences, Warrington, USA), suspended in BSA 20% (Sigma Aldrich, St-Louis, USA), was injected into the left ventricle until the pulmonary veins became colored. The lungs were then inflated via the trachea with optimal cutting temperature compound diluted in PBS, and then frozen in dry-ice–cold isopentane, as previously described\textsuperscript{13}.

**Proliferation assay \textit{in vivo}**

To examine cell proliferation in rats, we visualized the incorporation of exogenously supplied 5-ethyl-2’-deoxyuridine (EdU) to identify cells that underwent DNA replication. EdU was injected intraperitoneally into the rats at 5 μg/g, at 72 h before they were sacrificed. EdU staining was performed using a Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies, Saint Aubin, France) on frozen sections, according to the manufacturer’s instructions. To characterize EdU-positive cells, we performed double immunostains with rabbit anti-CD34 (Ref ab81289, Abcam, Paris, France) and FITC-labelled anti-α-SMA FITC (clone 1A4, Sigma-Aldrich, Lyon, France) after EdU staining. Sections were viewed under a LSM 700 microscope (Carl Zeiss, Le...
Pecq, France) equipped with 405-, 488-, 555-, and 639-nm lasers (Carl Zeiss). Images were recorded and analyzed with ZEN software (Carl Zeiss).

**Quantification of BMPRII and GCN2 lung expression by Western blotting**

Lung-tissue samples from rats and humans were prepared in lysis buffer containing 1% Igepal, 20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 2mM EDTA, 1 mM Na$_3$VO$_4$, leupeptin 10 μg/μL, lepstatine 10 μg/μL, aprotinin 10 μg/μL, and a protease-inhibitor cocktail (aprotinin, leupeptin and Pefabloc [Roche, Meylan, France]). Protein lysates (40 μg) were separated on SDS–PAGE and transferred to a PVDF membrane. After blocking, the membranes were incubated in Tris-buffered saline with Tween (T-TBS) and 5% non-fat milk overnight at 4°C with the primary antibodies, BMPRII (Ref MA515827, 1/1000, Thermo Scientific), GCN2 (Ref 3302, 1/1000, Cell Signaling, Danvers, USA), and phospho-smad1/5/8 (Ref D5B10, 1/800, Cell Signaling), as well as the mouse monoclonal antibody against β-actin (Sigma), diluted to 1:2000. Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse diluted to 1:10,000 (Cell Signaling) or with horseradish peroxidase-conjugated goat anti-rabbit diluted to 1:5000 (Cell Signaling), respectively. Antibodies were revealed using enhanced chemiluminescence (ECL) (Perkin Elmer). ImageJ software (http://rsb.info.nih.gov/ij/) was used to quantify the level of protein expression.

**Construction of network pathways**

We used Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, USA) as previously described$^{14}$.

**Statistical analyses**

Comparisons were made using the Mann–Whitney (comparison between two groups) or Kruskal–Wallis (comparison between more than two groups) test, followed by Dunn’s test. To
compare the survival between the two groups, the log-rank test was used. $P$-values of <0.05 were considered to be statistically significant.

Results

Description of MMC-induced PVOD in the French PH Registry

We identified seven cases of MMC-induced PVOD in the French PH Registry between June 2012 and December 2014. All patients presented with a diagnosis of squamous anal cancer; two of them were treated with MMC alone and five were treated with MMC plus 5-FU. A diagnosis of severe PVOD was definitively established in all patients based on clinical, functional, radiological, and hemodynamic characteristics (Table 1, Figure 1). All patients were tested for HIV infection, a common risk factor for PAH and anal cancer, and one test was positive (patient 2). No patient underwent a histological procedure because a lung biopsy was contraindicated in these fragile patients and lung transplantation was not proposed due to the recent history of anal cancer\(^1\). The median age of patients was 53 years (range: 42–59) with a predominance of females ($n=6$). The delay between the end of chemotherapy with MMC and the diagnosis of PVOD was 4 months (range: 2–12 months). Patients received between two and four cycles of chemotherapy except for one who also received eight cycles of 5-FU plus oxaliplatin (Table 1).

At diagnosis, MMC-induced PVOD patients had severe clinical, functional, and hemodynamic symptoms. Patients were within NYHA functional class III ($n=4$) or IV ($n=3$). Severe hypoxemia and a low diffusing capacity of the lung for carbon monoxide were observed in all patients (Table 1). Brain natriuretic peptide was increased at diagnosis in all patients (median 951ng/L, range: 452–1536 [normal value <100 ng/L]). Echocardiography suggested PH, impairment of right-ventricular function, and an absence of left-heart dysfunction. Right-sided
heart catheterization confirmed severe pre-capillary PH with a median mPAP of 41 mmHg (range: 36–64 mmHg), a decreased cardiac index of 2.1 L/min/m² (range: 1.6–4), and normal PCWP of <15 mmHg in all patients. Median pulmonary vascular resistance was 11,2 Wood units (range: 5.5–13.6). High-resolution CT of the chest identified septal lines, centrilobular ground-glass opacities, and lymph-enlargement in all patients (Figure 1). Pleural effusions were present in two of the seven patients but no cardiac effusions were observed. Genetic screening for EIF2AK4 and BMPR2 mutations was performed in four patients, and identified no mutations. Due to the severely impaired hemodynamic parameters and the contraindication for lung transplantation, specific PAH therapies were introduced either as a monotherapy (n=2) or as a combination of endothelin-receptor antagonists and phosphodiesterase type-5 inhibitors (n=4) or prostacyclins (n=1) (Table 1). Diuretics were initiated in all patients.

A fatal course of the disease was observed in four of the seven patients due to right-heart failure (n=2), local tumor progression (n=1), and severe pneumonia (n=1).

**Estimated incidence of MMC-induced PVOD in patients with anal cancer in France**

In cancer registries within France, the age-standardized incidence of squamous anal cancer is 0.2–0.7 per 100,000 cases per year in men, and 0.7–1.2 per 100,000 cases per year in women. Thus, we estimated that a maximum of 600 new cases of anal cancer are diagnosed each year in France. In the French PH network, we diagnosed seven cases of MMC-induced PVOD in a 3-year period. The estimated incidence of PVOD in patients displaying anal cancer was 3.9/1000 patients/year and was higher than the incidence of PVOD in the general population, which is estimated at 0.5/million persons/year. The incidence of PVOD in the population that have anal cancer is, therefore, >5000 higher than in those with idiopathic PVOD. Indeed, not all patients that displayed anal cancer received MMC, which led to potential underestimation of the
incidence of MMC-induced PVOD. Unfortunately, the exact number of patients treated with MMC in France was not available.

**MMC-induced pulmonary hypertension in rats with pulmonary vascular lesions resembling PVOD lesions**

When male and female rats were injected with a single dose of 4 mg/kg MMC, they developed PH. This was evident at 24 days later by a significant increase in mPAP, a decrease in CO, increased total pulmonary vascular resistance, and compensatory right-ventricle hypertrophy, as quantified by the Fulton index (RV/(LV+S)) (Figure 2). Due to the small size of our experimental groups, we did not find a significant statistical difference between being male versus female using the log-rank test (data not shown). However, female rats seem to be more sensitive to the toxicity of MMC exposure as only a few (3/10) survived until the endpoint of the study (as compared to 8/10 males surviving).

Specific lesions of human PVOD include septal-vein and pre-septal-venule muscularization and fibrous occlusion; however, another important sign is focal capillary proliferation within the interstitium. Compared to the controls (Figure 3A, B), we observed severe pulmonary vascular remodeling in rats with MMC-induced PH; this included i) smooth-muscle cell hypertrophy/hyperplasia in pulmonary arteries (Figure 3C), ii) neomuscularization of the small distal and normally not muscularized pulmonary vessels (Figure 3D), iii) vasculitis of the pulmonary arteries and veins (Figure 3E), iv) foci of pulmonary edema and capillaritis (Figure 3F–G), and v) foci of alveolar-wall thickening that resembled pulmonary capillary hemangiomatosis (Figure 3H).

Because female rats were more sensitive to MMC, we decided to treat females twice with lower doses (i.e., 2 and 3 mg/kg) of MMC, at one week apart. Rats exposed twice to MMC were
too fragile to undergo invasive hemodynamic measurements, so we evaluated the severity of PH using the Fulton Index (Supplementary Figure 1). At 30 days after the first injection, female rats exposed to 3 mg/kg MMC had severe PH when compared to controls and females given 2 mg/kg MMC. In animals that received 3 mg/kg MMC, we found severe obstructive lesions with pulmonary-artery adventitial and intimal thickening, and medial hypertrophy/hyperplasia (Figure 4A–D). There was also intimal thickening and eosinophilic inflammation of pulmonary veins (Figure 4E–H). More distally, in the vessels accompanying the terminal bronchioles, we found medial hypertrophy of the small pulmonary arteries and severe intimal lesions of small pulmonary veins still associated with extensive adventitial accumulation of eosinophils (Figure 4M–P) when compared to control vessels (Figure 5I–L). MMC-exposed arterioles (characterized by separated internal and external elastic lamina) and venules (characterized by a single external elastic lamina) arranged in the lung parenchyma, harbored neomuscularization, neointimal thickening, and eosinophilic inflammation (Figure 4Q–S), as did the distal pulmonary arteries and veins. In contrast, microvessels (arterioles and venules) present in the control rats' parenchyma were indistinguishable (both were characterized by extremely thin walls) (Figure 4T). The foci of pulmonary edema and capillaritis (Figure 4U–V) and the foci of alveolar-wall thickening were suggestive of pulmonary capillary hemangiomatics in the vicinity of the remodeled venule in MMC-exposed animals (Figure 4W–X): this strengthened the resemblance of this experimental model of PH to that of human PVOD.

To ascertain the post-capillary nature of the pulmonary micro-lesions found in MMC-exposed animals, we performed a retrograde injection of microbeads from the left ventricle into the venous bed of the lungs. The 10-μm-diameter of the beads did not allow them to cross the capillary bed. In control rats, beads-containing vessels displayed loose and slack walls, and large
lumens characteristic of veins and venules (Figure 5A–C). In MMC-exposed rats, the beads were plugged in severely remodeled microvessels, which we identified as post-capillary vessels (Figure 5D–F).

PVOD is characterized by numerous patch-like foci of capillary hemangiomatoses, which are produced by microvascular endothelial-cell proliferation (Figure 6B), and was almost undetectable in the control rats' lung parenchyma (Figure 6A). To examine cell proliferation in rats, we visualized the incorporation of exogenously supplied EdU, to identify cells undergoing DNA replication. In control lung parenchyma, we observed very few EdU-positive cells (Figure 6C), whereas there was a foci of intense microvascular endothelial-cell proliferation (parenchymal CD34+EdU+ cells) in MMC-exposed rats (Figure 6C-G). We also saw endothelial (luminal CD34+EdU+ cells) proliferation in pulmonary venules, which was identified by the presence of microbeads (retrograde injection) in the lumen (Figure 6E). We also observed adventitial-cell proliferation in the remodeled pulmonary microvessels of these animals (Figure 6D, F).

Kinetics of MMC-induced-pulmonary hypertension in rats

In order to assess the time course of PH development in MMC-exposed rats, we performed echocardiographic analyses at the time of MMC injection and then weekly for the five weeks. Pulmonary-artery acceleration time was significantly reduced from the fourth week onwards compared to the controls (medians: 25 vs 39 ms, \( p=0.0007 \)) and baseline values (medians: 25 vs. 30 ms, \( p=0.0035 \)). CO was significantly reduced from the fourth week compared to the controls (medians: 73 vs. 110 mL/min, \( p=0.0008 \)) and baseline values (medians: 73 vs. 91 mL/min, \( p=0.0056 \)). The RV-wall thickness was significantly increased compared to control (medians: 0.08 vs. 0.06 cm, \( p=0.0187 \)) and baseline values (medians: 0.08 cm vs. 0.06 cm, \( p=0.0047 \)).
ratio of RV to LV increased from the fifth week compared to baseline values (medians: 0.58 vs. 0.52, \( p=0.0326 \)). For some animals, we managed to have both echo readings and direct hemodynamic measurement by catheterization: both gave comparable results (Supplementary figure 2).

**MMC-induced PVOD in rats with reduced GCN2 expression and disruption of smad1/5/8 signaling**

On the one hand, our group demonstrated that \( EIF2AK4 \) (coding GCN2) is the major gene linked to PVOD\(^1 \); on the other hand, \( BMPRII \) mutations are strongly related to PAH susceptibility/development\(^16 \). We measured the protein expression of these two genes in MMC-exposed rats. We found that pulmonary protein level of BMPRII were unaffected in these animals, whereas protein levels of GCN2 were decreased (Figure 7A). Moreover, we found that GCN2 pulmonary protein levels were inversely related to the dose of MMC exposure: a high dose of MMC was related to higher PH and lower GCN2 levels (Figure 7B). Accordingly, explanted lungs from heritable PVOD, which we considered as 'pure' PVOD, showed null expression of GCN2, whereas BMPRII levels remained not statistically different from the control lungs (Figure 7C).

Although pulmonary protein level of BMPRII were unaffected in MMC-exposed rats and in human PVOD, both conditions were characterized by an almost total disruption of smad1/5/8 signaling (Figure 7D and 7E). Because the dysfunction of general control non-derepressible 2 (GCN2) signaling is crucial to the development of PVOD, we wondered if this pathway could modulate the smad cascade in the absence of BMPRII alteration, and thus explain, at least in part, our results. We used Ingenuity Pathway Analysis software (IPA), a web-based mammalian biology knowledgebase and analysis tool used for genomic data analysis, to decipher the
connections between both signals. We found a complex and unrevealed interplay between both signals (Supplementary figure 3).

**Amifostine prevented PVOD development when used in combination with MMC.**

Amifostine ameliorated MMC-induced PVOD with notable improvement in survival (Figure 8A), right-ventricle hypertrophy (Figure 8B), and pulmonary hemodynamics (increase in CO with a decrease in TPVR) in MMC + amifostine-treated rats (Figure 8C-E). The decrease of TPR in CP + amifostine treated rats was linked to a decrease in resistive FM-distal microvessels, and an increase in low-resistance NM distal microvessels (Figure 8F-H). The percentage of occluded distal microvessels was significantly lowered in the amifostine group (Figure 8I).

**Discussion**

Since 2012, the French PH network has identified seven cases of PVOD in patients treated with MMC for squamous anal cancer, which is the major indication for MMC in France. These PVOD patients typically present with pre-capillary pulmonary hypertension, radiological abnormalities seen on high-resolution CTs of the chest, a low diffusing capacity of the lung for carbon monoxide (DLCO), and severe hypoxemia. These patients had severe symptoms and marked hemodynamic symptoms at diagnosis. According to previous reports, the prognosis of these PVOD patients appeared very poor, and four of the seven patients died shortly after diagnosis from acute right-heart failure and/or tumor progression17–19. Indeed, two of the seven patients received only MMC, which strongly reinforced the hypothesis of a direct link between MMC and PVOD. Nevertheless, these findings should not obscure the possibility that several other drugs may also have had similar effects as MMC. A recent published analysis of the association between chemotherapeutic agents and PVOD suggests that alkylating agents may
represent a drug class at risk for PVOD\textsuperscript{6}.

In France, treatment with MMC is nearly exclusively used in the setting of anal cancer. Therefore, we used the number of patients treated in France for this indication and extrapolated these results to obtain an incidence rate for PVOD after exposure to MMC. The lower estimated incidence of PVOD in patients displaying anal cancer is excessively high (3.9 per 1000/year) when compared to the incidence of PVOD in the general population (<1 per million/year). However, MMC-induced PVOD has also been reported in the setting of non-small-cell lung cancer\textsuperscript{18}, gastric adenocarcinoma\textsuperscript{20}, and cervical carcinoma\textsuperscript{5}. MMC lung toxicity is mainly caused by acute or chronic interstitial lung diseases, with an estimated incidence of 1.8\textsuperscript{21}.

In this study, we have confirmed the role of MMC in the development of pulmonary vascular lesions in animal models. In rats, MMC exposure induced pulmonary hypertension with pulmonary vascular lesions that resembled PVOD lesions: i.e., i) foci of alveolar-wall thickening suggestive of pulmonary capillary hemangiomatosis, ii) foci of pulmonary edema and capillaritis, iii) vasculitis of pulmonary veins and arteries, iv) intimal and adventitial remodeling of pulmonary arteries and veins (especially after two exposures to MMC), iv) neomuscularization of small distal pulmonary microvessels, v) venule obstruction, and vi) medial hypertrophy/hyperplasia of pulmonary arteries. The post-capillary nature of the pulmonary micro-lesions found in MMC-exposed animals was ascertained though retrograde injection of microbeads from the left ventricle into the venous bed of the lungs. Subsequently, the beads became plugged into severely remodeled microvessels of treated rats, and were identified as post-capillary vessels. MMC-exposed lungs also displayed foci of intense microvascular endothelial-cell proliferation, mimicking the patch-like foci of capillary hemangiomatosis found in PVOD. We also observed endothelial and adventitial cell proliferation in the remodeled
pulmonary microvessels of these animals.

We assessed the time course of PH development in MMC-exposed rats though echocardiographic analyses at the time of MMC injection, and then every week for four weeks. Echocardiographic abnormalities appeared from the fourth week of follow-up. Females were more susceptible to MMC toxicity. Interestingly, while the female/male (F/M) ratio for anal cancer is 2/1 in the general population in France\textsuperscript{15,22}, the gender disequilibrium for MMC-induced PVOD was much stronger (F/M ratio: 6/1). Of note, the only man affected in our series was also HIV-infected, a risk factor for both PAH and anal cancer, whereas all six females were HIV negative. Even if there is no pathophysiological explanation to date, experimental and human data suggest an increased susceptibility of females to MMC-induced PVOD. The reasons for the association between female gender and MMC toxicity are poorly understood.

Only a small proportion of patients who received MMC (alone or in combination with other chemotherapeutic agents) will develop PVOD, although all the rats developed pulmonary venous remodeling in this study. This observation is in accordance with the low proportion of patients who develop PAH in other forms of drug-induced PAH (appetite suppressants: \(<1/10000\textsuperscript{23}\), dasatinib: \(<1/100\textsuperscript{24}\)), suggesting an individual susceptibility to pulmonary vascular drugs. In our series, 5 patients received MMC with 5-FU and one patient had a comorbid HIV infection, a well-known cause of PAH. We cannot exclude that multiple hits may explain the development of PVOD in these patients. Nevertheless, the occurrence of PVOD in 2 patients receiving only MMC (without any other known PAH risk factors) and the data obtained in animal models, suggest a direct role of MMC in pulmonary venular lesions. The multiple hits hypothesis has also been proposed in other pulmonary vascular diseases such as drug-induced PAH or heritable PAH. This susceptibility is not explained by mutations in \textit{EIF2AK4} gene\textsuperscript{4} in
our patients. These results do not exclude the involvement of other genetic characteristics and particular susceptibility polymorphisms in other genes. It is well accepted that several risk factors, such as gene mutations, auto-immunity, drugs, infection, may induce PAH. Similarly, we hypothesize that PVOD could result from different triggers, including chemotherapeutic agents, connective tissue disease (such as systemic sclerosis) or genetic susceptibility (biallelic EIF2AK4 mutations).

We cannot exclude that an associated treatment, such as chemoprotectant (amifostine) or corticosteroids, may also modulate pulmonary vascular injury and reduce the side effects of MMC toxicity in patients. None of our patients received amifostine. To investigate preventive strategies, we evaluated the impact of amifostine in MMC-induced PVOD in rats. In this animal model, amifostine markedly improved survival, and significantly decreased pulmonary distal vascular remodeling, total pulmonary resistance, RV hypertrophy, and RV function. These experimental data suggest that cytoprotective agents may have an ability to prevent PVOD in patients treated with MMC.

Interestingly, we demonstrated in animal models that MMC induced a PVOD-like disease and depletion of pulmonary GCN2 content in a dose-dependent manner. Although pulmonary protein level of BMPRII was unaffected in MMC-exposed rats and in human PVOD, both conditions were characterized by almost total disruption of smad1/5/8 signaling. Using IPA, we found a complex and unrevealed interplay between both signals, which improves our understanding of PH/PVOD pathobiology and justifies further investigations.

PVOD patients have a poor outcome, with a worse prognosis than PAH patients. To date, there is no cure for this devastating disease and the use of specific PAH therapies (prostacyclin, endothelin-receptor antagonists, or phosphodiesterase type 5 inhibitors) may induce life-
threatening pulmonary edema. Thus, the benefits of these treatments have not been demonstrated in PVOD; the only recommended treatment is lung transplantation for eligible PVOD patients. MMC-induced PVOD in rats represents a specific model for pulmonary venous involvement associated with reduced GCN2 expression: this suggests that it could represent a general model for idiopathic, heritable, or drug-induced PVOD. Indeed, this model constituted a unique opportunity to learn more about the pathophysiology of PVOD and to test an innovative therapy.

MMC is an alkylationing agent with antibiotic and antitumor characteristics. It has been previously used to treat pancreatic, stomach, and breast cancers; however, anal cancer remains the main indication for this drug therapy. Complications from this treatment are bone-marrow toxicity, renal damage, and interstitial lung diseases, caused by extensive tissue damage.

Pulmonary vascular injuries, including alterations to endothelial cells, have also been reported. MMC-related lung toxicity is a dose-dependent side effect. It was thought that MMC could have selective toxicity on cancer cells through specific tumor expression of MMC-activating enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1). NQO1 is a flavoenzyme that catalyzes the two-electron reduction of quinones and related compounds, such as MMC. NQO1 has been shown to be overexpressed in many types of cancer. However, Siegel et al. also demonstrated a high level of NQO1 protein expression in normal lung respiratory epithelium and pulmonary vascular endothelium. Hence, vascular cells in normal lungs contain marked NQO1 protein expression and may be damaged by MMC therapy, leading to specific pulmonary vascular toxicity.

Development of lung damage induced by MMC begins with irregular thickening of the endothelial cells of the capillaries and progresses to the release of a surfactant and to the
activation of alveolar macrophages. In a woman treated with MMC plus 5-FU, who developed progressive and fatal PH, scanning electron microscopy of the cast blood vessels showed distortion and destruction of alveolar capillaries that prohibited the passage of erythrocytes. Extensive capillary damage and venous occlusion were observed, resulting in excessive new capillary formation, sometimes in angiomatoid configurations, and hypertrophy of pulmonary veins and arteries. At the molecular level, it has been suggested that MMC-induced pulmonary vascular dysfunction may be related to its bifunctional alkylating property. Cultured pulmonary-artery endothelial cells exposed to MMC showed similar growth arrest and morphological alterations to monocrotaline pyrrole, another bifunctional alkylating substance known to be a potent trigger for experimental PH in rats. Both alkylating agents may compromise the capacity for replicative repair of endothelial cells, accounting for the delayed vascular injury induced by these molecules. Moreover, it has been shown that MMC results in a marked decrease in the biosynthesis of prostacyclin in cultured human umbilical-cord-vein endothelial cells. Lastly, MMC has the characteristics expected of a bio-reductive alkylating agent: activation of alkylating species occurs more readily under hypoxic conditions and the drug is selectively cytotoxic to hypoxic cells. The underlying hypoxic condition may, thus, increase the pulmonary vascular side effects of MMC.

Biallelic mutations of the EIF2AK4 gene (coding GCN2) have been identified as the predisposing gene related to heritable PVOD. According to our results, we can hypothesize that MMC depletes GCN2 in the lungs and mimics PVOD induced by EIF2AK4 mutations. Interestingly, Wilson et al. have demonstrated that the loss of GCN2 promotes oxidative stress and inflammatory-mediated DNA damage during asparaginase therapy; this suggests that patients with reduced GCN2 may be at risk of developing hepatic complications during
asparaginase treatment\textsuperscript{37}. Our results may suggest that MMC-induced GCN2 depletion could favor similar oxidative and inflammatory pulmonary injuries.

Recently, Lathen et al.\textsuperscript{38} demonstrated that the ERG–APLNR axis may play a role in the control of pulmonary-venule endothelial proliferation. These authors demonstrated that \textit{Erg} binds to and serves as a transcriptional activator of the G-protein-coupled receptor gene, \textit{Aplnr}, and that the knockout of either \textit{Erg} or \textit{Aplnr} results in pulmonary venous-specific endothelial proliferation \textit{in vitro}\textsuperscript{38}. Indeed, mice with a homozygous deletion of \textit{Erg} or \textit{Aplnr} develop remodeled post-capillary pulmonary venules. Interestingly, in human PVOD lung tissues, levels of ERG protein, APLNR-mRNA, and protein were significantly decreased compared to control lungs\textsuperscript{38}. Unlike our animal model of MMC-induced PVOD, which was characterized by a dose-dependent depletion in pulmonary GCN2 content, the authors did not find any difference in the expression of GCN2 in lung tissues from animals with homozygous or heterozygous deletion of \textit{Erg} or \textit{Aplnr}\textsuperscript{38}. These results may suggest that animal models of PVOD, based on the deletion of \textit{Erg} or \textit{Aplnr}, may induce pulmonary venous lesions independently of the \textit{EIF2AK4} pathway, the main predisposing gene factor for heritable PVOD\textsuperscript{4}. Relations between exposure to the alkylating agent \textit{EIF2AK4} and the ERG-APLN axis should better characterize the pathophysiology of PVOD. Interestingly \textit{Erg}-\textit{Aplnr}-related and MMC-induced PVOD models may allow us to address distinct aspects of PVOD. The first model is a model of pulmonary venular sprouting, whereas the second is an Eif2ak4-related model that reproduces the whole spectrum of PVOD lesions.

In conclusion, seven cases of MMC-induced PVOD have been identified in the French PH network in a 3-year period. Physicians should be aware of this rare complication of MMC therapy. Unexplained dyspnea after MMC therapy should raise the possibility of underlying
pulmonary vascular toxicity and PVOD should be screened for through a non-invasive work-up (DLCO, blood gases, Doppler echocardiography, and a high-resolution CT of the chest). Possible cases of PVOD at screening should undergo right-heart catheterization to confirm and evaluate PH severity. Our preclinical data confirm the links between MMC exposure and pulmonary venous lesions, and open up a new area of research. MMC-induced PVOD in rats is a novel animal model for human PVOD, which allows us to test innovative strategies to prevent and treat this devastating disease. Amifostine prevents MMC-induced PVOD in rats and should be tested as a preventive therapy of MMC-induced PVOD in humans.

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Table 1. Characteristics of the seven patients with MMC-induced PVOD identified in the French PAH network.

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Figure Legends:

Figure 1. High-resolution CT of the chest in a patient displaying MMC-induced PVOD. A, Presence of septal lines and centrilobular ground-glass opacities. B, subcarinal lymphadenopathy.

Figure 2. MMC induces pulmonary hypertension in rat. A, Mean pulmonary arterial pressure (mPAP: in mmHg) in control and MMC-exposed rats (MMC). B, Cardiac output (CO in mL/min). C, Total pulmonary vascular resistances (TRP: in mmHg.mL⁻¹.min). D, Right ventricular hypertrophy characterized by the Fulton Index (FI = right ventricle (RV)/left ventricle (LV) + septum (S) weight ratio). Black dots: males; red dots: females. Females were more sensitive to MMC than males: only 3/10 of females survived compare to 8/10 of males by day 24 after 4 mg/kg MMC. Due to the very severe PH, we could only obtain hemodynamic data from one female. Dot plots with medians; ***: P<0.001, ****: P<0.0001.

Figure 4. Rats exposed twice to 3 mg/kg of MMC had severe pulmonary arterial, capillary, and venous remodeling; the histopathological pattern of PVOD. A-H, M-S, and U-X, MMC-exposed lungs. I-L and T, Gender- and age-matched control lungs. A, B, Intimal remodeling (white double-headed arrow) of the muscular pulmonary artery accompanying the bronchus (external diameter >200 μm). Note the internal elastic (*). C, D, Adventitial remodeling (white double-headed arrow). C, Note the medial smooth-muscle cells (α-smooth-muscle actin, stained red) and D, fibrous remodeling of the adventitia (collagen in orange). E, F, Intimal thickening of pulmonary veins (external diameter >100 μm). G, H, Perivascular eosinophilic inflammation of the pulmonary vein (arrows). I, Control small pulmonary artery and vein (<100 μm). J, Control small pulmonary artery accompanying a terminal bronchiole with very thin muscular media. K,L, A small pulmonary vein with a typical thin loose, slack wall and a large lumen. M, A MMC-exposed small pulmonary artery accompanying a terminal bronchiole with hypertrophied muscular media that is delimited by apparent internal and external elastica. O, P, A MMC-exposed small pulmonary vein, typically delimited by a single elastica, with severe lumen obstruction and extensive perivascular eosinophilic inflammation. Q, R, S, MMC-exposed arteriole (K) and venule (S) arranged in lung parenchyma and harboring neomuscularization, neointimal thickening, and eosinophilic inflammation, as were the distal pulmonary arteries and veins (N, O). T, Microvessels (arterioles and venules, *) present in the control parenchyma are indistinguishable. U, V, Foci of pulmonary edema and capillaritis. W, X, Foci of alveolar-wall thickening suggestive of pulmonary capillary hemangiomatosis in the vicinity of the remodeled venule (*). A, B, D-X: Hematoxylin–eosin–safran + orcein staining, and C: α-smooth-muscle actin immunostained paraffin-embedded tissue sections. A, C, D, E, G, I, M, Q, T, U, W: Scale bar = 100 μm. F, H, J, K, N, O, R, S, X: Scale bar = 50 μm. B, L, P: Scale bar = 20 μm. U: Scale bar = 1000 μm.

Figure 6. Microvascular endothelial cell (MEV) proliferation in human PVOD and MMC-induced PVOD-like PH in rats. A-B, Human lungs, PCNA IHC stains (red stain = proliferating cells, blue counterstain: hematoxylin) on paraffin-embedded tissue sections. C-G, Rat lungs, click-iT EdU stain (green nuclei = proliferating cells) with CD34 (endothelial cells, in red) and α-smooth-muscle actin (smooth-muscle cells/α-SMA, in white) immunofluorescent staining of frozen sections and confocal imaging. Counterstain = 4’,6-diamidino-2-phenylindole (DAPI). A, Control human lung parenchyma displays very few cellular proliferation. B, Pulmonary capillary hemangiomatosis (PCH) from explanted PVOD lung is characterized by intense MEV proliferation. C, Control rat-lung parenchyma displays very little MEV proliferation. D-G, PCH-like foci from MMC-exposed rats display intense MEV proliferation (red cells with green nucleus). Note the microvessels coated with α-SMA (neomuscularization) displaying adventitial-cell proliferation (arrows); the venule is identified by the red autofluorescent microbead (*, retrograde injection), and displays important endothelial-cell proliferation. A-B: Scale bar = 100 μm. C-F: Scale bar = 50 μm. G: Scale bar = 20 μm.
Figure 7. Pulmonary levels of BMPRII GCN2 and p-smad1/5/8 in human PVOD and MMC-induced PVOD-like PH in rats as measured by Western blot analysis (WB). β-actin was used as loading control. Right panels: quantification of the WBs. A, BMPRII and GCN2 protein expression in lungs from controls or rats exposed to a single dose of MMC (4 mg/kg). B, GCN2 protein-expression in lungs from controls or rats exposed to two low (2 mg/kg) or high (3 mg/kg) doses of MMC. C, BMPRII and GCN2 protein expression in lungs from controls or human heritable PVOD (hPVOD = “pure” forms of PVOD) explanted lungs. D, p-smad1/5/8 phospho-protein expression in lungs from controls or rats exposed to a single dose of MMC (4 mg/kg). E, p-smad1/5/8 phospho-protein expression in lungs from controls or hPVOD explanted lungs. Box Plot (Min to Max); *: p<0.05, ****: p<0.0001, n=3-4/group.

Figure 8. MMC-induced PVOD is partially prevented by amifostine pretreatment. Amifostine was given i.p. at 200 mg/kg for 30 min prior to 3 mg/kg/week/2 weeks of MMC. Animals were sacrificed at 4 weeks after the second MMC injection. Survival, right heart hypertrophy, and hemodynamic parameters were evaluated. A, Survival curves for MMC, and MMC + amifostine groups. B, Fulton Index (FI = right ventricle (RV)/ left ventricle (LV) + septum (S) weight ratio). C, Right ventricular systolic pressure (RVSP) in mmHg. D, Cardiac output (CO) in mL/min. E, total pulmonary resistances (TPR) in mmHg.mL/min. MMC-induced pulmonary vascular remodeling is alleviated by amifostine pretreatment. Muscularization of distal vessels (DV) (<50μm) was evaluated. F, % of non-muscularized (NM) DV. G, % of partially muscularized (PM) DV. H, % of fully muscularized (FM) DV. I, % of occluded DV. Dot plots with medians; * p<0.05, ** p<0.01, **** p<0.0001.
Figure 1
Figure 2

(A) mPAP (mmHg)

(B) CO (ml/min)

(C) TPR (mmHg.ml-1.min)

(D) RV/(LV+S)

Control vs MMC: *** p < 0.001, **** p < 0.0001
Figure 8
Mitomycin-Induced Pulmonary Veno-Occlusive Disease: Evidence From Human Disease and Animal Models
Frédéric Perros, Sven Günther, Benoît Ranchoux, Laurent Godinas, Fabrice Antigny, Marie-Camille Chaumais, Peter Dorfmüller, Aurélie Hautefort, Nicolas Raymond, Laurent Savale, Xavier Jaïs, Barbara Girerd, Vincent Cottin, Olivier Sitbon, Gerald Simonneau, Marc Humbert and David Montani

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Expanded Methods

In vivo study design

Rats were housed at the Faculty of Pharmacy of Châtenay-Malabry (ANIMEX platform, Châtenay Malabry, France). Experiments were conducted according to the European Union regulations (Directive 86/609 EEC) for animal experiments and complied with our institution's guidelines for animal care and handling. The animal facility is licensed by the French Ministry of Agriculture (agreement N° B92–019-01). This study was approved by the Committee on the Ethics of Animal Experiments CEEA26 CAP Sud. Animal experiments were supervised by Dr. Frederic Perros (agreement delivered by the French Ministry of Agriculture for animal experiment N° A92–392). All efforts were made to minimize animal suffering.

Male and female Wistar rats (Janvier, eight weeks old) were subjected to different protocols to evaluate sex differences, dose–response relationships and the kinetics of PH development after MMC exposure.

Protocol 1: Gender-specific susceptibility. Male and female rats were randomly divided into saline (control, n=5) or MMC-exposed groups (Ametycin, Sanofi, 4 mg/kg, i.p., n=10). We performed haemodynamic measurements, assessed right-ventricle hypertrophy and tissues were collected at 24 days after the injection.

Protocol 2: Dose–response relationships. Females, which harboured a more severe response to MMC (than males), were used to study MMC-induced severe pulmonary vasculopathy induced by fractioned lower doses. Rats were randomly divided into saline (control, n=5) or MMC-exposed groups: i.e., 2 and 3 mg/kg/week/2 weeks (i.p. n=10 for each dose). Rats were sacrificed at 30 days after the first injection. Haemodynamic measurements were made, right-ventricle hypertrophy was assessed and tissue samples were collected.

Protocol 3: Kinetics of PH development. Female rats were randomly divided into saline (control, n=5) or MMC-exposed groups (3 mg/kg/week/2 weeks, i.p., n=10). We evaluated the haemodynamics using echocardiography at baseline, and at 1, 2, 3, 4 and 5 weeks after the first MMC injection (n=5–10 rats for each time point).

Protocol 4: Prevention study. Amifostine was given i.p. at 200mg/kg 30 minutes prior to 3 mg/kg/week/2 weeks of MMC (n=15). This group was compared to MMC only-exposed group (MMC, 3 mg/kg/week/2 weeks,
Survival was evaluated over five weeks. Survivors were sacrificed five weeks after the second CP injection for hemodynamic, RVH measurements, and tissue collection.

Regarding the doses used in this study, Warren et al.\(^1\) used MMC-DNA adducts as an indicator for the host’s exposure and capacity for metabolic activation, detoxification, and DNA repair. In mice, they found that MMC (4 mg/kg) was roughly equivalent to a conventional human chemotherapy dose. In rats, Mizushima et al.\(^2\) found that 2, 3, and 4 mg/kg MMC induced 0, 33 and 30% mortality, respectively at 28 days. Those doses corresponded in this species, to high-dose chemotherapy. We decided to use 4 mg/kg and lower (3 and 2 mg/kg) in rats as they seemed to be in the equivalent range of human chemotherapy.

As addressed by Andreollo et al.\(^3\), the differences in anatomy, physiology, development and biological phenomena must be taken into consideration when comparing time-lapse between rat and human. Rats rapidly develop during childhood and become sexually mature at about six weeks old, but reach social maturity five to six months later. In adulthood, every month of the animal is approximately equivalent to 2.5 human years. Several authors performed experimental studies in rats and estimated 30 days of human life for everyday life of the animal\(^3\). Regarding our observations in humans, the delay between initiation of MMC therapy and final diagnosis of PVOD was 5.3 months (range: 2–12 months). It is difficult to compare the development of pulmonary vascular damage in animals and in humans, but in each case the development of PVOD after exposure to chemotherapeutical drugs is rapid.

Quantification of pulmonary microvessels neomuscularization

OCT-embedded frozen lungs were sectioned at 6 µm thickness and stained in double immunofluorescence, for von Willebrand factor (vWF, Dako) and α-smooth muscle actin (α-SMA, clone 1A4, Sigma). 40 to 60 pulmonary microvessels (<50µm) were analyzed and categorized as muscularized when vWF+ endothelial cells in microvessels were coated with α-SMA+ smooth muscle cells (fully [FM] or partially [PM] when vessels contained at least one cell that was positive for α-SMA but lacked a continuous layer) or non-muscularized (NM) in the absence of this coating, to assess the degree of muscularization of these normally non-muscularized pre- and post-capillary vessels.
Supplementary legends and figures

Supplementary figure 1: MMC induces PH in a dose-dependent manner. Female rats harboured a more severe response to MMC than males: thus, they were chosen to study MMC-induced severe pulmonary vasculopathy after fractioned lower doses of MMC (2 and 3 mg/kg at one week apart). Rats exposed twice to MMC were too fragile to undergo invasive haemodynamic measurements, so we quantified PH though the Fulton Index =RV/(LV+S) at 30 days after the first injection. Rats exposed to the higher dose of MMC had more severe right-ventricular hypertrophy than those exposed to the lower dose.

Dot plots with means; *: P<0.05, ***: P<0.001

Supplementary figure 2: MMC exposed rat displaying features of PH. A. Four chambers view at day 28 with ratio RV/LV < 1. B. Four chambers view of the same rat at day 33 with a RV/LV ratio > 1. C. Pulmonary artery
acceleration time at day 1 (34.3 ms). D. Pulmonary artery acceleration time at day 28 (33.2 ms). E. Pulmonary artery acceleration time at day 33 (17.0) with mid-systolic notch. Veloticy-time integral allowed an estimation of cardiac output of 45.1ml/min. F. At day 33, a tricuspid regurgitation jet appeared. G. The sPAP estimated with tricuspid regurgitation jet was 50 mm Hg. Right heart catheterization in the same rat has given: sPAP/dPAP/mPAP as follow 49/13/31 mm Hg and CO of 45.6 ml/min.

**Supplementary figure 3:** Network analysis using Ingenuity pathway analysis (IPA) software. This figure depicts the merged networks of proteins/molecules belonging to the BMPRII (BMPR2 gene), and GCN2 (EIF2AK4 gene) signaling. IPA analysis highlights complex and unrevealed interplay between both signaling. Genes/proteins implicated in this network:

- **ARL4D:** ADP-ribosylation factor-like 4D
- **BMPR2:** bone morphogenetic protein receptor, type II (serine/threonine kinase)
- **CDKN2B:** cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
- **CFDP1:** craniofacial development protein 1
- **Eif2:** elf2, Initiation Factor M1
