Serum and pulmonary uric acid in pulmonary arterial hypertension

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Shareable abstract (@ERSpublications)
Uric acid (UA) level is associated with poor prognosis in PAH. Local UA production is increased in the remodelled pulmonary vasculature, and inhibition of UA incorporation in PA-SMCs reduces their proliferation and mildly reduces experimental PH. https://bit.ly/2Ll5qXM

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Abstract

Previous studies have suggested an association between uric acid (UA) and the severity of pulmonary arterial hypertension (PAH), but it is unknown whether UA contributes to disease pathogenesis.

The aim of this study was to determine the prognostic value of circulating UA in the era of current management of PAH and to investigate the role of UA in pulmonary vascular remodelling.

Serum UA levels were determined in idiopathic, heritable or anorexigen PAH at baseline and first re-evaluation in the French Pulmonary Hypertension Network. We studied protein levels of xanthine oxidase (XO) and the voltage-driven urate transporter 1 (URATv1) in lungs of control and PAH patients and of monocrotaline (MCT) and Sugen/hypoxia (SuHx) rats. Functional studies were performed using human pulmonary artery smooth muscle cells (PA-SMCs) and two animal models of pulmonary hypertension (PH).

High serum UA levels at first follow-up, but not at baseline, were associated with a poor prognosis. Both the generating enzyme XO and URATv1 were upregulated in the wall of remodelled pulmonary arteries in idiopathic PAH patients and MCT and SuHx rats. High UA concentrations promoted a mild increase in cell growth in idiopathic PAH PA-SMCs, but not in control PA-SMCs. Consistent with these observations, oxonic acid-induced hyperuricaemia did not aggravate MCT-induced PH in rats. Finally, chronic treatment of MCT and SuHx rats with benzbromarone mildly attenuated pulmonary vascular remodelling.

UA levels in idiopathic PAH patients were associated with an impaired clinical and haemodynamic profile and might be used as a non-invasive indicator of clinical prognosis during follow-up. Our findings also indicate that UA metabolism is disturbed in remodelled pulmonary vascular walls in both experimental and human PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a severe and devastating condition characterised by progressive structural and functional remodelling of distal pulmonary arteries leading to progressive right heart failure,

functional decline and, ultimately, death [1]. Recent therapeutic advances in PAH management underlined the need for new tools for risk stratification at baseline and during follow-up [2]. There is increasing interest in the use of biomarkers for delineating the severity and prognosis of disease, and for monitoring the course of PAH and its response to therapy [3].

The process underlying the irreversible remodelling of the pulmonary vasculature in PAH is ascribed to the increased proliferation, migration and survival of pulmonary vascular cells within the pulmonary artery wall, including of endothelial and smooth muscle cells in the pulmonary vascular wall [4]. Although the exact mechanisms remain unclear, accumulating data suggest that oxidative stress and metabolic dysfunction are the major contributors to the development and progression of PAH. Despite the association of levels of serum uric acid (UA), the end product of purine compound metabolism, with several cardiovascular diseases [5], including PAH [6–11], it remains controversial as to whether UA modulates pathogenic mechanisms underlying the progression of these diseases. Elevated UA levels have long been considered a poor prognostic sign in idiopathic PAH (iPAH), before the era of current therapeutic management [9]. Recently, circulating UA levels have been documented as a prognostic factor in other forms of PAH, *e.g.* PAH associated with connective tissue disease [7]. Moreover, evidence supporting a direct pathogenic role for UA in PAH pathophysiology has accumulated over the years. A recent metabolomic analysis identified xanthine, an immediate precursor of UA, as a prognostic factor in PAH independent of renal function and diuretic use [12]. Nevertheless, it is unknown whether UA acts directly on pulmonary artery smooth muscle cells (PA-SMCs) to contribute to pulmonary vascular remodelling.

The objectives of this translational study were to analyse the prognostic impact of UA at baseline and during follow-up in the French Pulmonary Hypertension Network in patients with iPAH, heritable PAH or anorexigen-associated PAH and to determine whether UA and its voltage-driven urate transporter 1 (URATv1) contribute to the pulmonary vascular remodelling in PAH.

Methods

Study population

All experimental studies using human samples complied with the Declaration of Helsinki and were approved by the local ethics committee (Comité de Protection des Personnes, Ile-de-France VII, France). All patients gave informed consent before the study. The data collected were anonymised and complied with the requirements of the Commission Nationale Informatique et Libertés, the organisation dedicated to privacy, information technology and civil rights in France. The committee approved the methods used to collect and analyse the data on May 24, 2003 (approval number 842063).

Incident, treatment-naïve patients with iPAH, heritable PAH or anorexigen-induced PAH recorded in the French Pulmonary Hypertension Network registry from 2006 to 2016 were retrospectively reviewed and enrolled if a dosage of circulating UA was available at baseline and/or at the time of the first follow-up. Patients were eligible for inclusion if they had baseline right heart catheterisation confirming newly diagnosed precapillary pulmonary hypertension (known as PAH), defined by a resting mean pulmonary artery pressure (mPAP) $\geqslant$25 mmHg, pulmonary artery wedge pressure $\leqslant$15 mmHg and pulmonary vascular resistance (PVR) >3 Wood units, in the absence of other known causes of pulmonary hypertension (PH). Characteristics, including demographics, cardiovascular comorbidities and variables related to PAH (Modified New York Heart Association (NYHA) functional class, physical examination, routine blood tests, non-encouraged 6-min walk distance (6MWD) and standard cardiopulmonary haemodynamic variables assessed by right heart catheterisation), were recorded at the same time as UA level measurement. A risk assessment was performed according to the 2015 European Society of Cardiology (ESC)/European Respiratory Society (ERS) PH guidelines [13]. We evaluated the presence of four low-risk criteria, which were defined as 1) World Health Organization (WHO)/NYHA functional class I or II, 2) 6MWD >440 m, 3) right atrial pressure (RAP) <8 mmHg and 4) cardiac index $\geqslant$2.5 $L \cdot min^{-1} \cdot m^{-2}$, as previously described [2]. Patients were classified according to the number of low-risk criteria present at baseline (*i.e.* at the time of PAH diagnosis) and at the time of re-evaluation.

Animals and in vivo *treatment*

The *in vivo* protocol was approved by the Animal Ethics Committee of the Université Paris-Saclay, Le Plessis-Robinson, France. Young male Wistar rats (100 g, Janvier Labs, Saint Berthevin, France) were injected with monocrotaline (MCT) or were subjected to the vascular endothelial growth factor (VEGF) receptor antagonist SU5416 followed by 3 weeks of hypoxia (SuHx) to induce severe forms of PH. Male rats were used to minimise hormonal effects (*e.g.* of oestrogen). For the MCT model, a single subcutaneous injection of vehicle or MCT (40 $mg \cdot kg^{-1}$; Sigma-Aldrich) was performed. Then, rats were assigned to either an untreated control group or a group that received a daily oral administration of vehicle

(dimethyl sulfoxide:water, 1:4, v:v) or benzbromarone (BBR) (10 mg·kg^{-1}·day^{-1} for 3 weeks) (figure 1a). BBR is a uricosuric agent that exerts its hypouricaemic action solely by blocking tubular reabsorption of UA by inhibiting the URATv1 transporter. An additional group of MCT rats was treated daily with oxonic acid (OA) at a dose of 750 mg·kg^{-1}·day^{-1} (drinking water) for 3 weeks. OA is an inhibitor of uricase, and results in an increase in UA level in rats. OA treatment was initiated 1 week before MCT injection to ensure an optimal increase in serum UA level (figure 2a).

To validate our findings obtained in the MCT rat model, a second rat model of severe PH was used. Briefly, rats received a single subcutaneous injection of SU5416 (20 mg·kg^{-1}) and were exposed to normobaric hypoxia for 3 weeks before a return to room air for 5 weeks. At 5 weeks post-SU5416 injection, pulsed-wave Doppler during transthoracic echocardiography was used to validate the presence of established PH by assessing pulmonary artery acceleration time to right ventricular ejection time ratio (AT/ET), using Vivid E9 (GE Healthcare, Velizy-Villacoublay, France). Then, SuHx rats were randomised to receive vehicle or BBR (10 mg·kg^{-1}·day^{-1} for 3 weeks).

At the end of treatment protocols, rats were blindly analysed under inhalation of isoflurane (2.0% in room air), and a polyvinyl catheter was introduced into the right jugular vein and pushed through the right ventricle into the pulmonary artery to measure the mPAP. Cardiac output (CO) in rats was measured using the thermodilution method. After measurement of haemodynamic parameters, the thorax was opened and the left lung immediately removed and frozen. The right lung was fixed in the distended state with formalin buffer. Right ventricular hypertrophy was assessed by the Fulton index and the percentage of wall thickness ((2×medial wall thickness/ external diameter)×100) and percentages of wall thickness and of muscularised vessels were determined.

UA assay, Western blot and immunostaining

Serum UA concentrations were evaluated using a specific uric acid assay kit (QuantiChrom™ Uric Acid Assay Kit, BioAssays Systems, Hayward, CA, USA) according to the manufacturer's instructions. Human

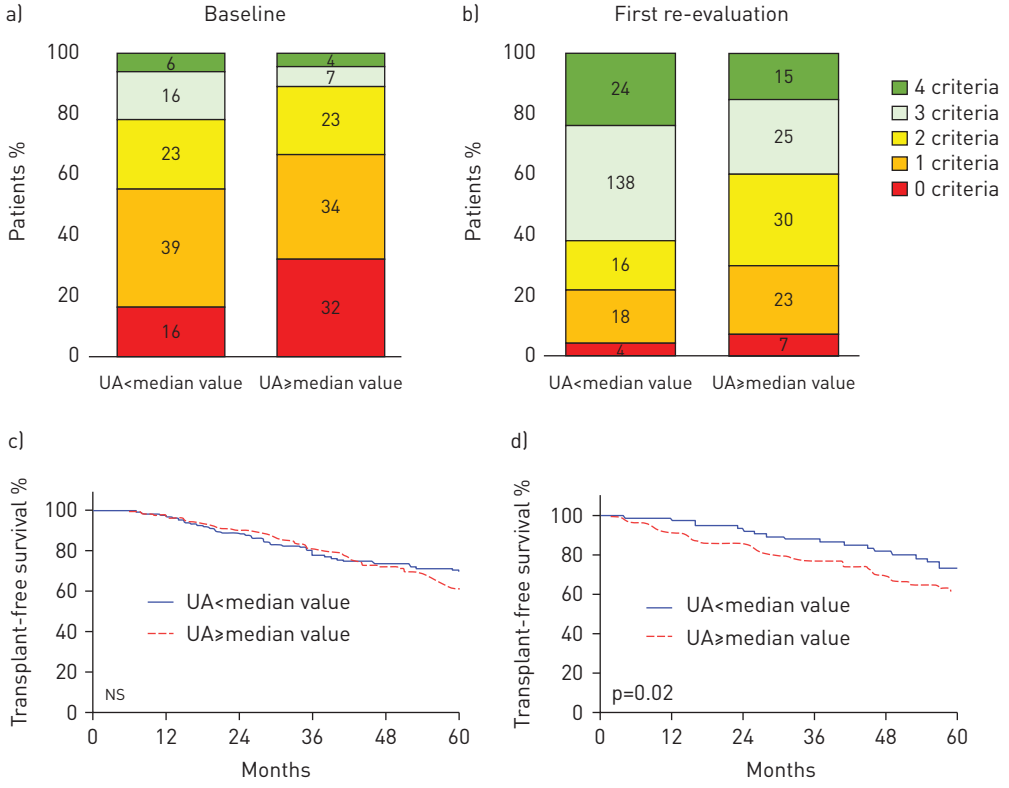


FIGURE 1 Number of low-risk criteria (a, b) and survival (c, d) according to serum uric acid (UA) level at baseline (a, c) and at first follow-up (b, d) in incident patients with idiopathic, heritable or anorexigen-associated pulmonary arterial hypertension. NS: nonsignificant.

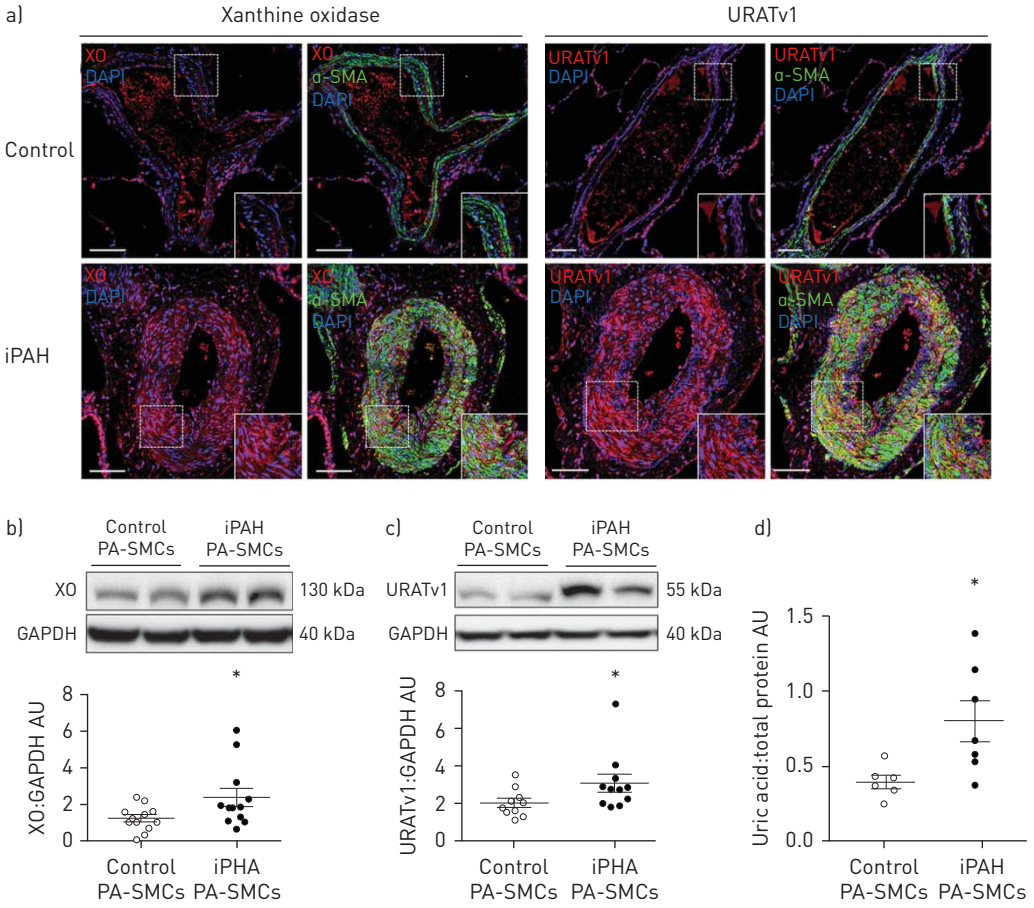


FIGURE 2 Increased expressions of xanthine oxidase (XO) and URATv1 in human pulmonary artery smooth muscle cells (PA-SMCs) in idiopathic pulmonary arterial hypertension (iPAH). a) Representative images of XO (red) and URATv1 (red) in PA-SMCs (positive for α-smooth muscle actin (α-SMA); green) in lungs from control subjects and patients with iPAH (n=3–5). Scale bars: 100 µm. b, c) Representative Western blots and quantification of the XO:GADPH (b) and URATv1:GAPDH (c) ratios (arbitrary units (AU)) in cultured PA-SMCs derived from control and iPAH patients (n=10–12). d) Conditioned media from 24 h serum-starved PA-SMCs was measured for secreted uric acid (UA) levels using a specific UA assay. Data are presented as mean±SEM (n=6–7). Comparisons were made using the non-parametric Mann–Whitney U test. *: p<0.05 *versus* control PA-SMCs.

lung tissue samples were obtained from Hôpital Marie Lannelongue, Le Plessis-Robinson, France. For the preparation of whole lung homogenates, tissues were homogenised and sonicated in RIPA buffer containing protease and phosphatase inhibitors, and 30 µg of protein was used to detect URATv1 (1:400) (Cat#:BMP027; MBL), xanthine oxidase (XO) (1:400) (Cat#:SC20991; Santa Cruz Biotechnology), and β-actin (1:50 000). Immunohistochemistry staining for URATv1 and XO were performed in human and rat lung paraffin sections. Briefly, lung sections (5-µm thickness) were deparaffinised and stained with haematoxylin and eosin (Sigma-Aldrich) or Sirius red, or incubated with retrieval buffer. Then sections were saturated with blocking buffer and incubated overnight with specific antibodies, followed by corresponding secondary fluorescent-labelled antibodies (Thermo Fisher Scientific). Mounting was done using ProLong Gold Antifade reagent containing DAPI (Thermo Fisher Scientific). Images were taken using an LSM700 confocal microscope (Carl Zeiss). Other lung sections were used for immunochemistry using a VECTASTAIN ABC kit according to the manufacturer's instructions (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich). Images were taken using an Eclipse 80i microscope (Nikon Instruments).

Isolation and culture of human PA-SMCs and pulmonary endothelial cells

Human PA-SMCs were isolated from explants of small pulmonary arteries (0.5-mm internal diameter) microdissected from lung explants of PAH patients (n=11) or from lung specimens obtained during

lobectomy or pneumonectomy at a distance from the tumour foci in control subjects (n=10; Hôpital Marie Lannelongue, Le Plessis-Robinson, France). Preoperative echocardiography was performed in these control patients to rule out PH, and the absence of tumoural infiltration was retrospectively established in all tissue sections by histopathological analysis. Briefly, as previously described [14], small pieces of freshly isolated arteries were cultured in DMEM media supplemented with 10% FCS, 2 mM L-glutamine and antibiotics. The isolated PA-SMCs were strongly positive for α-smooth muscle actin (α-SMA), smooth muscle-specific SM22 protein and calponin and negative for von Willebrand factor (vWF) and CD31. Human pulmonary endothelial cells (ECs) were isolated and cultured as previously described [3–5]. The isolated ECs were strongly positive for acetylated low-density lipoprotein coupled to Alexa 488 (Alexa488-Ac-LDL), vWF, CD31 and *Ulex europaeus* agglutinin-1 (UEA-1) and negative for α-SMA. Cells were used at passage <7.

Cell proliferation assays

Cells proliferation was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation and by cell counting. BrdU staining was measured by the DELFIA cell proliferation kit (PerkinElmer, Courtaboeuf, France) and a time-resolved fluorometer EnVision™ Multilabel Reader (PerkinElmer). To suppress URATv1 expression, PA-SMCs were transfected using lipofectamine RNAiMAX with 100 nM of URATv1 siRNA or with a scrambled sequence (Invitrogen, Cergy-Pontoise, France). The cells were studied within 3 days after transfection. Suppression of URATv1 levels was documented 72 h after transfection.

Measurement of reactive oxygen species

The superoxide indicator dihydroethidium (DHE) (Thermo Fisher Scientific, Saint Aubin, France) was used to evaluate intracellular reactive oxygen species (ROS) in cultured human PA-SMCs. Cells (10 000 cells·mL^{-1}) were seeded in four-well Minicell EZ slides (Merck Millipore, Molsheim, France) and treated with increasing doses of UA (3, 6, 9, 12 mg·dL^{-1}) in media containing 0.1% bovine serum albumin (BSA) for 30 min at 37°C. DHE (10 µM) was added and incubated for 20 min at 37°C. After incubation, the cells were washed twice with PBS and the Lab-Tek chamber slide was mounted with ProLong Gold Antifade reagent containing DAPI (Thermo Fisher Scientific) and the mean fluorescence, which is proportional to the intracellular superoxide production, was determined.

Statistical analyses

Continuous variables are expressed as mean±SD or SEM for normally distributed variables or median (interquartile range (IQR)) for non-normally distributed variables. Categorical variables are expressed as number of patients (n) and relative frequencies (%). Differences in continuous variables were compared using an independent t-test for normally distributed variables and the Mann–Whitney U test for non-normally distributed variables. All comparisons were two sided with a p-value <0.05 considered significant.

Data were collected from the web-based French registry (PAH Tool; Inovultus, Santa Maria da Feira, Portugal) and were stored in a personal computer-based data spreadsheet. The median value of UA at baseline was determined distinctly in males and females. Patients were classified according to the level of circulating UA: higher or lower than the median value at first evaluation. Clinical characteristics, comorbidities and haemodynamic parameters were compared between the two groups. Overall survival time was calculated from the date of PAH diagnosis or first re-evaluation to the date of last follow-up or death. The Kaplan–Meier method was used to estimate the proportion of patients surviving at each time point. Survival curves were compared with log-rank test.

Univariable analysis based on the Cox proportional hazards model was used to examine the relationship between survival and selected demographic variables, medical history and variables including UA level at baseline and at first re-evaluation after PAH therapy initiation. Multivariable analyses based on the Cox proportional hazards regression model were used to examine the independent effect of each variable on survival, controlling for possible confounding variables. We incorporated into the multivariable models modifiable and non-modifiable prognostic parameters commonly used in PAH risk assessment as well as variables that can impact UA level. Potential confounders of the relationship between UA and survival were limited to one per 10 events. Variables were eligible for entry into the multivariable models only if they were not highly correlated (absolute value of Pearson's or Spearman's r<0.6) with other continuous variables and if <25% of individuals had missing values for that variable. Results with a p-value <0.05 were considered statistically significant.

Results

Between January 1, 2006, and March 30, 2016, we identified 330 incident patients with iPAH (n=238), heritable PAH (n=38) or anorexigen-induced PAH (n=54) enrolled in the French registry who had both a baseline measurement of UA and a baseline evaluation including right heart catheterisation, 6MWD and WHO/NYHA functional class. At baseline, the median UA level was 8.2 mg·dL^{-1} (IQR 6.9–9.7 mg·dL^{-1}) in male and 7.3 mg·dL^{-1} (IQR 5.9–8.8 mg·dL^{-1}) in female patients. 163 patients had a re-evaluation of PAH within 1 year after diagnosis and a serum UA measurement. The median time between baseline and first re-evaluation was 4.4 months (IQR 3.8–6.5 months). At first follow-up, the median UA level was 8.3 mg·dL^{-1} (IQR 7.2–9.8 mg·dL^{-1}) in male and 6.8 mg·dL^{-1} (IQR 5.4–7.9 mg·dL^{-1}) in female patients.

UA and PAH severity

Comparison of baseline characteristics between patients with UA levels higher and lower than the median value is reported in table 1. Patients with a high serum UA level at the time of PAH diagnosis had significantly higher body mass index (BMI) (p=0.03), more frequent diabetes (p=0.007) and lower 6MWD (p=0.005). Haemodynamic measurements showed a higher RAP (p=0.002) and a lower cardiac index (p=0.006) and biological measurements showed a higher proportion of high B-type natriuretic peptide (BNP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) levels (p=0.03) in patients with a high level of UA. At diagnosis, the proportion of patients with no or one low-risk criteria was 55% in the group with a low level of UA and 66% in the group with a high level of UA (figure 1a).

At first re-evaluation, patients with a high level of UA were significantly older (p=0.01) and more frequently had diabetes (p<0.001) and hypertension (p=0.01). 6MWD was significantly lower in patients with a persistent high level of UA (p=0.001). A high level of UA was also associated with a higher proportion of patients with no or one low-risk criteria (30% *versus* 22%) (figure 1b).

TABLE 1 Characteristics at baseline and first follow-up according to UA level

	Baseline			First re-evaluation		
	UA<baseline median[#]	UA⩾baseline median[#]	p-value	UA<baseline median[#]	UA⩾baseline median[#]	p-value
Patients n	165	165		82	81	
Age years	57±18	58±17	0.62	50±18	57±17	0.01
BMI kg·m^{-2}	27±6	29±7	0.03	27±6	29±7	0.06
Hypertension	76 (46)	82 (50)	0.5	28 (34)	43 (53)	0.01
Diabetes	10 (6)	25 (15)	0.007	7 (9)	24 (30)	<0.001
NYHA-FC						
I/II	53 (30)	38 (19)	0.06	58 (71)	52 (64)	0.3
III/IV	112 (70)	127 (81)		24 (29)	29 (36)	
6MWD m	339±127	297±142	0.005	427±128	359±134	0.001
BNP⩾50 ng·L^{-1} or NT-proBNP⩾300 ng·L^{-1}	101/133 (76)	118/137 (86)	0.03	30/72 (42)	49/74 (66)	0.18
Haemodynamics						
RAP mmHg	8±5	10±4	0.002	6±4	8±5	0.08
mPAP mmHg	50±13	50±11	0.76	42±12	44±10	0.24
PAWP mmHg	10±4	10±4	0.66	10±4	10±5	0.66
CO L·min^{-1}	4.4±1.2	4.2±1.3	0.15	5.7±1.9	5.4±1.5	0.24
Cardiac index L·min^{-1}·m^{-2}	2.5±0.6	2.3±0.6	0.006	3.2±0.9	2.9±0.8	0.1
PVR Wood units	10±5	11±5	0.08	6.3±3.2	6.8±2.9	0.31
PAH therapy						
Monotherapy				38 (46)	28 (36)	0.12
Combo-therapy				44 (54)	53 (64)	
Diuretics	91 (55)	127 (77)	<0.001	46 (56)	60 (74)	0.03

Data are presented as mean±SD or n (%), unless otherwise indicated. UA: uric acid; BMI: body mass index; NYHA-FC: modified New York Heart Association functional class; 6MWD: 6-min walk distance; BNP: brain natriuretic peptide; NT-proBNP: N-terminal pro-brain natriuretic peptide; RAP: right atrial pressure; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary artery wedge pressure; CO: cardiac output; PVR: pulmonary vascular resistance; PAH: pulmonary arterial hypertension. [#]: the baseline median UA level was 8.2 mg·dL^{-1} (interquartile range (IQR) 6.9–9.7 mg·dL^{-1}) in male patients and 7.3 mg·dL^{-1} (IQR 5.9–8.8 mg·dL^{-1}) in female patients.

UA and survival

The median time of follow-up after PAH diagnosis was 58 months (IQR 35–77 months). During this period, 137 events were recorded including seven lung transplantations and 130 deaths. A UA level higher than the median value at the time of PAH diagnosis was not associated with higher mortality or transplantation (p=0.33) (figure 1c). Univariable analysis for clinical, haemodynamic and biological variables including UA are reported in supplementary table S1. UA level, analysed as a continuous variable, was not associated with prognosis (p=0.18). Moreover, no relationship between UA level and survival without transplantation was found after adjustment for demographic characteristics and comorbidities (*i.e* age, sex, BMI, diabetes and hypertension), for haemodynamic variables (RAP, pulmonary capillary wedge pressure, mPAP and cardiac index), for non-invasive prognostic factors listed in ESC/ERS guidelines (NYHA functional class, 6MWD and BNP/NT-proBNP) or for therapies (diuretics and PAH therapies) (table 2).

The median time of follow-up after first re-evaluation was 57 months (IQR 35–72 months). During this period, 60 events were recorded including seven lung transplantations and 53 deaths. A UA level higher than the median value was associated with higher mortality at first re-evaluation (p=0.02) (figure 1d). In addition, univariable analysis found a significant relationship between UA level, analysed as continuous variable, and survival (HR 1.004, 95% CI 1.002–1.006, p=0.003) (supplementary table S1). The prognostic value of UA was attenuated after adjustment for age, sex and comorbidities (HR 1.002, 95% CI 1.000–1.005, p=0.067), and persisted after adjustment for haemodynamic variables (HR 1.004, 95% CI 1.001–1.006, p=0.0019) and for therapies (HR 1.003, 95% CI 1.001–1.005, p=0.004). However, the prognostic value of UA did not persist after adjustment for non-invasive prognostic factors listed in ERS/ESC guidelines (6MWD, NYHA functional class and BNP/NT-proBNP) (table 2).

TABLE 2 Multivariable analysis determining prognostic value of UA at baseline, follow-up and for overall survival

	Baseline		First re-evaluation	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Prognostic value of UA adjusted for age, sex, BMI and comorbidities[#]				
UA mg·dL^{-1}	1.000 (0.999–1.002)	0.52	1.002 (1.000–1.005)	0.067
Age years	1.048 (1.034–1.062)	<0.0001	1.037 (1.016–1.058)	0.0004
Female *versus* male sex	0.671 (0.475–0.947)	0.0234	1.088 (0.617–1.918)	0.77
BMI kg·m^{-2}	0.998 (0.968–1.029)	0.89	0.941 (0.896–0.989)	0.016
Hypertension yes *versus* no	1.036 (0.713–1.506)	0.85	1.664 (0.864–3.195)	0.13
Diabetes *yes versus no*	1.050 (0.591–1.866)	0.86	1.331 (0.649–2.725)	0.43
Prognostic value of UA adjusted for haemodynamic variables[¶]				
UA mg·dL^{-1}	1.000 (0.999–1.002)	0.43	1.004 (1.001–1.006)	0.0019
RAP mmHg	1.034 (0.995–1.073)	0.09	0.991 (0.904–1.086)	0.842
PAWP mmHg	0.985 (0.939–1.034)	0.54	1.089 (1.009–1.174)	0.028
mPAP mmHg	0.981 (0.965–0.997)	0.0177	1.005 (0.976–1.034)	0.76
Cardiac index L·min^{-1}·m^{-2}	0.861 (0.629–1.179)	0.35	0.603 (0.411–0.883)	0.0094
Prognostic value of UA adjusted for non-invasive prognostic variables[+]				
UA mg·dL^{-1}	1.000 (0.999–1.001)	0.98	1.001 (0.999–1.003)	0.45
NYHA-FC, III–IV *versus* I–II	1.117 (0.683–1.828)	0.66	2.242 (1.274–3.952)	0.0051
6MWD m	0.995 (0.994–0.997)	<0.0001	1.004 (1.001–1.006)	0.0021
BNP or NT-proBNP high *versus* low	1.499 (0.805–2.785)	0.20	3.144 (1.433–6.896)	0.0043
Prognostic value of UA adjusted for therapies[§]				
UA mg·dL^{-1}	1.001 (0.998–1.005)	0.30	1.003 (1.001–1.005)	0.004
Diuretics yes *versus* no	1.901 (0.562–6.493)	0.38	0.545 (0.282–1.057)	0.07
Combo-therapy yes *versus* no	-	-	1.128 (0.651–1.952)	0.69

For each model, the hazard ratio represents the hazard ratios for each variable with adjustment for all other listed variables. UA: uric acid; BMI: body mass index; RAP: right atrial pressure; PAWP: pulmonary artery wedge pressure; mPAP: mean pulmonary arterial pressure; NYHA-FC: modified New York Heart Association functional class; 6MWD: 6-min walk distance; BNP: brain natriuretic peptide; NT-proBNP: N-terminal pro-brain natriuretic peptide. [#]: baseline n=328, first re-evaluation n=154; [¶]: baseline n=314, first re-evaluation n=153; [+]: baseline n=256, first re-evaluation n=142; [§]: baseline n=310, first re-evaluation n=161.

Local UA production in the pulmonary vasculature of patients with iPAH

To test whether dysfunctional pulmonary vascular cells in iPAH produce UA *in vivo*, confocal microscopic analyses and double labelling with either XO or for the efflux transporter URATv1 (GLUT9/SLC2A9) was performed in paraffin-embedded lung specimens from iPAH and control patients. We found strong staining for XO, the enzyme known to catalyse the hydroxylation of hypoxanthine to xanthine and xanthine to UA, and URATv1 in the smooth muscle cell layer of remodelled distal pulmonary arteries in iPAH when compared with controls (figure 2a), suggesting abnormal local UA production and overabundance in iPAH. These *in situ* observations were replicated *in vitro*, with cultured PA-SMCs from iPAH patients exhibiting increased protein levels of XO and URATv1 compared with control PA-SMCs (figure 2b). Consistent with upregulated local UA production in iPAH, we found higher UA levels in conditioned media from cultured human PA-SMCs derived from iPAH patients compared to those of control PA-SMCs (figure 2c).

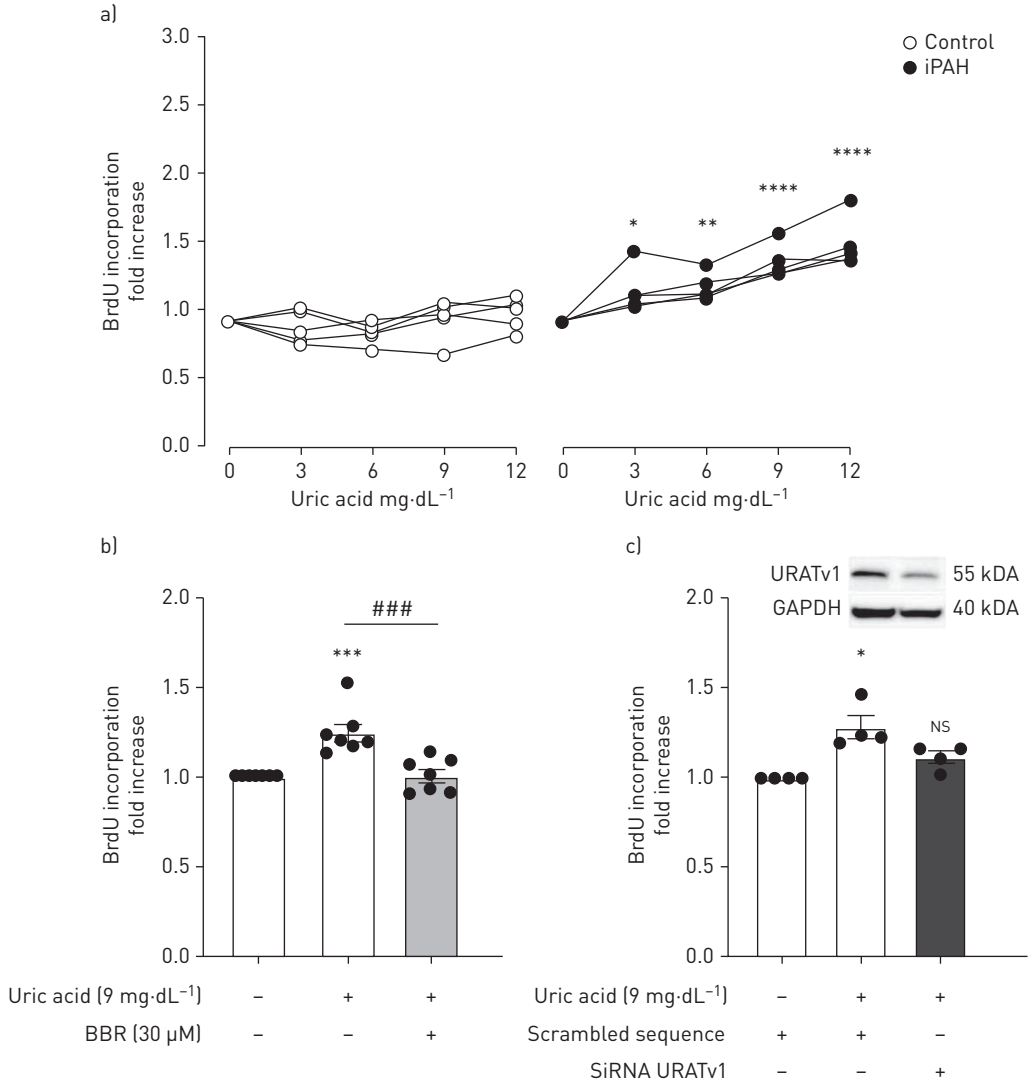


FIGURE 3 High uric acid (UA) concentrations promote cell growth in pulmonary artery smooth muscle cells (PA-SMCs) derived from patients with idiopathic pulmonary arterial hypertension (iPAH). a) Proliferation of PA-SMCs derived from control subjects (n=4) and patients with iPAH (n=4) in response to increasing doses of UA. b, c) Proliferation of iPAH PA-SMCs in response to 9 mg·dL^{-1} of UA in the presence of 30 µM of benzbromarone (BBR) (b) or 48 h after transfection with URATv1 siRNA or scrambled sequence (c) assessed by BrdU incorporation (n=7 and n=4, respectively). Data are presented as mean±SEM. Comparisons were made using one-way ANOVA with Tukey's *post hoc* tests. NS: nonsignificant. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 *versus* vehicle-treated cells; ###: p<0.001 *versus* PA-SMCs treated with 9 mg·dL^{-1} of UA.

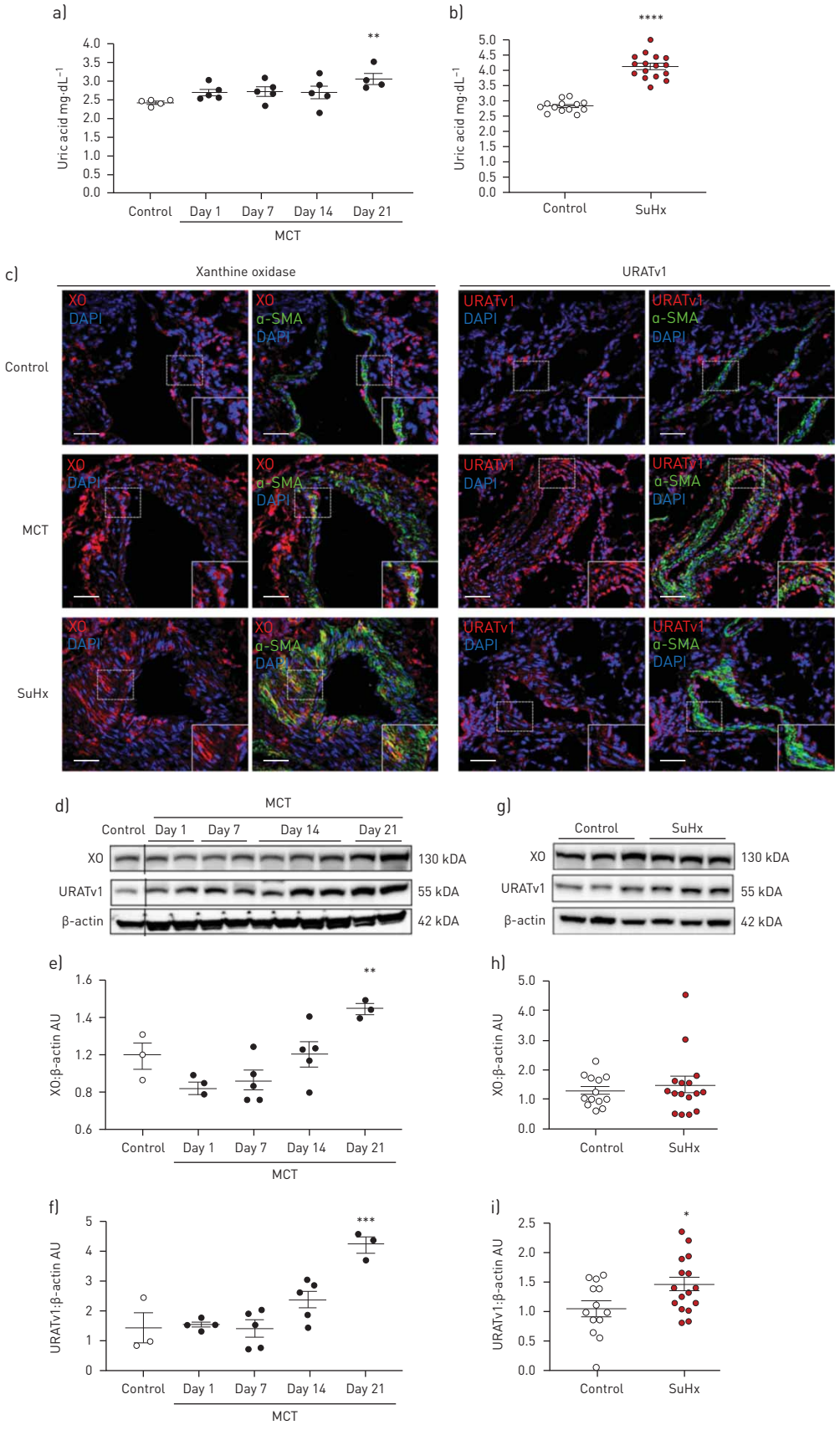


FIGURE 4 Hyperuricaemia and increased lung expression of xanthine oxidase (XO) and URATv1 in two animal models of pulmonary hypertension. a) Levels of uric acid (UA) in the serum of control rats and monocrotaline

(MCT) rats at various time points post-MCT injection (n=5 in all groups; one rat died prematurely at day 19 in the group Day 21). b) Serum UA levels in control and Sugen/hypoxia (SuHx) rats (n=13–16). c) Representative images of XO (red) and URATv1 (red) in pulmonary artery smooth muscle cells (positive for α-smooth muscle actin (α-SMA); green) in lungs from control, MCT and SuHx rats (n=3–5). Scale bars: 50 μm. d–i) Representative Western blots and quantification of the XO:β-actin and URATv1:β-actin ratios (arbitrary units (AU)) in lungs of control, MCT (d–f) and SuHx (g–i) rats (n=3–5). Horizontal lines indicate the mean±SEM. Comparisons were made using one-way ANOVA with Tukey's *post hoc* tests or the non-parametric Mann–Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 compared with control.

Consistent with our results obtained with iPAH PA-SMCs, a higher intracellular UA level was noted in PA-ECs in iPAH combined with an increased protein level of XO. However, a decreased protein level of URATv1 was found in iPAH pulmonary ECs compared with control pulmonary ECs (supplementary figure S1).

High UA concentrations promote cell growth and ROS production in iPAH PA-SMCs

Given that proliferation and oxidative stress of PA-SMCs contribute to vascular remodelling in PAH [4, 15, 16], we next questioned whether this local overabundance of UA in the remodelled pulmonary vasculature could directly modulate PA-SMC behaviour. We examined the effect of increasing doses of UA on the proliferation of cultured PA-SMCs derived from iPAH patients (n=4) and controls (n=4). Although UA had no effect on cell growth in control PA-SMCs, BrdU incorporation was increased 1.2-, 1.2-, 1.4- and 1.5-fold in iPAH PA-SMCs exposed to UA at 3, 6, 9 and 12 mg·dL^{-1}, respectively (figure 3a). We also found that BBR (30 μM), a uricosuric agent and non-competitive inhibitor of urate transporters, and URATv1 siRNA attenuated iPAH PA-SMC proliferation induced by 9 mg·dL^{-1} of UA (figure 3b, c).

To monitor intracellular oxidative stress, we used redox-sensitive fluorophore hydroethidine (DHE) and noted a significant increase in intracellular ROS production in cultured iPAH PA-SMCs exposed to high UA concentrations (supplementary figure S2).

Local UA production and secretion are increased in two animal models of severe PH

To better understand the role of UA in the pathogenesis of the disease, we next examined serum UA levels and XO and URATv1 protein levels in lungs of rats with severe PH induced by MCT and SuHx. Consistent with our data obtained in human samples, circulating UA levels were found to be significantly elevated 3 weeks after MCT injection compared with control rats, although they were similar to control at days 1, 7 and 14 (figure 4a and supplementary figures S3 and S4). Interestingly, the increase in circulating UA levels was also found in SuHx rats with established PH compared with control rats (figure 4b). As observed in human lung specimens, we also found increased levels of XO and URATv1 proteins in lungs of MCT and SuHx rats compared with control rat lungs (figure 4c–i and supplementary figures S3 and S4). At days 1, 7 and 14, XO and URATv1 expression were similar in rats treated by MCT and controls (figure 4d–f).

OA-induced hyperuricaemia did not aggravate the progression of MCT-induced PH in rats

Based upon the above observations, subsequent studies were carried out to determine whether increasing UA levels with the uricase inhibitor OA would result in worsening of MCT-induced PH in rats. We therefore induced mild hyperuricaemia in rats by providing low doses of OA for 1 week prior to randomisation and assigned these rats to either a vehicle-treated control group or a group that received a single subcutaneous injection of MCT to induce experimental PH (figure 5a, b). After 3 weeks of OA treatment, pulmonary haemodynamic parameters and structures were examined. A similar decrease in AT/ET ratio and increase in mPAP, transcatheter pulmonary valve replacement (TPVR) and Fulton index was observed between OA-treated MCT rats and vehicle-treated MCT rats (figure 5a, c–g). Consistent with these results, a similar increase in the percentages of medial wall thickness and of muscularised distal pulmonary arteries was noted between OA-treated MCT rats and vehicle-treated MCT rats (figure 5h–j). Taken together, these results support the notion that the increase in serum UA levels has no effect on pulmonary vascular remodelling in PAH.

Chronic treatments of MCT and SuHx rats with BBR mildly attenuate PH

To assess the role of the increased local UA production in the pulmonary vasculature in PAH, we next tested the effect of BBR, a uricosuric agent and inhibitor of XO, in the two preclinical models of severe PH (figure 6). First, we examined the efficacy of BBR treatments against the development of PH in the MCT rat model (preventive protocol) and we next studied its efficacy against the progression of PH in the

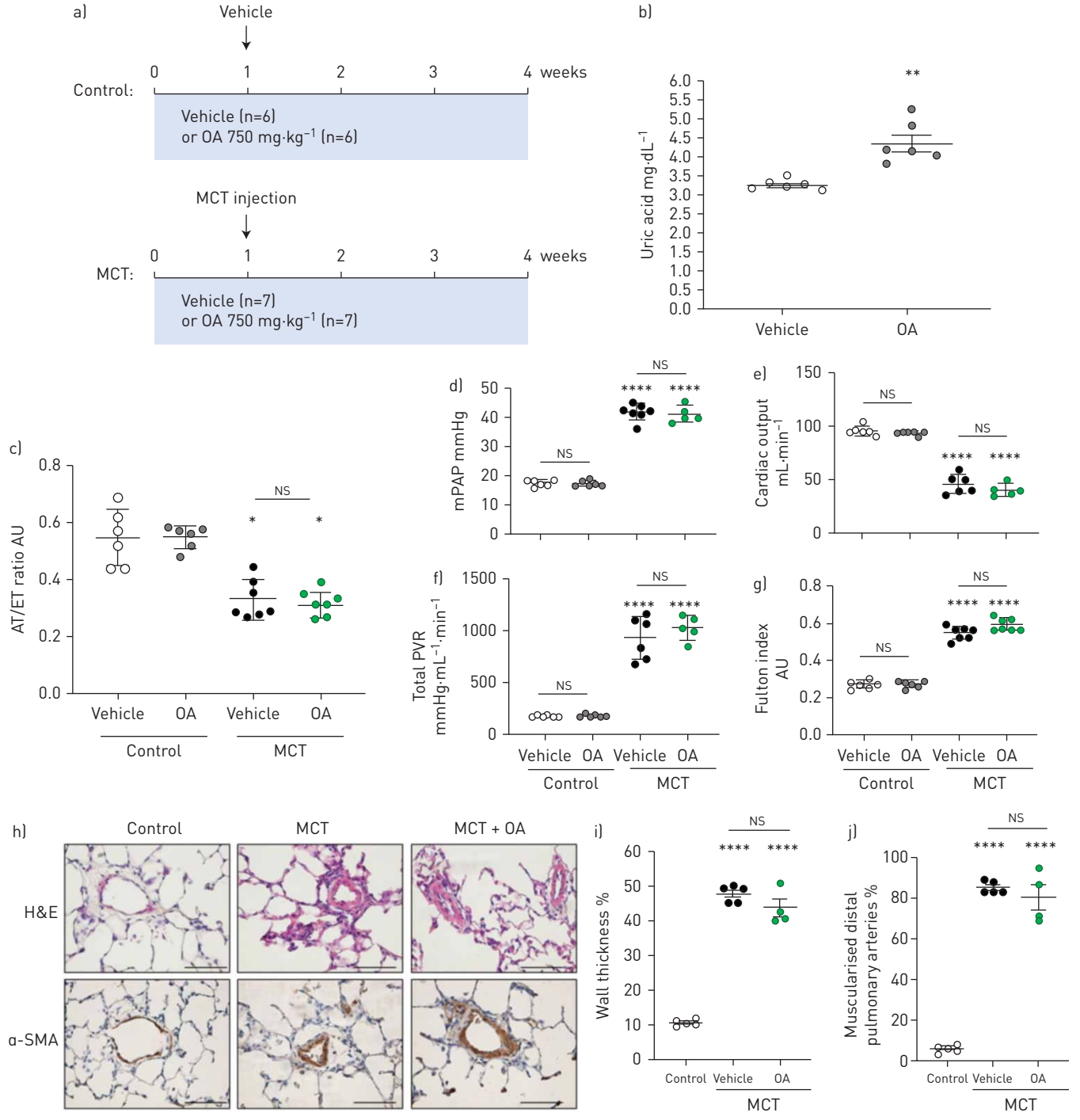
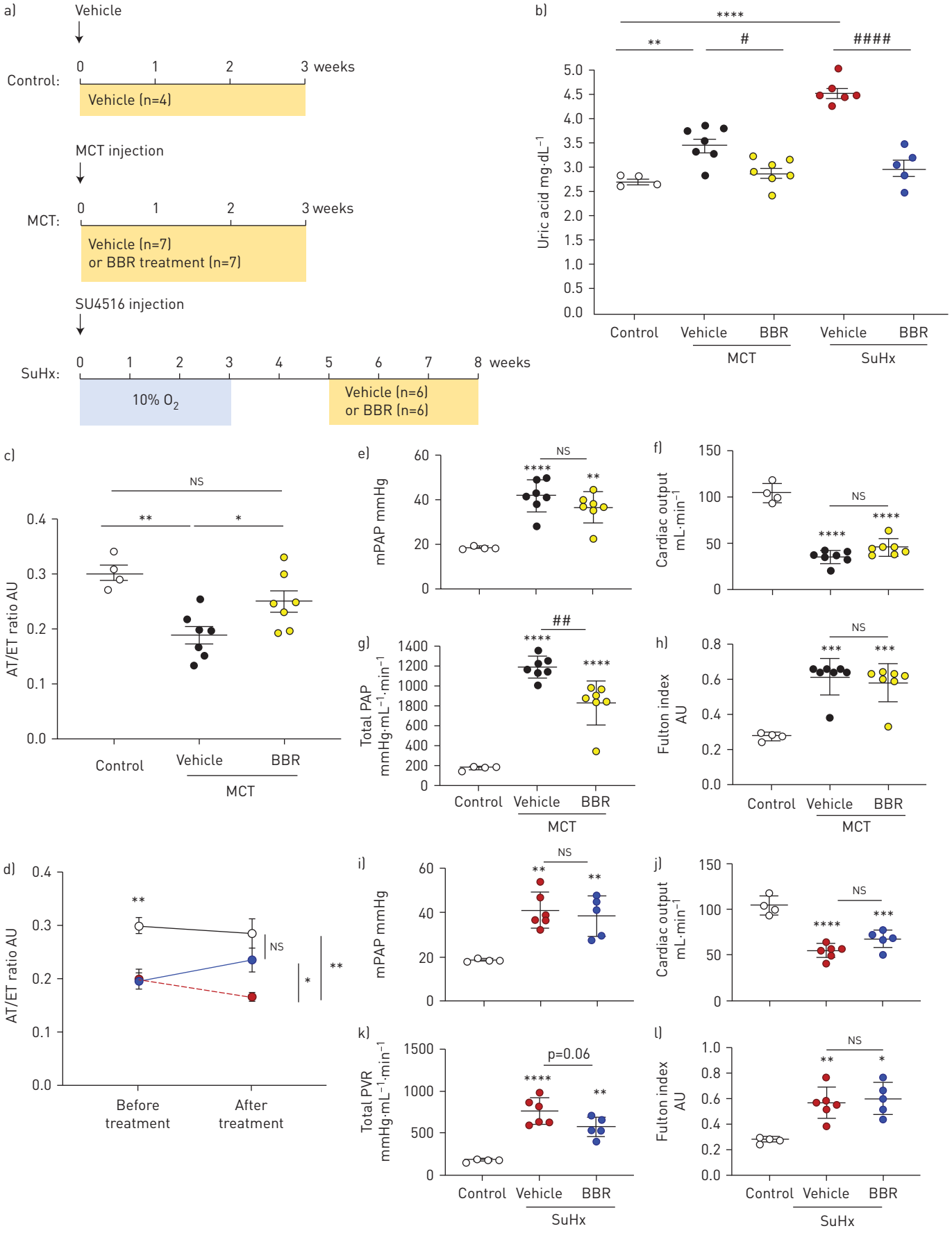


FIGURE 5 Oxonic acid (OA)-induced hyperuricaemia did not aggravate the progression of pulmonary hypertension induced by monocrotaline (MCT) in rats. a) Experimental scheme and number of rats in each group. b) Serum uric acid (UA) levels in control rats chronically treated with vehicle or OA at week 4 (n=6). c) Acceleration time (AT)/ejection time (ET) ratio obtained by transthoracic echocardiography. d–g) Mean pulmonary arterial pressure (mPAP) (d), cardiac output (e), total pulmonary vascular resistance (PVR) (f) and Fulton index (g) in control and MCT rats treated with OA at the indicated doses (two haemodynamic assessments in the group MCT+OA and one in the group MCT+Vehicle are lacking owing to complications related to right heart catheterisation). h) Representative images of haematoxylin and eosin (H&E)- and α-smooth muscle actin (α-SMA)-stained sections of distal pulmonary arteries and quantification of the i) percentage of wall thickness and j) muscularised distal pulmonary arteries in lungs of control and MCT rats treated with vehicle or OA. Scale bar: 20 µm. Data are presented as mean±SEM (n=4–9). Comparisons were made using one-way ANOVA with Tukey's *post hoc* tests or the non-parametric Mann–Whitney U test. AU: arbitrary units; NS: nonsignificant; *: p<0.05; **: p<0.01; ****: p<0.0001 compared with control rats treated with vehicle.


a)
Vehicle
Control:
0 1 2 3 weeks
Vehicle (n=4)
MCT injection
MCT:
0 1 2 3 weeks
Vehicle (n=7)
or BBR treatment (n=7)
SU4516 injection
SuHx:
0 1 2 3 4 5 6 7 8 weeks
10% O2
Vehicle (n=6)
or BBR (n=6)
b)
Uric acid mg·dL−1
Control
Vehicle
BBR
Vehicle
BBR
MCT
SuHx
c)
AT/ET ratio AU
Control
Vehicle
BBR
MCT
d)
AT/ET ratio AU
Before treatment
After treatment
e)
mPAP mmHg
f)
Cardiac output mL·min−1
g)
Total PAP mmHg·mL−1·min−1
h)
Fulton index AU
i)
mPAP mmHg
j)
Cardiac output mL·min−1
k)
Total PVR mmHg·mL−1·min−1
p=0.06
l)
Fulton index AU
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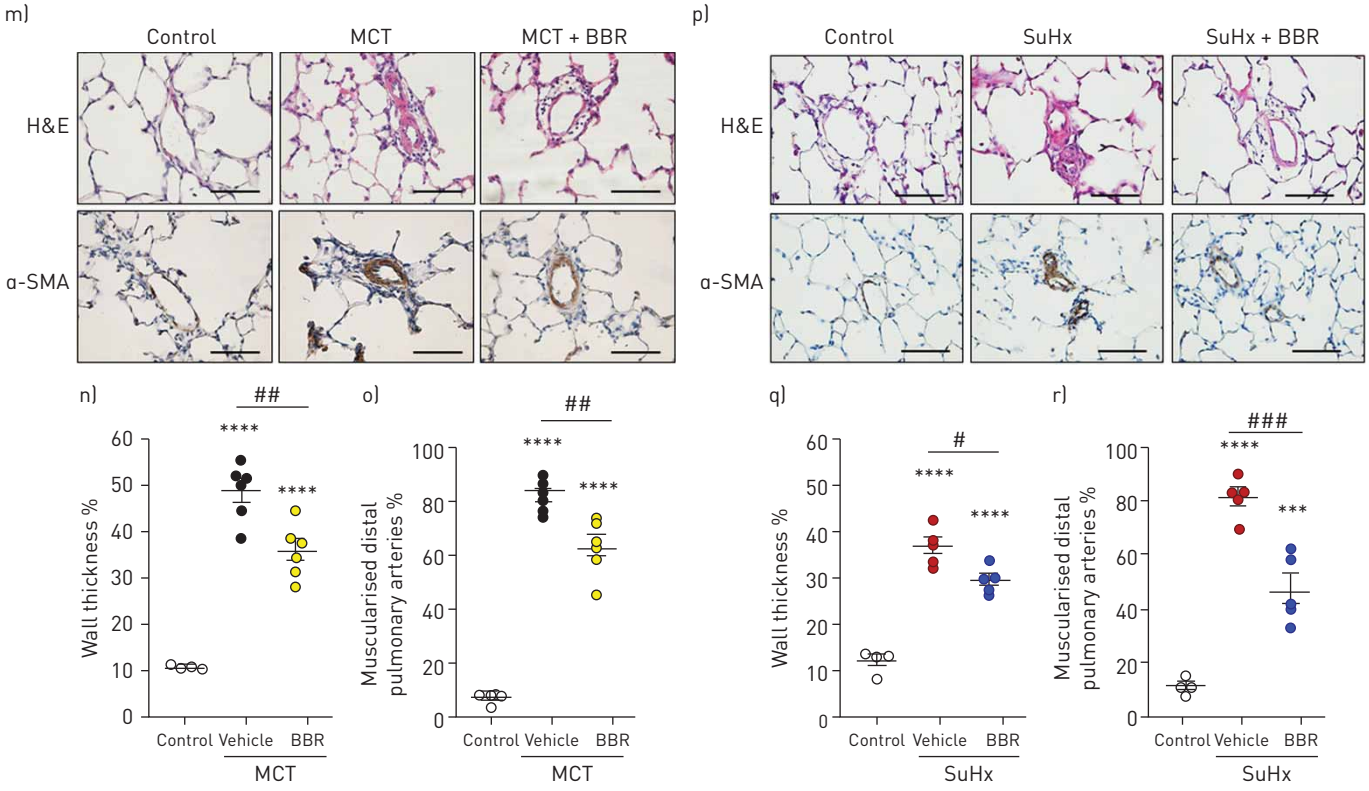

FIGURE 6 Efficacy of chronic treatment with benzbromarone (BBR) in the monocrotaline (MCT) and in Sugen/Hypoxia (SuHx) rat models of severe pulmonary hypertension. a) Experimental scheme and number of rats in each group. One rat died prematurely at day 20 in the group SuHx and BBR. b) Serum uric acid levels in control rats chronically treated with BBR at the end of these protocols (n=4–7). c, d) Acceleration time (AT)/ejection time (ET) ratio obtained by transthoracic echocardiography (n=4–7) in MCT (c) and SuHx (d) rats. e–l) Mean pulmonary arterial pressure (mPAP), cardiac output, total pulmonary vascular resistance (PVR) and Fulton index in control, MCT (e–h) and SuHx (i–l) rats treated with BBR or vehicle. m–r) Representative images of haematoxylin and eosin (H&E)- and α-smooth muscle actin (α-SMA)-stained sections of distal pulmonary arteries and quantification of the percentage of wall thickness and of muscularised distal pulmonary arteries in lungs of control and MCT (m–o) and SuHx (p–r) rats treated with vehicle or BBR (n=4–7). Scale bar: 20 µm. Data are presented as mean±SEM. Comparisons were made using one-way ANOVA with Tukey's *post hoc* tests. AU: arbitrary units; NS: nonsignificant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 compared with control rats treated with vehicle; #: p<0.05; ##: p<0.01; ###: p<0.001 *versus* vehicle-treated rats.

SuHx rat model (curative protocol) (figure 6a). At the end of these protocols, we found a significant reduction in serum UA concentrations in MCT and SuHx rats treated with BBR compared with MCT and SuHx rats treated with vehicle (figure 6b). At 3 and 8 weeks post-SU5416 injection, pulsed-wave Doppler during transthoracic echocardiography indicated a decrease in pulmonary AT/ET ratio in MCT and SuHx rats treated with vehicle, respectively. However, chronic treatment of MCT and SuHx rats with BBR significantly attenuated the decrease in pulmonary AT/ET ratio (figure 6c, d). Right heart catheterisation indicated that the increase in TPVR in MCT and SuHx rats treated with BBR was less pronounced than in MCT rats and SuHx rats treated with vehicle (figure 6g, k). However, no significant difference in mPAP, CO or Fulton index was observed between MCT and SuHx rats treated with BBR and those treated with vehicle (figure 6e, f, h–j, l). Consistent with these observations, a mild reduction in the muscularisation of pulmonary arteries was observed in MCT and SuHx rats treated with BBR compared with MCT and SuHx rats treated with vehicle in the MCT rat model (figure 6o, r). These results indicate a mild protective effect of BBR treatment in these two animal models of severe PH.

Discussion

Although PAH is often associated with hyperuricaemia, it is not known whether serum UA levels at baseline and during follow-up are useful for risk stratification in the era of modern management, or if UA contributes to the progression of pulmonary vascular remodelling. In the present study, our data show that, in patients with PAH, high serum UA levels at first follow-up after PAH therapy initiation are associated with a poor prognosis, whilst high levels at baseline are not. We obtained additional evidence supporting

the notion that UA may represent a biomarker for PH severity, because its metabolism is disturbed in the remodelled pulmonary vessels of PAH patients.

BNP and its N-terminal pro-hormone fragment NT-proBNP are the only two biomarkers listed in the latest guidelines (at the time of writing) for PAH risk stratification at baseline and during follow-up [13]. Therefore, there is a clear need to develop new biomarkers that can be routinely used in clinical practice. Because earlier studies have suggested an association between serum UA levels and the severity of PAH [6–11], we studied the prognostic value of circulating UA levels at baseline and during follow-up in our large cohort of PAH patients. Our data show that patients with a high level of UA at baseline had more severe haemodynamic impairment (*i.e.* higher level of RAP and lower cardiac index), as well as more severe 6MWD impairment. Paradoxically, a high UA level at baseline was not associated with poorer prognosis, in contrast to what was described prior to the modern management of PAH [8, 9]. However, the persistence of a high UA level at first follow-up after the initiation of PAH-targeted therapies is associated with more severe prognosis. It is important to underline that the initial treatment strategy may explain the lack of independent association between baseline UA level and outcomes, given that disease severity has likely influenced treatment decisions. Similarly, it has been observed that haemodynamic variables are useful for guiding the choice of first-line therapy, but they are now considered more effective for risk stratification at first follow-up after PAH-targeted therapy initiation [2, 17]. We also used univariable and multivariable analysis to investigate the usefulness of UA in the risk stratification method using haemodynamic variables, NYHA functional class and 6MWD at first follow-up. UA was independently associated with patient prognosis when adjusted for haemodynamic variables. In a non-invasive approach, including BNP/NT-proBNP levels, NYHA functional class and 6MWD, UA was not an independent predictor of mortality. In this method of risk stratification, BNP/NT-proBNP seemed to be more robust for predicting patient survival.

We recognise that there are limitations inherent to the retrospective nature of this study, and the lack of data for non-invasive variables in 23% of patients could limit the power of the multivariable analysis. Moreover, a substantial proportion of patients had UA measurement at baseline but not at follow-up. Another limitation of our study is the lack of information on renal function, which could also influence the level of UA.

UA is a heterocyclic compound that is produced during the breakdown of purines, which represents the last product of purine metabolism in humans. A high-purine diet, obesity, insulin resistance, dyslipidaemia, diabetes, use of diuretics, chronic renal failure and high alcohol intake are known common causes of hyperuricaemia [18–21]. PAH registries and clinical trials indicate that the proportion of elderly patients with cardiovascular risk factors is progressively increasing [22–24]. Elevated UA level is also associated with a higher frequency of cardiovascular risk comorbidities, *e.g.* hypertension or diabetes, that could also influence the prognosis of patients. Because UA levels are higher in men and postmenopausal women, due to an oestrogen-induced increase in renal urate elimination [25], we separately analysed the median value of UA in each cohort according to patient sex.

Both vasoconstriction and enhanced accumulation of PA-SMCs within the vascular wall contribute to the progressive course of PH/PAH. It has been reported that induction of mild hyperuricaemia in rats induced by OA is associated with the development of small-vessel disease in the kidney, consisting of thickening of the preglomerular arteries with smooth muscle cell proliferation [26, 27]. These observations strongly suggest that UA may contribute to progressive renal disease. Based on these findings and the observation that elevated UA is associated with increased PAH severity, we questioned whether UA may have a direct pathogenic role and contribute to the pulmonary vascular remodelling associated with PAH. In line with this notion, UA can stimulate SMC proliferation by increasing the production of angiopoietin 2, cyclooxygenase 2, endothelin-1, platelet-derived growth factor (PDGF) and ROS, and activation of pathways including the extracellular signal-regulated protein kinase and p38 kinase pathways and the NF-κB and c-Jun/activator protein-1 pathways [27–35]. However, our *in vitro* studies indicate that UA exerts a significant but rather small pro-proliferative effect on PA-SMCs derived from iPAH patients and has no effect on control PA-SMCs. To explain these differences between control and iPAH PA-SMCs, we explored the expression of XO and URATv1 and found that OA and URATv1 were upregulated in the smooth muscle layer of remodelled vessels in iPAH and in the two animal models of severe PH. Hypoxia, tumour necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-6, IL-1 and dexamethasone are known modulators of XO expression and activity [36–39], but further studies are needed to identify the mechanisms underlying this XO and URATv1 upregulation in remodelled vessels in PAH. Our results indicate that pulmonary ECs derived from iPAH patients also exhibit higher intracellular UA levels together with elevated XO levels and lower URATv1 levels. Given that high UA concentrations have been

reported to modulate the amount of nitric oxide released, the expression of endothelial nitric oxide synthase protein, the production of ROS, the secretion of inflammatory cytokines (IL-6 and TNF-α) and the expression of adhesion molecules (intracellular adhesion molecule 1 and vascular cell adhesion molecule 1) in human umbilical vein ECs and other EC types [40–42], further work is needed to investigate the role of UA in pulmonary endothelial dysfunction in PH/PAH.

To assess the role of elevated UA levels in the progression of PAH, we treated MCT rats with OA and studied the effect of mild hyperuricaemia on the severity of experimental PH by assessing pulmonary haemodynamic variables and vascular remodelling. Our data indicate that OA-induced hyperuricaemia does not aggravate the progression of MCT-induced PH in rats. A similar increase in the percentages of medial wall thickness and of muscularised distal pulmonary arteries was found in MCT rats treated with OA compared to vehicle-treated MCT rats, supporting the conclusion that *in vivo* high UA concentrations do not contribute to the MCT-induced vascular remodelling. Because no SuHx rats were treated with OA, we cannot exclude the possibility that OA-induced hyperuricaemia would have accelerated vascular remodelling in SuHx rats. In line with this notion, we found that chronic administration of BBR in MCT and SuHx rats mildly attenuated PH. BBR is a known uricosuric agent and a non-competitive inhibitor of XO [43]. In the MCT rat model, chronic BBR treatment partially prevented the increase in total PVR in MCT rats, with a trend towards a decrease in mPAP and an increase in CO. These data were consistent with the observation that chronic administration of BBR in SuHx rats with established PH led to a trend towards improved haemodynamic parameters and significant reductions in the percentages of medial wall thickness and of muscularised arterioles. However, recent evidence suggests that these observed beneficial effects of BBR treatment could also be due to inhibition of the Ca^{2+}-activated Cl^- channel TMEM16A [44]. BBR is indeed an inhibitor of TMEM16A at micromolar concentrations [45]. Papp *et al.* [44] reported that chronic administration of BBR partially reverses PH in both the chronic hypoxia mouse and MCT rat models through inhibition of TMEM16A. Using cultured PAH PA-SMCs, these authors demonstrated that BBR normalises membrane potential and blocks PDGF-induced proliferation. Despite the fact that BBR has multiple modes of action, our results indicate that elevated UA levels have a negligible role on the pulmonary vascular remodelling in PAH.

In summary, we show that UA is increased in patients with PAH and correlates with survival after PAH therapy initiation and might be used as a non-invasive indicator of disease severity. Although experimental hyperuricaemia did not influence pulmonary vascular remodelling in MCT rats, our findings indicate that production and metabolism of UA are disturbed in the wall of remodelled pulmonary arteries in MCT and SuHx rats as well as in iPAH patients.

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