# A potential therapeutic role for angiotensin-converting enzyme 2 in human pulmonary arterial hypertension

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**The ACE2–Ang-(1–7)–Mas1 axis is probably involved in the pathophysiology of human pulmonary arterial hypertension** http://ow.ly/pgS530jOxnd

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ABSTRACT Pulmonary arterial hypertension (PAH) is a deadly disease with no cure. Alternate conversion of angiotensin II (AngII) to angiotensin-(1–7) (Ang-(1–7)) by angiotensin-converting enzyme 2 (ACE2) resulting in Mas receptor (Mas1) activation improves rodent models of PAH. Effects of recombinant human (rh) ACE2 in human PAH are unknown. Our objective was to determine the effects of rhACE2 in PAH.

We defined the molecular effects of Mas1 activation using porcine pulmonary arteries, measured AngII/Ang-(1–7) levels in human PAH and conducted a phase IIa, open-label pilot study of a single infusion of rhACE2 (GSK2586881, 0.2 or 0.4 $mg \cdot kg^{-1}$ intravenously).

Superoxide dismutase 2 (SOD2) and inflammatory gene expression were identified as markers of Mas1 activation. After confirming reduced plasma ACE2 activity in human PAH, five patients were enrolled in the trial. GSK2586881 was well tolerated with significant improvement in cardiac output and pulmonary vascular resistance. GSK2586881 infusion was associated with reduced plasma markers of inflammation within 2–4 h and increased SOD2 plasma protein at 2 weeks.

PAH is characterised by reduced ACE2 activity. Augmentation of ACE2 in a pilot study was well tolerated, associated with improved pulmonary haemodynamics and reduced markers of oxidant and inflammatory mediators. Targeting this pathway may be beneficial in human PAH.

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## Introduction

Pulmonary arterial hypertension (PAH) is a highly morbid disease that primarily affects young females with progressive pulmonary vascular obliteration resulting in right heart failure and death. While much progress has been made in improving outcomes in PAH, therapies are imperfect and there is presently no cure. Newer, more effective treatments are needed that address key disrupted pathways in PAH.

Although the renin–angiotensin–aldosterone system is known to be activated in PAH [1–4], trials of angiotensin-converting enzyme (ACE) inhibitors in this disease have not demonstrated benefit [5–8], and although angiotensin II (AngII) type 1 ($AT_1$) receptor antagonism has benefit in the monocrotaline rat model of pulmonary hypertension, this drug class has not been trialled in humans with PAH [1]. The observation has led to the hypothesis that alternate hydrolysis of AngII to angiotensin-(1–7) (Ang-(1–7)) *via* ACE2 may be a more effective therapeutic intervention. Ang-(1–7) activates the Mas receptor (Mas1), which is present on endothelial cells, and has vasodilatory, anti-inflammatory and antifibrotic effects [9, 10], functionally antagonising the effects of $AT_1$ receptor stimulation [11–13]. Finally, activation of the ACE2–Ang-(1–7) axis reduces oxidant stress in diabetes mellitus [14], suggesting impact on pathways of relevance to PAH [15–17]. Thus, the ACE2–Ang-(1–7)–Mas1 axis may be a promising therapeutic pathway in PAH.

We and others have demonstrated that both infusion of ACE2 and direct activation of Mas1 ameliorate rodent models of PAH [18–23], likely through improved cytoskeletal function, which is consistent with prior work on ACE2 [24]. Furthermore, the ACE2–Ang-(1–7) axis has been studied in a right ventricular failure model in which ACE2 peptide administration resulted in reduced right ventricular hypertrophy and fibrosis with improved function [19], suggesting potentially beneficial effects on both the pulmonary vasculature and also right ventricular load stress responses. In human patients, there are no US Food and Drug Administration-approved mechanisms to stimulate Mas1 and there are no direct Mas1 agonists approved for human use. ACE2 enzymatic activity can be augmented by administration of an intravenous formulation of the soluble recombinant human form of the naturally occurring enzyme (rhACE2) with existing safety data in healthy volunteers and acute respiratory distress syndrome (GSK2586881; ClinicalTrials.gov identifier NCT01597635) [25].

We tested the hypotheses that ACE2 activity is reduced in human PAH compared with healthy controls and that short-term ACE2 administration may be safe in a proof-of-concept pilot study of GSK2586881 in PAH patients. We further sought to identify short-term markers of Mas1 activation, suggesting molecular drug effect, which may facilitate future studies of Mas1 activation in PAH.

## Methods

### *Animal experiments*

All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center (VUMC), Nashville, TN, USA.

#### *Pulmonary arterial isolation and cannulation*

Pulmonary arteries (80–300 μm diameter) were dissected from portions of piglet lungs using previously described methods [26]. RNA sequencing experiments were performed as previously reported [27, 28]. See supplementary material for full details.

### *Observational studies of ACE2 in PAH patients*

Human studies were approved by the VUMC Institutional Review Board (numbers 9401 and 151388) and registered at ClinicalTrials.gov (identifier NCT01884051). All patients gave written informed consent prior to inclusion in the study. Idiopathic and heritable PAH patients, aged ⩾18 years, defined according to standard criteria by expert clinicians, were included [29]. See supplementary material for details of renin–angiotensin system (RAS) peptide measurement and aptamer-based superoxide dismutase 2 (SOD2) protein measurement.

### *Pilot trial of GSK2586881*

The study was a phase I dose-escalation, open-label proof-of-concept study in patients with idiopathic or heritable PAH, functional class I–III. The primary end-point was safety, with secondary end-points of change in disease biomarkers (brain natriuretic peptide, AngII/Ang-(1–7) ratio, SOD2 activity and nitrotyrosine activity), and systemic and pulmonary haemodynamics and echocardiographic metrics of PAH including right ventricular function. We planned three patients at 0.2 $mg \cdot kg^{-1}$ based on prior safety data with an escalation to 0.4 $mg \cdot kg^{-1}$ if no dose-limiting toxicity occurred. See supplementary material for detailed inclusion and exclusion criteria.

Patients were recruited from the pulmonary hypertension clinic at VUMC from March 2016 to December 2016. Study procedures are outlined in supplementary table S3. Briefly, patients underwent right heart catheterisation. Haemodynamics were recorded 1 h prior to drug administration (−1 h), immediately prior to drug administration (0 h), and 1, 2 and 4 h after drug administration. Patients were observed overnight with the 6-min walk test (6MWT) and echocardiography measured 24 h after drug administration. Patients returned 2 weeks after drug administration for safety assessment, 6MWT and clinical evaluation. Safety end-points assessed included change in functional class, development of right heart failure signs or symptoms, 6MWT distance, plasma electrolytes and markers of renal function, complete blood count, and echocardiography-derived right ventricular function.

See supplementary material for details of RAS peptide measurement, SOD2 ELISA, nitrotyrosine dot-blot assay, cytokine Luminex assay, and isoprostane and isofuran measurement

### *Statistical analysis*

Continuous variables of demographic data are reported as mean with standard deviation. The Wilcoxon rank-sum test and the Mann–Whitney U-test were used to compare differences between groups. Categorical variables were compared between groups using the Chi-squared test or Fisher's exact test. A p-value of <0.05 was considered statistically significant. Data from the pilot study were compared using the paired two-tailed t-test. Statistical analyses were performed using Prism version 5.0 (GraphPad, La Jolla, CA, USA) and R version 3.0.1 (www.r-project.org).

## Results

### *ACE2 activity in human PAH*

We first tested the hypothesis that ACE2 activity is suppressed in human PAH patients compared with controls. We enrolled 11 consecutive heritable or idiopathic PAH patients and eight healthy controls (two males and six females) (supplementary table S1). We measured AngII and Ang-(1–7) in plasma, and found a significant decrease in ACE2 activity as reflected by the AngII/Ang-(1–7) ratio in PAH compared with controls ($p=0.01$) (figure 1a–c). This difference was driven by an increase in plasma AngII, which was nearly four-fold higher in PAH than controls ($p<0.003$), with a less significant difference in Ang-(1–7) levels in PAH *versus* controls.

### *Biochemical effects of Mas1 activation*

We next sought to define the molecular consequences of Mas1 activation. As ACE2 requires AngII as substrate and Ang-(1–7) has a short half-life, the use of ACE2 in isolated artery studies is challenging. Subsequently we used AVE0991, a direct Mas1 agonist, to determine if Mas1 activation results in pulmonary artery vasodilation (figure 2a). Using AVE0991 in porcine pulmonary arteries pre-constricted with endothelin-1 [30], we found a dose-dependent increase in percentage dilation as a function of AVE0991, demonstrating that Mas1 activation has similar physiological effects in this model as seen in rodent models [18, 20, 21].

To determine the acute changes in gene expression as a result of Mas1 activation in this isolated artery model, we isolated RNA from arteries exposed to AVE0991 and controls, and performed RNA sequencing. Significantly changed genes are presented in supplementary table S2. Using a gene ontology analysis approach (figure 2b), we found significant changes in several pathways, including genes associated with pressure regulation ($p<10^{-5}$), inflammatory responses ($p=10^{-4}$), and leukocyte and cell migration ($p<10^{-4}$), again suggesting potential effects of Mas1 activation on inflammation and cytoskeletal function. In order to define a potential marker for Mas1 activation in the pulmonary artery, we compared pulmonary arterial gene expression levels before and after AVE0991 (figure 2c). Several genes were upregulated as a result of AVE0991 exposure, including *TGIF1* (transforming growth factor-β-induced factor homeobox 1), *CCL2* (C-C motif chemokine ligand 2) and *SOD2*. CCL2 is known to be important in PAH, suggesting potential relevance of this research to the field [31]. SOD2, a mitochondrial protein catalysing production of hydrogen peroxide and oxygen from superoxide, was an attractive marker based on prior publications [32–34], and a known effect of Mas1 activation in reducing oxidative stress in rodent models of diabetes [14], suggesting potential biological plausibility and also its significant upregulation after AVE0991 exposure. Validation using quantitative PCR in pulmonary arteries can be found in supplementary figure S1. Furthermore, SOD2 expression was high in the control pulmonary arteries and demonstrated marked increase in gene expression after Mas1 activation, suggesting it may be a biologically important and sensitive marker of increased ACE2 activity.

### *SOD2 in human PAH*

We next sought to determine if SOD2 levels were different in human PAH and control patients. We used an aptamer-based proteomic profiling approach to measure relative plasma SOD2 protein content in

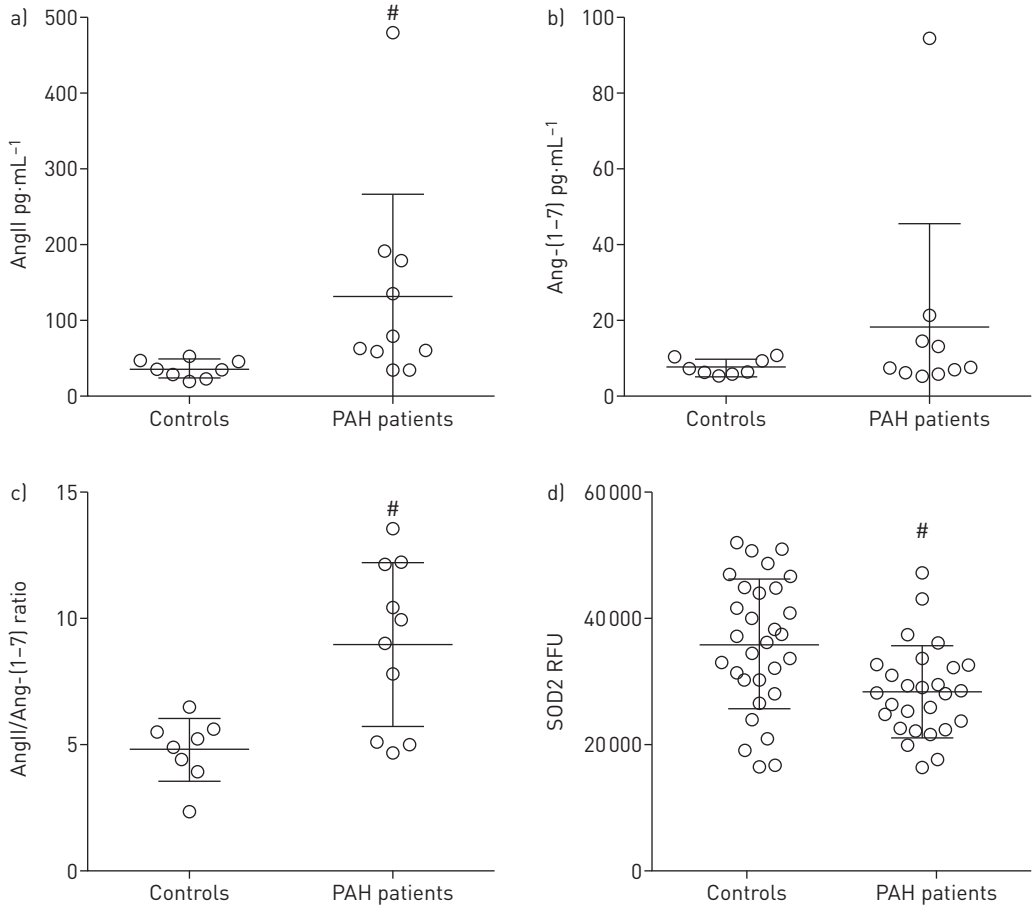


FIGURE 1 Evidence of insufficient angiotensin-converting enzyme 2 (ACE2) activity in human pulmonary arterial hypertension (PAH). a–c) Plasma AngII and Ang-(1–7) levels were measured in 10 PAH patients and eight healthy controls as a marker of ACE2 activity. Log-transformed values are normally distributed by the Shapiro–Wilk W-test for a) AngII, b) Ang-(1–7) and c) AngII/Ang-(1–7) ratio, allowing parametric testing. a) AngII was increased in PAH ($^{\#}$: p<0.003) and c) AngII/Ang-(1–7) ratio was increased in PAH ($^{\#}$: p=0.01), suggesting reduced conversion of AngII to Ang-(1–7) by ACE2 in the plasma of PAH patients. d) SOD2 protein level in plasma was measured by aptamer-based proteomic assay in 30 PAH patients and 27 age-, sex- and body mass index-matched controls. SOD2 protein was reduced in PAH. $^{\#}$: p=0.002. AngII: angiotensin II; Ang-(1–7): angiotensin-(1–7); SOD2: superoxide dismutase 2; RFU: relative fluorescence units.

fasting peripheral blood in 30 PAH patients (age 49.5±13.2 years, body mass index (BMI) 29.8±8.2 kg·m$^{-2}$, three males) and 27 matched controls (age 46.7±11.8 years, BMI 29.1±6.7 kg·m$^{-2}$, five males) (figure 1d). We found levels of SOD2 protein were ~25% lower in PAH plasma compared with controls (28 489±7112 *versus* 35 930±10 202 relative fluorescence units; p=0.002).

### *Pilot trial of GSK2586881 in PAH*

We next sought to determine the safety and potential acute haemodynamic and biochemical effects of a single *i.v.* infusion of GSK2586881 administration in human PAH patients (see supplementary table S3 for details of the protocol). We enrolled five PAH patients and used two different GSK2586881 doses (patients 1–3, 0.2 mg·kg$^{-1}$; patients 4 and 5, 0.4 mg·kg$^{-1}$) (table 1). All patients were receiving at least two PAH-directed therapies including prostaglandin pathway treatments. All patients completed the study protocol and there were no serious adverse effects with study drug administration. One patient experienced dizziness without change in vital signs (patient 1) within 1 h of drug administration and one patient reported leg tingling during the overnight observation period (patient 5) that did not require intervention.

There were no statistical or clinically significant changes in other safety parameters, including 6MWT distance (supplementary table S4). All patients were functional class II at baseline and four were functional class II at the 2-week follow-up, one patient was functional class I. With the exception of a small but statistically significant decrease in creatinine from baseline to 24 h (1.03±0.4 *versus* 0.99±0.4 mg·dL$^{-1}$;

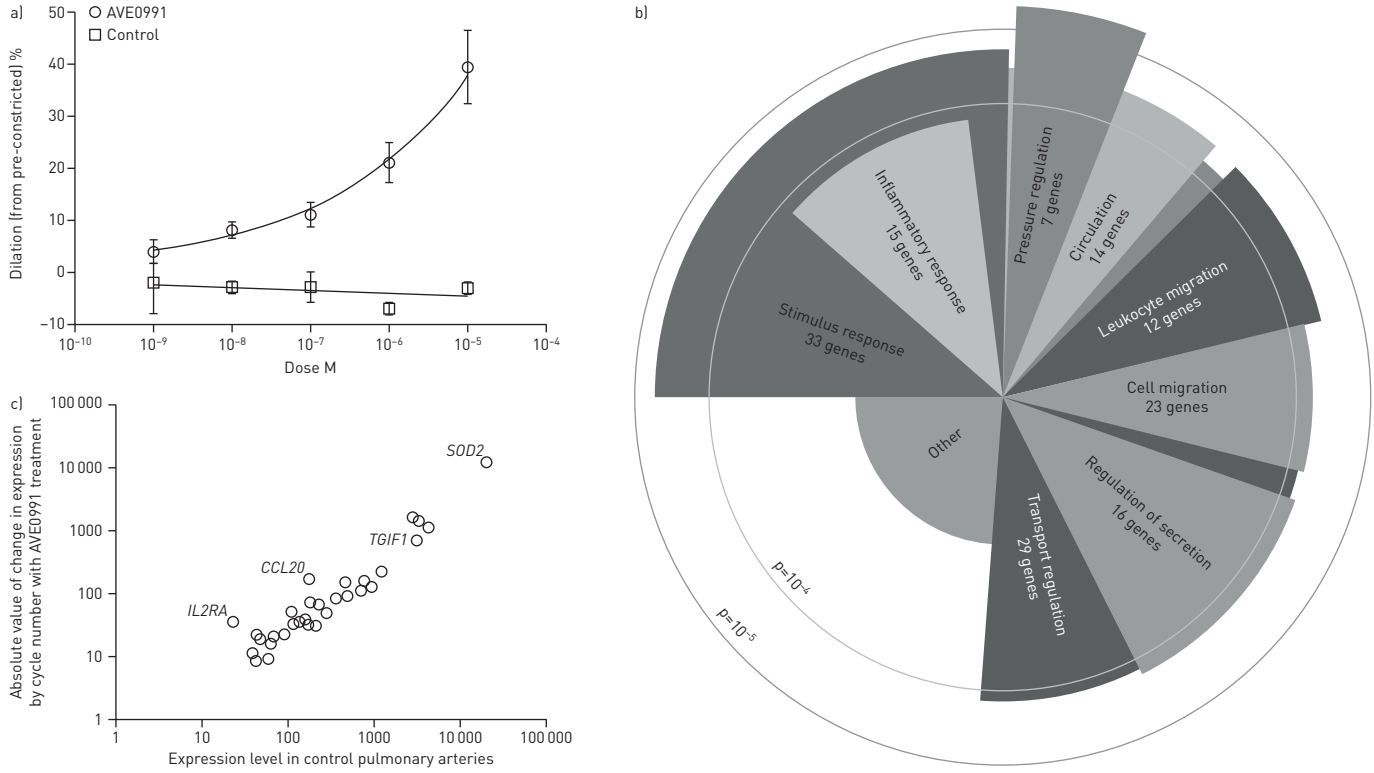


FIGURE 2 Acute molecular effects of Mas receptor (Mas1) activation in a porcine pulmonary hypertension model. a) AVE0991, a direct Mas1 agonist, was administered to pig arteries pre-constricted with endothelin-1. There was a dose-dependent increase in percentage dilation with AVE0991 administration. No dilation was seen with exposure to a biologically irrelevant control compound. b) RNA was isolated from pig arteries with and without AVE0991 exposure, and RNA sequencing was performed. Gene ontology analysis demonstrated significant differences in gene expression in several pathways, including inflammatory responses ($p=10^{-4}$), cell and leukocyte migration ($p<10^{-4}$), and pressure regulation ($p<10^{-5}$). c) When absolute change in gene expression with drug exposure was plotted as dependent on expression level in control arteries, *SOD2* expression was strongly upregulated in response to Mas1 activation. n=8 vessels.

p=0.03), there were no significant changes in laboratory values (data not shown). There were no differences in echocardiographic metrics of left ventricular or right ventricular function at 24 h (supplementary table S5). No antidrug antibodies were detected at 2 weeks in any patient (data not shown). Pharmacokinetic profiles are listed in supplementary table S6.

A single dose of GSK2586881 had no effect on mean pulmonary arterial pressure (supplementary table S7 and figure 3a); however, we did observe a statistically significant increase in cardiac output (5.3±1.4 *versus* 6.1±1.3 $L \cdot min^{-1}$; p=0.008) 4 h after study drug administration, representing an average increase of 40% (figure 3b). Pulmonary vascular resistance (PVR) would be expected to decrease when cardiac output increases without a change in mean pulmonary arterial pressure. We observed substantial variability in PVR with low numbers of enrollees, but when comparing combined time-points prior to drug

TABLE 1 Characteristics of patients enrolled in the GSK2586881 trial

| Patient | Age years | Sex | BMI $kg \cdot m^{-2}$ | PAH type | Disease duration months | Current therapy | Comorbid disease |
|---|---|---|---|---|---|---|---|
| **1** | 28 | Female | 27.5 | Idiopathic | 13 | PP, PDE-5I | None |
| **2** | 39 | Female | 27.8 | Idiopathic | 60 | PP, PDE-5I, ERA | None |
| **3** | 57 | Male | 32.5 | Idiopathic | 91 | Selexipag, PDE-5I | DM2, systemic hypertension, hyperlipidaemia |
| **4** | 41 | Female | 27.4 | Heritable | 92 | Inhaled treprostinil, PDE-5I, ERA | None |
| **5** | 68 | Female | 28.3 | Idiopathic | 65 | PP, PDE-5I, ERA | DM2, systemic hypertension |

BMI: body mass index; PAH: pulmonary arterial hypertension; PP: parenteral prostaglandin; PDE-5I: phosphodiesterase type 5 inhibitor; ERA: endothelin receptor antagonist; DM2: type 2 diabetes mellitus. Patients 1, 2 and 3 received 0.2 $mg \cdot kg^{-1}$ GSK2586881; patients 4 and 5 received 0.4 $mg \cdot kg^{-1}$ GSK2586881.

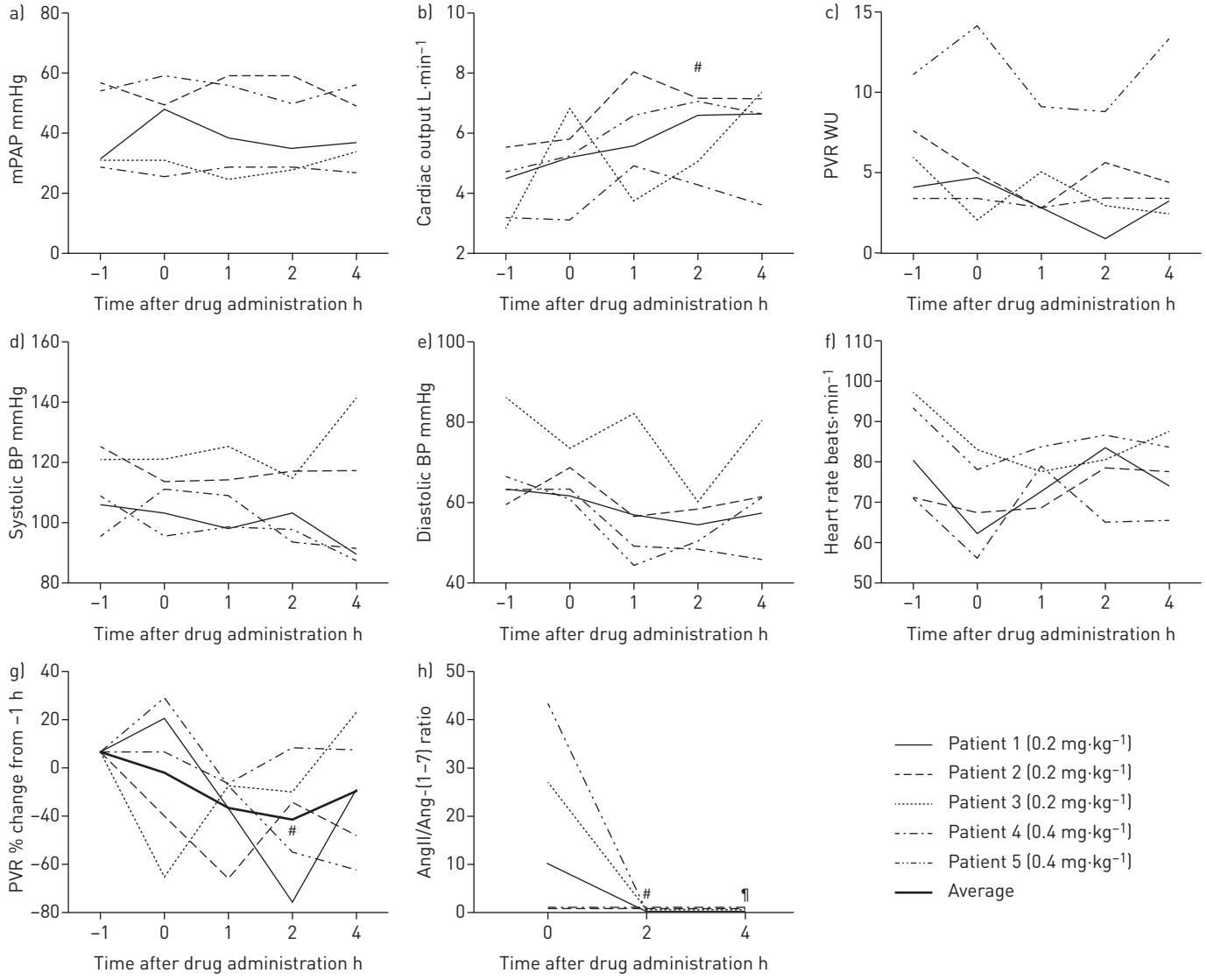


FIGURE 3 Pulmonary and systemic haemodynamic effects of intravenous GSK2586881. A pulmonary artery catheter was placed and haemodynamics measured (−1 h). Just prior to GSK2586881 exposure, pulmonary haemodynamics were recorded again (0 h), and then at 1, 2 and 4 h after drug administration. n=5. a) There was no significant change in mPAP. b) There was a significant increase in cardiac output 2 h after drug administration. #: p=0.008. c–f) Individual raw data on c) PVR, d) systolic BP, e) diastolic BP and f) heart rate. g) Percentage change in PVR showed a significant decrease when comparing time-points before drug administration (−1 and 0 h) with those after drug administration (1, 2 and 4 h). #: p<0.05. h) AngII and Ang-(1–7) levels were measured just prior to drug administration (0 h), and 2 and 4 h after drug adminstration. In the three patients with elevation in the AngII/Ang-(1–7) ratio at baseline, there was a significant decrease in the ratio, suggesting effective angiotensin-converting enzyme 2 augmentation with administration of GSK2586881. #: p=0.009 and ¶: p=0.008 *versus* 0 at 2 and 4 h, respectively. mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; WU: Wood unit; BP: blood pressure; AngII: angiotensin II; Ang-(1–7): angiotensin-(1–7).

administration with after infusion in normalised PVR, we found a statistically significant decrease in PVR, reaching a nadir 2 h after administration (p<0.05) (figure 3g). There were no significant effects on systemic haemodynamics (figure 3d–f).

Plasma AngII and Ang-(1–7) levels were measured as a marker of clinical pharmacology. A decrease in the AngII/Ang-(1–7) ratio suggests increased activity of ACE2 and thereby effective augmentation by the study drug. There was significant variability in the AngII/Ang-(1–7) ratio at baseline (figure 3h). Three patients had substantial elevations in this ratio at baseline and, in these patients, there was a reduction to a nearly undetectable ratio with drug administration at 2 h (26.5±16.5 *versus* 0.2±0.2; p=0.009) that was sustained at 4 h (0.3±0.2; p=0.008). Full details of changes in RAS peptides are in shown in supplementary figure S3.

### *Effect of GSK2586881 on SOD2 in human PAH*

As our *ex vivo* animal model had shown induction of SOD2 expression with Mas1 activation, we tested the hypothesis that SOD2 protein content in PAH patient plasma would be induced by GSK2586881

administration (figure 4a). Using an ELISA assay, we found that there was significant induction of plasma SOD2 protein levels by 2 weeks (p=0.009 *versus* baseline values) suggesting induction in the enzymatic activity by GSK2586881, corroborating our findings in the animal model and indicating that this may be a marker of Mas1 activation in human PAH.

### *Effects of GSK2586881 on plasma markers of inflammation*

Our animal microarray data further suggested that Mas1 activation potentially acts through altering the pattern of pro-inflammatory gene expression. We next tested the hypothesis that this improvement would occur in human PAH patients after acute GSK2586881 administration. As markers of reduced pro-inflammatory signalling, we measured a plasma cytokine array and markers of oxidant stress. We utilised a Luminex platform to assess the effect of GSK2586881 on plasma cytokines (table 2). Consistent with prior reports [35], there were increased levels in six out of nine measured cytokines at time-point 0 h compared with controls (p<0.05 for interleukin (IL)-10, IL-1β, tumour necrosis factor (TNF)-α, IL-13, IL-8 and IL-4). With GSK2586881 administration, there was suppression of measured cytokines including IL-10, IL-1β, IL-2 and TNF-α that could be detected as early as 2 h after drug administration, and was

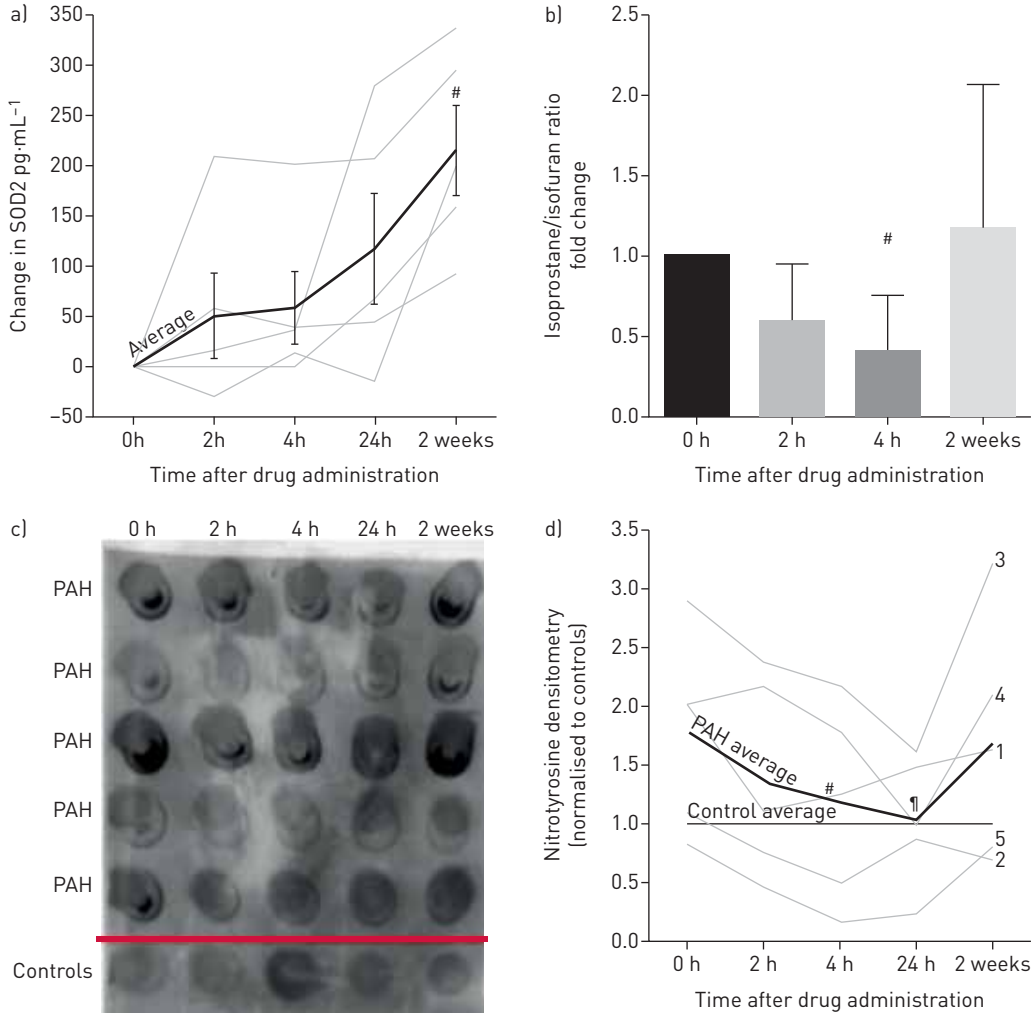


FIGURE 4 Effect of GSK2586881 on markers of oxidant stress. a) Plasma SOD2 protein levels were measured by ELISA assay just prior to drug administration (0 h), and 2, 4 and 24 h, and 2 weeks after drug administration. n=5. There was a significant increase in plasma SOD2 levels at 2 weeks. #: p=0.009. b) We measured plasma isoprostanes and isofurans as markers of oxidant stress. Isofurans are lipid peroxidation products similar to isoprostanes, but are formed in times of oxygen availability. There was a decrease in the isoprostane/isofuran ratio, suggesting improved oxygenation. #: p<0.05. c) Nitrotyrosine dot-blot assay from the plasma of five healthy controls and five study enrollees (PAH), and d) quantification of densitometry. In PAH patients 1–5, there was a decrease in plasma nitrotyrosine levels to those of controls by 4 h (#: p=0.004) that was sustained at 24 h (¶: p=0.02). See supplementary figure S1 for full original blot. SOD2: superoxide dismutase 2; PAH: pulmonary arterial hypertension.

TABLE 2 Effect of GSK2586881 on plasma cytokines

| | Controls | Time after drug administration | | | | |
|---|---|---|---|---|---|---|
| | | 0 h | 2 h | 4 h | 24 h | 2 weeks |
| IFN-γ pg·mL$^{-1}$ | 8.4±5.1 | 17.1±10.9 | 14.9±12.9 | 15.4±9.8$^{¶}$ | 20.5±17.8 | 15.7±11.2 |
| IL-10 pg·mL$^{-1}$ | 6.6±5.5 | 15.9±5.3$^{\#}$ | 12.0±3.8$^{¶}$ | 13.8±6.6 | 19.1±10.4 | 13.6±9.1 |
| IL-13 pg·mL$^{-1}$ | 11.6±9.5 | 39.1±15.0$^{\#}$ | 30.3±14.3 | 32.9±14.2$^{¶}$ | 41.0±22.2 | 34.9±18.6 |
| IL-1β pg·mL$^{-1}$ | 1.3±0.5 | 3.8±2.0$^{\#}$ | 2.6±1.8$^{¶}$ | 3.0±1.7$^{+}$ | 4.3±3.2 | 3.0±1.8$^{¶}$ |
| IL-2 pg·mL$^{-1}$ | 3.1±1.4 | 6.3±3.0 | 4.8±3.0 | 5.6±2.8$^{¶}$ | 7.1±5.2 | 5.6±2.6 |
| IL-4 pg·mL$^{-1}$ | 14.0±11.4 | 42.8±26.5$^{\#}$ | 34.5±23.3 | 35.7±19.7 | 50.0±35.7 | 36.9±27.3 |
| IL-6 pg·mL$^{-1}$ | 2.5±1.8 | 4.6±1.8 | 4.0±1.9 | 9.2±6.7 | 6.4±6.4 | 3.3±1.5$^{+}$ |
| IL-8 pg·mL$^{-1}$ | 5.0±1.6 | 8.6±1.5$^{\#}$ | 7.5±2.5 | 8.5±1.6 | 9.2±2.9 | 6.9±1.4$^{¶}$ |
| TNF-α pg·mL$^{-1}$ | 4.8±3.2 | 9.1±1.8$^{\#}$ | 5.6±3.0$^{¶}$ | 6.8$^{+}$±1.9 | 9.6±5.3 | 5.2±1.5$^{¶}$ |

Data are presented as mean±SD for five healthy controls and five pulmonary arterial hypertension patients treated with angiotensin-converting enzyme 2. IFN: interferon; IL: interleukin; TNF: tumour necrosis factor. $^{\#}$: p<0.05 *versus* controls by paired t-test with normalised data; $^{¶}$: p<0.05 *versus* pre-treatment (time-point 0 h) by paired t-test with normalised data; $^{+}$: p<0.01 *versus* pre-treatment (time-point 0 h) by paired t-test with normalised data.

associated with sustained anti-inflammatory effects with reduced levels of IL-1β, IL-6, IL-8 and TNF-α at 2 weeks (p<0.05). These data collectively suggested a broad suppression of inflammatory cytokines with rhACE2 administration in PAH.

### *Effect of ACE2 on oxidant stress*

Plasma levels of oxidised lipids are known to be increased in PAH as a marker of oxidant stress [16, 17]. Isofurans are lipid peroxidation products similar to isoprostanes, but isofurans exhibit favoured formation in the presence of increased oxygen availability [36]. The ratio of isoprostanes to isofurans is a useful index of oxygen availability. We measured isoprostanes and isofurans in the plasma of our trial participants, and found a significant decrease in the isoprostane/isofuran ratio 4 h after GSK2586881 administration (p<0.05) (figure 4b).

To further assess the effect of GSK2586881 on markers of oxidant stress, we quantified plasma 3-nitrotyrosine levels by dot-blot assay in five healthy controls and in our study participants (figure 4c and d). We found that nitrotyrosine was increased in PAH patients compared with controls (p=0.02) at baseline. With GSK2586881 administration, plasma nitrotyrosine levels fell in PAH patients at both 4 and 24 h (p=0.004 and p=0.02, respectively). At the 24-h time-point, PAH nitrotyrosine levels were no different from controls after GSK2586881 administration, suggesting a significant reduction in whole-body oxidant stress.

## Discussion

Our study sought to 1) demonstrate reduced ACE2 activity in human PAH, 2) define robust readouts of Mas1 agonism in animal models and determine if these readouts are potentially relevant in human PAH, and 3) perform a pilot trial of GSK2586881 in PAH patients to determine if this biological is potentially safe in human PAH and may demonstrate either haemodynamic or biochemical efficacy. We aimed to derive a marker of Mas1 agonism in our pig pulmonary artery model that was of relevance to PAH, and to test the haemodynamic and pharmacodynamics effects of GSK2586881 in human PAH patients. These experiments demonstrate that rhACE2 administration in human PAH may have potential biochemical and haemodynamic efficacy through Mas1 activation. A visual summary of the findings and interpretations is shown in figure 5.

Given compelling pre-clinical data in rodent models of PAH [18–21] and pathology specimens demonstrating upregulation of the RAS in PAH [3, 4], interventions on the RAS pathway would seem likely to have benefit. After demonstrating relevance of this pathway to human PAH and defining inflammatory markers and SOD2 as potential markers of drug effect relevant to pulmonary vascular disease, we proceeded with a pilot study to assess the safety of GSK2586881 in PAH, restricting entry to idiopathic and heritable PAH as our rodent model experiments were most relevant to these PAH subgroups [18]. We enrolled five patients with advanced disease, as evidenced by multiple PAH-directed therapies including those targeting the prostacyclin pathway. We did not identify any safety concerns, although we enrolled relatively low numbers of patients. Even in this small study there were potential

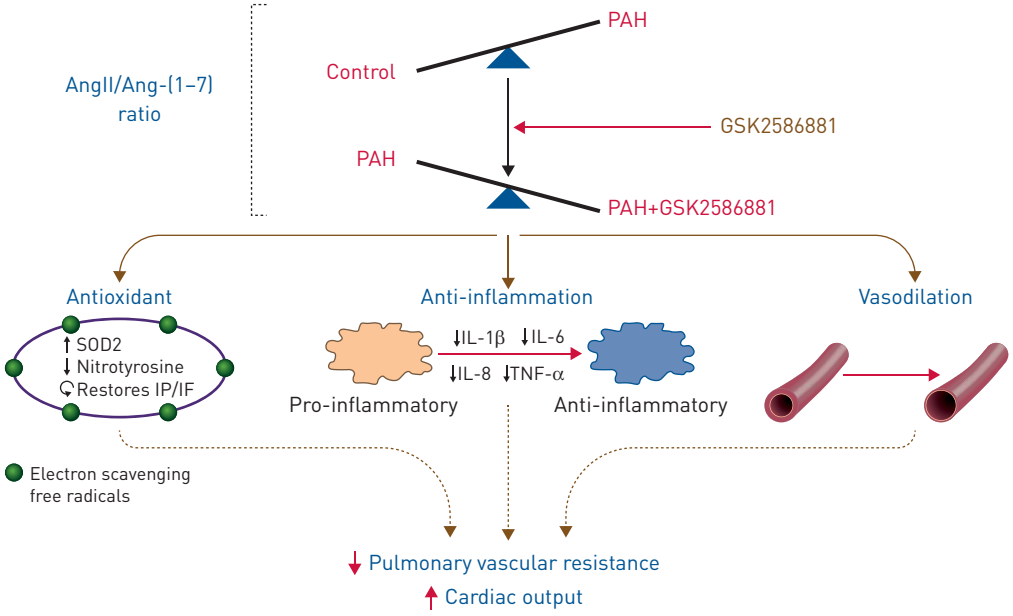


FIGURE 5 Schematic of study findings. PAH: pulmonary arterial hypertension; AngII: angiotensin II; Ang-(1–7): angiotensin-(1–7); SOD2: superoxide dismutase 2; IP: isoprostane; IF: isofuran; IL: interleukin; TNF: tumour necrosis factor.

haemodynamic markers of efficacy, including improved cardiac output and PVR. Furthermore, there was additional biochemical data supporting improved cardiac output: the drop in creatinine may be consistent with improved renal perfusion and the reduced isoprostane/isofuran ratio may indicate improved oxygen delivery to the systemic circulation.

A major hindrance to drug development in PAH is the lack of effective biological markers of drug efficacy. We sought to overcome this limitation by first developing markers of drug effect in relevant tissue, *i.e.* the pig pulmonary artery. We identified *SOD2* and inflammatory pathway genes as having robust changes in response to AVE0991 exposure. SOD2 plays a role in animal models of PAH, although data in human PAH are limited [32–34]. Using proteomic data, we showed that SOD2 protein was reduced in PAH plasma compared with controls, suggesting potential relevance in humans with detectable protein levels. Human and rodent data show a role for inflammation in PAH [35, 37, 38], thus we tested cytokine levels in our own PAH population and found elevations in several cytokines in plasma compared with controls. After demonstrating the potential relevance of SOD2 and inflammation in PAH, we next tested the potential effect of Mas1 activation on these markers in human PAH. We found that rhACE2 augmentation resulted in similar effects in the plasma of PAH patients as direct Mas1 activation in pig arteries. These findings correlated with improved cardiac output at the time of maximum suppression of plasma cytokines and maximum reduction in plasma nitrotyrosine levels, a downstream marker of SOD2 activity. SOD2 levels rose at a later time-point in the plasma, but this was expected as SOD2 is located in the mitochondria and cell turnover would be required prior to detection of a change in plasma levels. Although the AngII/Ang-(1–7) ratio also reflected an effective increase in plasma ACE2 activity, these changes in SOD2, nitrotyrosine and cytokines are likely more reflective of activation of Mas1 with changes in downstream signalling. Indeed, prior work has demonstrated that Ang-(1–7) inhibits leukocyte migration and cytokine expression [39, 40]. While inflammation and SOD2 are potential markers of drug effect, their levels may be altered by other exposures and pathology, making the relative change with drug exposure particularly of interest. It is possible that future drug trials in this pathway may use these markers of Mas1 agonism as early readouts of biochemical efficacy in adaptive trial designs or to define patients most likely to benefit from these interventions.

Oxidant stress has long been implicated in PAH [33, 41–46]. Whole-body markers of oxidant stress include F2-isoprostanes, formed through free radical peroxidation of essential fatty acids, and nitrotyrosine, a product of tyrosine nitration by reactive nitrogen species. Both nitrotyrosine and isoprostanes have been implicated in PAH [17, 44, 47]. Isofurans, in contrast, are nonenzymatic compounds formed through free radical peroxidation of arachidonic acid, and indicate increased production of oxidants at the level of the mitochondria and thus require oxygen to produce. We hypothesised that increased Mas1 signalling would reduce the total burden of oxidant stress in PAH

patients. We found a reduction in plasma nitrotyrosine by dot-blot assay, consistent with this hypothesis. There was a significant decrease in the plasma isoprostane/isofuran ratio at 4 h that correlated with the time-point of greatest improvement in cardiac output, suggesting increased delivery of oxygen to tissues and potentially greater oxygen-dependent respiration reflected in reduction in this ratio, although this hypothesis requires further confirmation. Overall, the data were reassuring that there was not an increase, and rather a decrease, in total body oxidant stress as measured by nitrotyrosine levels that showed a reduction to normal levels at 4 and 24 h after drug administration.

Our study is limited by the low number of trial participants; however, our intention was to perform a proof-of-concept study and to identify potential markers of drug efficacy that can be used in a larger trial. Our RNA expression markers of AVE0991 effects in the pig vasculature were reduced by the known transcriptome of pigs, which is limited compared with humans, and thus there may be more significantly changed transcripts that could not be identified by our methodology; nonetheless, we were able to detect robust readouts. There are other members of the RAS peptide family that would be informative regarding ACE2 activity, including angiotensin-(1–9) and alamandine [48]; however, these compounds rapidly degrade and thus could not be measured in our samples. Also, we did not administer rhACE2 to healthy controls to measure the effect on oxidant stress of inflammatory compounds; however, literature supports comparing an individual's own cytokine panel before and after intervention, rather than comparing groups given the variability in these measurements [49, 50].

In conclusion, our data show evidence of reduced ACE2 activity in human PAH accompanied by reduced SOD2 activity, increased cytokine expression and increased oxidant stress. Infusion of a single dose of rhACE2 (GSK2586881) was well tolerated and may have potential haemodynamic benefit. GSK2586881 administration was associated with increased SOD2 levels, reduction in markers of inflammation and reduced plasma oxidant stress. Further study of GSK2586881 as a therapeutic in PAH is warranted.

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