Prolonged overcirculation-induced pulmonary arterial hypertension as a cause of right ventricular failure

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Aims
Three-month chronic systemic-to-pulmonary shunting in growing piglets has been reported as an early pulmonary arterial hypertension (PAH) model with preserved right ventricular (RV) function. We sought to determine whether prolonged shunting might be associated with more severe PAH and RV failure.

Methods and results
Fourteen growing piglets were randomized to a sham operation or the anastomosis of the left innominate artery to the pulmonary arterial trunk. Six months later, the shunt was closed and the animals underwent haemodynamic evaluation followed by tissue sampling for pathobiological assessment. Prolonged shunting had resulted in increased mean pulmonary artery pressure (22 ± 2 versus 17 ± 1 mmHg) and pulmonary arteriolar medial thickness, while cardiac output was decreased. However, RV-arterial coupling was markedly deteriorated, with a ~50% decrease in the ratio of end-systolic to pulmonary arterial elastances (Ees/Ea). Lung tissue expressions of endothelin-1, angio- poietin-1, and bone morphogenetic protein receptor-2 were similarly altered compared with previously observed after 3-month shunting. At the RV tissue level, pro-apoptotic ratio of Bax-to-Bcl-2 expressions and caspase-3 activation were increased, along with an increase in cardiomyocyte size, while expressions in voltage-gated potassium channels (Kv1.5 and Kv2.1) and angiogenic factors (angiopoietin-2 and vascular endothelial growth factor) were decreased. Right ventricular expressions of pro-inflammatory cytokines [interleukin (IL)-1α, IL-1β, tumour necrosis factor-α (TNF-α)], and natriuretic peptide precursors (NPPA and NPPB) were increased. There was an inverse correlation between RV Ees/Ea and pro-apoptotic Bax/Bcl-2 ratios.

Conclusions
Prolonged left-to-right shunting in piglets does not further aggravate pulmonary vasculopathy, but is a cause of RV failure, which appears related to an activation of apoptosis and inflammation.

Keywords
Pulmonary arterial hypertension • Left-to-right shunt • Congenital heart disease • Right ventricle • Apoptosis

Introduction
Pulmonary arterial hypertension (PAH) is a rare dyspnoea-fatigue syndrome caused by a progressive increase in pulmonary vascular resistance (PVR) and eventual right ventricular (RV) failure.¹ In spite of progress achieved during the last two decades with the advent of therapies targeting the pulmonary vascular diseases process, based on the administration of prostacyclins, endothelin receptor antagonists (ERAs), and phosphodiesterase-5 inhibitors (PDE-5i) therapies, the prognosis of PAH remains poor, with limited survival and low quality of life in too many patients.²,³ This may be related to limited efficacy of targeted therapies in decreasing PVR. New approaches are therefore being developed. It has been recently better realized that PAH symptomatology is
largely determined by RV function adaptation to increased afterload.\textsuperscript{4,5} It is thus possible that an improved understanding of the pathobiology of load-induced RV failure by studies using realistic animal models of the disease might contribute to the development of efficient specific cardiac therapies for PAH patients.\textsuperscript{6}

Aorta-pulmonary shunting in growing piglets is a recognized model of congenital cardiac left-to-right shunt-induced PAH.\textsuperscript{7} The pulmonary circulation of piglets appears to be particularly sensitive to the mechanical stress of increased pulmonary artery flow and pressure, reproducing in a few months, a pulmonary vasculopathy and a preload/afterload-induced RV failure that may take decades to develop in humans born with left-to-right cardiac shunts.\textsuperscript{7,8} We reported that three months of shunting through a modified Blalock–Taussig procedure in piglets, or the anastomosis of the left innominate artery to the pulmonary arterial trunk, is associated with an approximate doubling of PVR and medial hypertrophy, making it a suitable model of early PAH.\textsuperscript{9–11} In these studies, RV function adaptation to afterload evaluated by the coupling of end-systolic to arterial elastances (Ees/Ea) appeared to be preserved, without or with preventive administration of the ERA bosentan, the PDE-5i sildenafil, or the angiotensin II antagonist losartan.\textsuperscript{9–11}

In the present study, we doubled the duration of systemic-to-pulmonary shunting in growing piglets, extending it to six months, aiming at a model of more advanced PAH with RV failure as a more adequate reproduction of clinical PAH. The results show the appearance of severe RV failure with unchanged progression of pulmonary vasculopathy.

### Methods

Fourteen piglets 18 ± 1 days old and weighing 5.3 ± 0.2 kg were included in this study, which was approved by our institutional committee on animal welfare. The animals were randomized to a sham operation (n = 7) or to an anastomosis between the left innominate artery and the pulmonary trunk (n = 7) as previously reported.\textsuperscript{9–11} The animals were followed during 6 months, and then anaesthetized for haemodynamic and lung and myocardial tissue sampled for pathobiological analysis.

### Haemodynamic evaluation

As previously reported,\textsuperscript{7–11} the animals were anaesthetized, equipped with catheters and an ultrasonic flow probe on the pulmonary artery for measurements of mean systemic arterial pressure (mPsa), mean pulmonary artery pressure (mPpa), occluded Ppa (Ppao), cardiac output (CO), pulmonary arterial flow (Q), and blood gases. Because PVR is a flow-sensitive variable, the measurements were done after shunt closure, and the flow-resistive properties of the pulmonary vessels were further assessed by multipoint (mPpa/Q) plots obtained by rapid inflation of the inferior vena cava balloon.\textsuperscript{12} A ‘single beat method’ was used to estimate RV afterload by pulmonary arterial elastance (Ea) and RV contractility by end-systolic elastance (Ees), and the adequacy of RV systolic function adaptation to afterload by the Ees/Ea ratio.\textsuperscript{13}

Haemodynamic and blood gases measurements were obtained after ensuring steady-state conditions (stable heart rate, Psa, and Ppa) for 60 min, after shunt closure in the shunted animals. After the measurements, the animals were killed with an anaesthetic overdose. Pulmonary, right, and left myocardial tissue samples were immediately harvested, snap-frozen in liquid nitrogen and stored at −80 °C for biological experiments or after overnight fixation, embedded in paraffin for histopathological evaluations.

### Morphometry—Immunohistochemistry

Pulmonary arterial morphometry was performed as previously described.\textsuperscript{9–11} Only arteries with an external diameter (ED) of <500 μm and a complete muscular coat were measured and assigned to five groups according to ED: 0–75, 76–150, 151–225, 226–300, and 301–500 μm. Medial thickness (MT) was related to arterial size with the following formula: %MT = (2xMT/ED) x 100 and performed

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**Table 1** Primers used for real-time quantitative polymerase chain reaction (RTQ-PCR) in porcine tissues

<table>
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<th>Genes</th>
<th>Primer sequences</th>
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<tr>
<td>Bax</td>
<td>Sense 5′-GCATTGGAGATGAACCTGG-3′</td>
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<tr>
<td></td>
<td>Antisense 5′-CGCCACTGGAAGAAGACT-3′</td>
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<td>Bel-2</td>
<td>Sense 5′-TGATGCTATTTCTCCTCC-3′</td>
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<td></td>
<td>Antisense 5′-GCTCAAACAGAGCAAACC-3′</td>
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<td>Interleukin-1 alpha (IL-1α)</td>
<td>Sense 5′-CCATATGGCCATGCTTTCCC-3′</td>
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<td></td>
<td>Antisense 5′-GCTCAAACAGAGCAAACC-3′</td>
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<td>Potassium voltage-gated channel 1.5 (KCNA5, Kv1.5)</td>
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<td></td>
<td>Antisense 5′-AGTGGGCTCTTGTAGTCG-3′</td>
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<tr>
<td>Potassium voltage-gated channel 2.1 (KCNB1, Kv2.1)</td>
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<td>Natriuretic peptide precursor A (atrial natriuretic peptide, ANP)</td>
<td>Sense 5′-TGCTCAATGCGAGGACCTAG-3′</td>
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<td></td>
<td>Antisense 5′-GGGCGGATGGCCATGCTTCT-3′</td>
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<td>Natriuretic peptide precursor B (brain natriuretic peptide, BNP)</td>
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<td>Antisense 5′-GCATCTAGCAGCAACAGGT-3′</td>
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</tr>
<tr>
<td></td>
<td>Antisense 5′-GCACCAATGCTTCACTTC-3′</td>
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by counting at least 300 pulmonary arteries per lung section from each pig.

Myocardial tissue were fixed and embedded in paraffin as previously described, and stained with haematoxylin–eosin for overall morphology. Mean cross-sectional areas of cardiomyocytes were calculated by measuring from 50 to 150 cells for each myocardial sample. Morphological differences were independently assessed in a blind fashion by two individuals. Immunohistochemical analysis was performed using ImageJ analysis software. From these haematoxylin–eosin stained myocardial sections, structural capillary density (number of capillaries per mm²) was evaluated using at least 40 microscopic fields (in × 400 total magnification) of myocardial sections randomly selected. Masson’s Trichrome staining was used to assess the presence of collagen accumulation and fibrosis within the myocardial sections.

Real-time quantitative polymerase chain reaction
Total RNA was extracted from snap-frozen pulmonary and myocardial tissue using the QIAGEN RNeasy® Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. RNA concentration was determined by standard spectrophotometric techniques and RNA integrity was assessed by visual inspection of ethidium bromide-stained agarose gels. Reverse transcription was performed using random hexamer primers and Superscript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

For real-time quantitative polymerase chain reaction (RTQ-PCR), sense and antisense primers were designed using Primer3 program for porcine Bax, Bcl-2, interleukins (IL-1α, IL-1β, IL-6, and IL-10), voltage-gated potassium channels (Kv1.5 and Kv2.1), natriuretic peptide precursors A and B (NPPA and NPPB) and insulin growth factor (IGF) and IGF receptor mRNA sequences (Table 1). To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected when exon sequences were known. For each sample, amplification reaction was performed in triplicate using SYBR-Green PCR Master Mix (Quanta Biosciences, Gaithersburg, MD, USA), specific primers and diluted template cDNA. Result analysis was performed using an iCycler System (BioRad Laboratories). Relative quantification was achieved with the comparative 2^(-ΔΔCt) method by normalization with the housekeeping gene (hypoxanthine phosphoribosyltransferase, HPRT). Results were expressed as relative fold increase over the mean value of RV relative mRNA expression of sham-operated group arbitrary fixed to 1.

Western blotting
Proteins were extracted from snap-frozen myocardial tissue by homogenization in an appropriate ice-cold lysis buffer. After centrifugation, protein concentration was determined using the Bradford protein assay. Protein extracts (50 μg) were resolved on 4–12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and electro-transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA). After blocking with 5% non-fat milk in 0.1% Tween-TBS (Tris-HCl pH 8.0; NaCl 150 mM), membranes were incubated with mouse anti-caspase-3 (1:500; Millipore, Temecula, CA, USA) monoclonal antibody. After incubation with secondary HRP-conjugated anti-mouse antibody (1:25 000; ThermoScientific, Rockford, IL, USA), immunoreactive bands were detected using SuperSignal® WestPicoChemiluminescent substrate (ThermoScientific, Rockford, IL, USA) and quantified by laser densitometry. Relative quantification was performed by normalization with β-actin (Sigma-Aldrich, St Louis, MO, USA).

Enzyme-linked immunosorbent assay
Interleukin-1β and tumour necrosis factor-α (TNF-α) protein levels were determined respectively, with Quantikine® Porcine IL-1beta/IL-1F12 and TNF-α/TNFFSF1A enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) in myocardial protein extracts (200 μg) and in systemic arterial plasma, according to manufacturer’s instructions. Pro-inflammatory IL-1β and TNF-α concentrations were obtained by referring to a standard curve realized in parallel. Results represented the mean value of two separate measurements performed in duplicate.

Statistical analysis
Values are expressed as mean ± SEM. Statistical analyses were performed using StatView 5.0 Software. The haemodynamic and biological

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**Table 2** Haemodynamic effects of 6-month shunting in overcirculation-induced experimental pulmonary arterial hypertension in piglets

<table>
<thead>
<tr>
<th></th>
<th>6-month sham, n = 7</th>
<th>6-month shunt, n = 7</th>
</tr>
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<tbody>
<tr>
<td>Weight, kg</td>
<td>51 ± 3</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>HR, b.p.m.</td>
<td>80 ± 6</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>Q, L min⁻¹ m⁻²</td>
<td>3.4 ± 0.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Psa, mmHg</td>
<td>103 ± 8</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>Ppa, mmHg</td>
<td>17 ± 1</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Ppao, mmHg</td>
<td>7 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>PVR, mmHg/L⁻¹ m²</td>
<td>2.8 ± 0.3</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>Ees, mmHg/mL</td>
<td>0.83 ± 0.12</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>Ea, mmHg/mL</td>
<td>0.58 ± 0.06</td>
<td>1.15 ± 0.16</td>
</tr>
<tr>
<td>Ees/Ea</td>
<td>1.42 ± 0.12</td>
<td>0.66 ± 0.07</td>
</tr>
</tbody>
</table>

HR, heart rate; Q, cardiac index; Psa, mean systemic arterial pressure; Ppa, mean pulmonary arterial pressure; Ppao, occluded Ppa; PVR, pulmonary vascular resistance; Ees, end-systolic elastance; Ea, pulmonary arterial elastance. Values are expressed as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 versus 6-month sham-operated pigs. NS means not significant.
Results
Six months after surgery, the weight of the animals was ~50 kg, and was not different in 6-month shunted versus 6-month sham animals (Table 2). Arterial blood gases and haematocrit were normal and were not different in the two study groups (Table 2). The ratio of pulmonary to systemic blood flow before closure of the shunt was 1.6 ± 0.1.

Haemodynamic evaluation
When compared with the 6-month sham group, six-month aortapulmonary shunting was associated with increased mPpa, PVR, and Ppao and decreased HR and Q, with no change in mPsa (Table 2). Five-point mPpa-Q plots were obtained in all animals, and always best described by a linear approximation, with correlation coefficients >0.95. A linear regression was calculated on each of them by the least squares method, and mPpa interpolated at flow levels of 2 and 5 L min⁻¹ m⁻².

Pulmonary hypertensive disease: morphometry and pathobiological evaluation
After 6 months of systemic-to-pulmonary shunting, pulmonary arterial medial thickness increased compared with the 6-month sham group (Figure 2A and C) and this effect was more pronounced in the smallest pulmonary arterioles (Figure 2C), with increased accumulation of α-smooth muscle positive stained cells (Figure 2B), suggesting smooth muscle cell proliferation within the media.

As illustrated in Figure 3, 6-month systemic-to-pulmonary shunting increased lung gene expressions in the precursor of endothelin-1 (the preproendothelin-1, PPET-1) and the endothelin receptor type B (ET-B), while expressions in the endothelin-converting enzyme-1 (ECE-1) and the endothelin receptor type A (ET-A) did not change (Figure 3A) compared with the 6-month sham operation. When compared with the 6-month sham group, pulmonary angiopoietin (Ang)-1, endothelial Tie2 receptor, and vascular endothelial growth factor (VEGF) gene expressions increased, while Ang-2 and VEGF receptors type 1 and type 2 (VEGFR1/Flt-1 and VEGFR2/flk-1) gene expressions did not change (Figure 3B). In the 6-month shunted group, lung gene expressions in bone morphogenetic protein receptor (BMPR)-2 decreased and in inducible nitric oxide synthase (iNOS) increased, with no changes in BMPR-1A and endothelial nitric oxide synthase (eNOS) gene expressions compared with the 6-month sham group (Figure 3C and D).

Right ventricular apoptosis: regulation and execution of apoptotic pathways
When compared with 6-month sham operation, at the gene level, 6 months of shunting decreased RV expression of anti-apoptotic Bcl-2 mitochondrial member, while pro-apoptotic Bax expression did not change (Figure 4A). Gene expressions of Bcl-2 and Bax mitochondrial members did not change within the left ventricle (LV) in the 6-month shunted compared with the 6-month sham group (Figure 4A). The resulting pro-apoptotic Bax/Bcl-2 gene ratio increased within the RV, while it did not change in the LV (Figure 4B). This RV pro-apoptotic Bax/Bcl-2 gene expression ratio was inversely correlated to Ees/Ea ratio (Figure 4C). At protein level, 6-month shunting increased the activation by cleavage of the final apoptotic executioner pro-caspase-3 within the RV of 6-month shunted compared with 6-month sham-operated pigs (Figure 4D). No increased activation of the pro-caspase-3 was found within the LV in both groups (Figure 4D).

Right ventricular hypertrophy and capillary density
The RV of the 6-month shunted pigs presented obvious cardiomyocyte hypertrophy characterized by the increase in cell surface area compared with the 6-month sham group (Figure 5A and B). The hypertrophy of LV cardiomyocytes was also exacerbated in the 6-month shunted compared with the 6-month sham-operated pigs (Figure 5A and B). No significant differences were seen between the RV capillary densities of the 6-month
shunted and the 6-month sham pigs (30 ± 5 versus 26 ± 4 capillaries/mm² myocardial tissue section, *P* > 0.05). After staining with Masson’s Trichrome to detect fibrotic areas, myocardial interstitial fibrosis normally detected with ageing of the animals was significantly exacerbated in sections of the RV myocardium by the 6-month shunting compared with 6-month sham operation (Figure 5C), mostly in perivascular areas.

**Heart disease—pathobiological evaluation**

As illustrated in Figure 6, 6-month shunting increased RV gene expressions of the ET-A receptor compared with the 6-month sham operation, while the expressions in PPET-1, ECE-1, and ET-B receptor did not change (Figure 6A). Gene expressions of Ang-2 and VEGF decreased, while Ang-1 expression did not change between the two groups (Figure 6B). When compared with the 6-month sham group, there were increased gene expressions of IL-1α and IL-1β in both right and left ventricles of 6-month shunted pigs, while gene expressions of IL-6 and the anti-inflammatory member, the IL-10, did not change (Figure 6C). At the protein level, 6-month shunting increased IL-1β in both right and left ventricles compared with the 6-month sham operation (Figure 7A), while the pro-inflammatory cytokine involved in a wide spectrum of biological processes including apoptosis, the TNF-α, only increased in the RV of the 6-month shunted pigs (Figure 7B). Gene expression patterns of voltage-gated potassium channels, including Kv1.5 and Kv2.1, decreased in the RV of the 6-month shunted compared with the 6-month sham-operated pigs (Figure 6D). Real-time quantitative polymerase chain reaction analyses confirmed that the right ventricles from the 6-month shunted pigs underwent an adverse cardiac remodelling in the molecular level. The expression levels of NPPA and NPPB (Figure 6E), markers for adverse hypertrophic remodelling, and IGF and associated receptor, the IGFR (Figure 6F) were increased in the RV of 6-month shunted compared with 6-month sham-operated pigs.

**Serum cytokine levels**

At systemic level, serum concentration of TNF-α protein was significantly raised from undetectable levels in the 6-month sham-operated group to 10.1 ± 3.8 pg/mL in the 6-month shunted group (*P* < 0.05). Interleukin-1β remained undetectable at the systemic level (in the serum) in both groups. There were no significant correlations between these cytokine levels and haemodynamic measurements.

**Discussion**

The present results show that prolonged systemic-to-pulmonary shunting in growing piglets does not aggravate pulmonary arteriolar remodelling, but induces a RV failure with pronounced RV-arterial uncoupling that appears related to a hypertrophic...
response with extracellular matrix accumulation, and activation of apoptotic pathways in association with increased expression of pro-inflammatory cytokines and decreased expression of Kv channels.

The piglets with 6-month systemic-to-pulmonary shunting presented with a pulmonary vasculopathy that was strikingly similar to previously reported after three months of shunting in terms of increased PVR, which was approximately doubled, medial hyperplasia, with medial thickness increased, and up-regulation of ET-1/ET-B, Ang-1/Tie2, and VEGF signalling pathways, and decreased BMPR-2 expression. The increase in mPpa was less prominent than previously reported after three months of shunting, but refined measure of resistance by multipoint mPpa/Q relationships, showed that this was entirely explained by a decreased CO.

The cause of a decreased CO observed after shunt closure in the prolonged shunted piglets is not clearly apparent. An acute decrease in preload which was increased during 6 months because of left-to-right shunting, is unlikely as a similar manoeuvre of shunt closure after three months of shunting did not decrease CO when compared with sham-operated controls. However, prolonged increase in preload, while maintaining CO as long as the shunt was open, probably added to the mechanical stress of increased PVR-related afterload. Measurements of the RV Ees/Ea ratio suggest an uncoupling of RV function from the hypertensive pulmonary circulation, which was not previously observed after three months of shunting. The porcine heart may not be intrinsically capable to sustain a chronic increase in CO, together with an increased afterload for the RV. More athletic canine or human species are probably better designed to sustain more prolonged increased RV loading conditions, although the biological basis for the difference remains unknown.

It is possible to speculate that RV failure with decreased pulmonary flow would have decreased the mechanical stress on the pulmonary circulation, thereby limiting the progression of pulmonary hypertensive disease, with arteriolar remodelling restricted to medial thickness, corresponding to the most early and still reversible pathology. This evolution resembles the limitation or prevention of pulmonary arteriolar remodelling obtained by pulmonary arterial banding as a palliative intervention in infants born with congenital cardiac disease and with high flow and pressure left-to-right shunting.

The pathobiology of RV failure remains largely unknown. An activation of apoptotic pathways has been recently reported in experimental models of acute and chronic RV failure induced by increased preload/afterload. In the present study, there were increases in the pro-apoptotic Bax/Bcl-2 ratio and activation of caspase-3, the final executioner of apoptosis, confirming the activation and the execution of apoptosis. In addition, there was a significant inverse correlation between RV Ees/Ea and Bax/

Figure 3 Relative lung mRNA content for the endothelin signalling pathway (A), including the preproendothelin-1 (PPET-1) the endothelin-converting enzyme-1 (ECE-1), the endothelin A (ET-A), and the endothelin B (ET-B) receptors; the angiogenic factor signalling pathways (B), including the angiopoietin (Ang)-1 and -2, the endothelial Tie-2 receptor, the vascular endothelial growth factor (VEGF) and the vascular endothelial growth factor receptors type 1 (VEGFR-1/Fli1) and type 2 (VEGFR-2/Flik-1); the bone morphogenetic protein signalling pathway (C), including the bone morphogenetic protein receptor type 1A (BMPR-1A) and bone morphogenetic protein receptor type 2 (BMPR-2) and the nitric oxide synthase pathway (D), including the inducible nitric oxide (NO) synthase and the endothelial nitric oxide (NO) synthase of 6-month sham-operated (n=7; white bars) and 6-month shunted (n=7; black bars) pigs. Values are expressed as mean ± SEM. *0.01< P< 0.05; **0.001< P< 0.01; ***P< 0.001, 6-month sham-operated versus 6-month shunted pigs.
Bcl-2 ratios, in keeping with the notion that cardiomyocyte apoptosis plays a role in RV-arterial uncoupling after prolonged increases in flow output against an increased PVR.

Prolonged exposure to afterload induces ventricular hypertrophy, which decreases wall tension through increased thickness, and helps to preserve RV-arterial coupling. The process may become maladaptive. In the present study, there was an increase in cardiomyocyte size in the RV, but also in the LV. A hypertrophic growth is an expected finding in conditions of chronic overload, with increased preload and afterload for the RV, but also an increased preload of the LV required for a chronically shunt-related increase in CO. The 6-month shunted RV myocardium exhibited increased accumulation of extracellular matrix, like previously reported in RV endomyocardial biopsies from PAH patients.20

In the present experimental model of RV failure, an altered mechanical stress and strain would be expected to induce altered expressions of genes that affect cardiac remodelling and cardiomyocyte contractile function. The gene expression of the ET-A receptor was down-regulated within the failing compared with the normal RV, while expressions of the precursor and the enzyme implicated in ET-1 synthesis and the ET-B receptor did not change. Myocardial release of ET-1 has been shown to have a direct influence on cardiomyocytes, including hypertrophic, anti-apoptotic, chronotropic, and inotropic effects,21–25 mainly via the activation of ET-A receptor. Numerous studies have shown increased plasma and myocardial levels of ET-1 and an increased ET-A expression in human and experimental models of LV failure,26–28 with no change in ET-B receptors.27 In the present study, despite the up-regulation of ET-A receptors, the inotropic response was insufficiently effective in the failing RV, as assessed by the decreased Ees/Ea ratio. Increased levels of ET-1 found after 3-month shunting9 returned to normal after 6-month shunting, while the decoupling between the RV and the pulmonary circulation evolved, suggesting a possible implication of the ET-1 signalling pathway in this maladaptive process of the failing RV.

Reduced capillary density and VEGF expression are known to play causative roles in pressure overload-induced LV failure.29–31

**Figure 4** Relative myocardial mRNA content for pro- and anti-apoptotic mitochondrial members of Bcl-2 protein family, respectively, Bax and Bcl-2 (A), as well as pro-apoptotic Bax/Bcl-2 ratio (B) in right (white bars) and left ventricles (black bars) from 6-month sham-operated (n = 7) and 6-month shunted (n = 7) pigs. Correlation between Ees/Ea and right ventricular pro-apoptotic Bax/Bcl-2 ratio (C). Relative caspase-3 activation in right (white bars) and left ventricles (black bars) from 6-month sham-operated (n = 7) and 6-month shunted pigs (D). Values are expressed as mean ± SEM. *0.01 < P < 0.05; **0.001 < P < 0.01, 6-month sham-operated versus 6-month shunted pigs.
Up-regulated after few days, VEGF signalling pathway was down-regulated within the LV after 2 weeks and contributed to decreased cardiac microvascular density and systolic dysfunction in a model of aortic constriction. Chronic RV overload in monocrotaline-induced pulmonary hypertension was associated with a reduced capillary density and reduced VEGF expression, which were actually increased in chronic hypoxic pulmonary hypertension. We show that gene expressions of angiogenic factors (VEGF and angiopoietin-2) were decreased in the failing RV, while capillary density and angiopoietin-1 expression did not change. The mechanisms by which VEGF and angiopoietin-2 may influence cardiomyocyte hypertrophy and contractility remain unknown. However, 3-month shunted pigs presented preserved RV function with increased VEGF gene expression, whereas after 6-month shunting, VEGF signalling became insufficient and was associated with RV dysfunction. Moreover, restoration of VEGF signalling has been previously shown to lead to a normalization of capillary density and an improvement in systolic function.

This data might be relevant to recent studies showing reversal of experimental pulmonary hypertension by tyrosine and multikinase inhibitors and already on-going clinical trials of these drugs in PAH patients. Cardiac complications of multikinase inhibitors in the setting of pressure overload-induced left heart failure have already been reported. Our results may be in keeping with the notion that pharmacological interference with some signalling pathways may improve pulmonary vascular remodelling yet be deleterious to RV adaptation to abnormal loading conditions.

Development and progression of RV failure has been associated with an early increase in RV inflammation in the monocrotaline-induced pulmonary hypertension model, but not in chronic overload-induced RV failure in piglets. Elevated levels of IL-1 and IL-6 have been identified as contributing factors to the development of cardiac remodelling and advanced LV failure in humans. These inflammatory cytokines can depress myocardium contractility by inducing hypertrophy and promoting apoptosis. In the present study, expressions of IL-1α and IL-1β increased in the RV and LV of the 6-month shunted pigs, while IL-6 and anti-inflammatory IL-10 gene expressions did not change. These increased expressions of pro-inflammatory cytokines could be related to the altered RV contractility and hypertrophy. Moreover, the fact that expressions of interleukins are increased in LV may also be indicative of the changes within

**Figure 5** Representative slides of haematoxylin–eosin staining of the right and left ventricular myocardium (magnification: ×400) (A). Representative slides of Masson Trichrome staining (to detect fibrotic areas) of the right ventricular myocardium (magnification: ×400) (B). Cardiomyocyte hypertrophy (assessed by cardiomyocyte area in square micrometer) was exacerbated in the right and the left ventricles of the 6-month shunted pigs compared with the 6-month sham-operated pigs (C). Values are expressed as mean±SEM. *0.01 < P < 0.05, 6-month sham-operated versus 6-month shunted pigs.
the LV towards the progression of severe global heart failure. Contrary to previous studies in PAH patients, serum levels of IL-1β remain undetectable in both shunted and sham-operated pigs, suggesting a local myocardial production of IL-1β. Increased serum levels of a broad range of inflammatory cytokines have recently been described in patients with idiopathic and heritable PAH, and identified as important biomarkers in risk stratifying these patients. In the present study, serum levels of TNF-α increased in the 6-month shunted compared with the 6-month sham pigs, but failed to correlate with any haemodynamic parameters, consistently with previous results in PAH patients. Even, if elevated serum TNF-α levels have been previously shown to be correlated with mortality in left heart failure patients, anti-TNF-α therapy in these patients failed to demonstrate efficacy.

Generation of cardiac action potential depends on a coordinated activation and inactivation of various channel conductances. A major current contributing to the early repolarization is the transient outward potassium current. Impaired early repolarization was associated with desynchronized calcium release from the sarcoplasmic reticulum, action potential prolongation, and reduction in the transmural gradient of repolarization. Down-regulation of potassium currents, which is commonly found in pathological LV hypertrophy with or without heart failure,
therefore significantly contribute to the pathogenesis of excitation contraction abnormalities and cardiac arrhythmias. In the present study, gene expressions of Kv1.5 and Kv2.1 were decreased, respectively, in the RV and LV and in the RV in the 6-month shunted pigs. This is consistent with previous results obtained in the chronic hypoxic model of pulmonary hypertension, showing reduced RV Kv1.5 expression and function.51

Natriuretic peptides, which are important regulators of blood volume and arterial pressure, have also been associated with anti-hypertrophic and antifibrotic properties within the heart.52 In the present study, increased gene expressions of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were found in the failing RV of the 6-month shunted pigs. These findings are consistent with the notion that ANP and BNP expressions are predominantly synthesized by cardiomyocytes when cardiac pressure and volume overload increased.53 Natriuretic peptides, which induce the activation of the particulate guanylate cyclase, therefore increase intracytoplasmic levels of cGMP and in turn activate the protein kinase G, protecting the heart against apoptosis, promoting hypertrophic response, and affecting myocardial contractility.6 Moreover, increased BNP production might also be stimulated by proinflammatory cytokines, such as IL-1β.54

Pressure-mediated hypertrophy and mechanical stretch have been recently implicated in the generation of a subinflammatory low level of IL-1β, which constitutively causes IGF-1 production to maintain adaptable compensation hypertrophy and inhibit interstitial fibrosis.55 Moreover, chronic treatment with IGF-1 has been shown to enhance intrinsic cardiomyocyte function, via an enhanced intracellular calcium handling.56 Here, we showed increased expressions of IGF-1 and related receptor, the IGFR1. This phenomenon might be implicated in the transition from adaptive to maladaptive processes of the failing RV.

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