



## BASIC SCIENCE ARTICLE

# Simvastatin attenuates lung functional and vascular effects of hyperoxia in preterm rabbits

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**BACKGROUND:** Bronchopulmonary dysplasia (BPD) remains a frequent complication following preterm birth, affecting respiratory health throughout life. Transcriptome analysis in a preterm rabbit model for BPD revealed dysregulation of key genes for inflammation, vascular growth and lung development in animals exposed to hyperoxia, which could be prevented by simvastatin.

**METHODS:** Preterm rabbits were randomized to either normoxia (21% O<sub>2</sub>) or hyperoxia (95% O<sub>2</sub>) and within each condition to treatment with 5 mg/kg simvastatin daily or control. Lung function, structure and mRNA-expression was assessed on day 7.

**RESULTS:** Simvastatin partially prevented the effect of hyperoxia on lung function, without altering alveolar structure or inflammation. A trend towards a less fibrotic phenotype was noted in simvastatin-treated pups, and airways were less muscularized. Most importantly, simvastatin completely prevented hyperoxia-induced arterial remodeling, in association with partial restoration of VEGFA and VEGF receptor 2 (VEGFR2) expression. Simvastatin however decreased survival in pups exposed to normoxia, but not to hyperoxia.

**CONCLUSION:** Repurposing of simvastatin could be an advantageous therapeutic strategy for bronchopulmonary dysplasia and other developmental lung diseases with pulmonary vascular disease. The increased mortality in the treated normoxia group however limits the translational value at this dose and administration route.

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## INTRODUCTION

BPD is still a major cause of morbidity in very to extremely premature infants (<32 weeks gestation). It predisposes to increased mortality, prolonged initial hospitalization and long-term compromise of respiratory, cardiovascular and neuro-developmental outcomes. Recent advances in perinatal care like antenatal corticosteroids, surfactant therapy, or more gentle ventilation strategies did improve the survival rate of preterm infants, but did not reduce BPD incidence rates.<sup>1,2</sup> More bench to bedside research is needed to identify the pathophysiological pathways involved in BPD and treatments directly targeted at these disrupted pathways.

While mouse and rat models rely on the effects of hyperoxia alone to disrupt further development of structurally immature lungs,<sup>3</sup> the rabbit model combines hyperoxia with structural and functional prematurity.<sup>4</sup> However not as close to the human setting as preterm sheep and baboons, preterm rabbits exhibit respiratory distress at birth and have immature defense mechanisms against oxidative stress, just like preterm humans do.<sup>5</sup> Previously, we have documented the functional and structural changes in preterm rabbit pups exposed to hyperoxia, which depict similarities with the clinical and pathological features of BPD.<sup>6,7</sup> Transcriptome analysis of whole lungs has been performed in this rabbit model of prematurity- and hyperoxia-induced lung damage and demonstrated dysregulations in 2217 genes. Major dysregulations were found in genes

involved in inflammation, lung development, vascular development and reactive oxygen species (ROS) metabolism.<sup>8</sup> Based on these data a pathway analysis predicted that simvastatin would reverse the dysregulation of several key genes involved in inflammation, vascular growth and lung development, such as IL8, chemokine CC-motif ligand 2 (CCL2), IL1B, VEGFA, angiopoietin 2 (ANGPT2) and secreted phosphoprotein (SPP1) (Supplemental Fig. S1). This prompted the hypothesis that simvastatin reduces certain aspects of hyperoxia-induced lung injury in preterm rabbits.

Simvastatin is a molecule of the statin family. Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and are used on a large scale for hypercholesterolemia. Their pleiotropic effects have made them popular for repurposing in many different fields of research. Also for respiratory conditions such as acute lung injury (ALI) and chronic obstructive pulmonary disease (COPD), observational studies and clinical trials have suggested beneficial effects of statins.<sup>9,10</sup> Preclinical evidence even suggests benefits in perinatal lung diseases such as congenital diaphragmatic hernia and persistent pulmonary hypertension of the newborn. More specifically, in animal models for both conditions treatment with simvastatin resulted in a marked effect on pulmonary artery remodeling.<sup>11,12</sup> Furthermore, in an adult hyperoxia-based mice model for ALI, simvastatin resulted in decreased lung inflammation and endothelial permeability.<sup>13</sup>

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In this study we tested if simvastatin attenuates hyperoxia-induced lung injury in the preterm rabbit model.

### METHODS

#### Animal protocol

Time-mated pregnant does (New Zealand White and Dendermonde hybrids) were obtained from the *animalium* of the group Biomedical Sciences at the KULeuven. The Ethics committee for Animal Experimentation of the Faculty of Medicine at KULeuven approved the experiments (P059/2016). All animals were treated according to current guidelines of animal wellbeing. Does were housed in separate cages before delivery, with free access to water and chow and a light-dark cycle of 12 h.

#### Cesarean section

To obtain preterm rabbit pups, cesarean sections were performed at 28 days of gestation (term = 31 days). Does were sedated by intramuscular ketamine (35 mg/kg; Nimatek®; Eurovet Animal Health BV, Bladel, The Netherlands) and xylazine (6 mg/kg; XYLM®; VMD, Arendonk, Belgium), placed in supine position and euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium and 5 mg tetracain hydrochloride (intravenous bolus of 1 ml T61; Intervet Belgium, Mechelen, Belgium). The abdomen was opened through a low midline abdominal incision and the pups were rapidly extracted after hysterotomy. At delivery, pups were dried, stimulated and placed in an incubator at 95% O<sub>2</sub>, 32 °C and 50% humidity.

#### Randomization and treatment

After 1 h, surviving pups were weighed and marked for inclusion in the experiment. Pups were randomly assigned to either normoxia (N; 21% O<sub>2</sub>) or hyperoxia (H; >95% O<sub>2</sub>) conditions, and within each condition to a group receiving 5 mg/kg of simvastatin per day (N + SS5 and H + SS5) or to a control group (N and H) (Fig. 1a). In a second round of experiments, a higher dose of

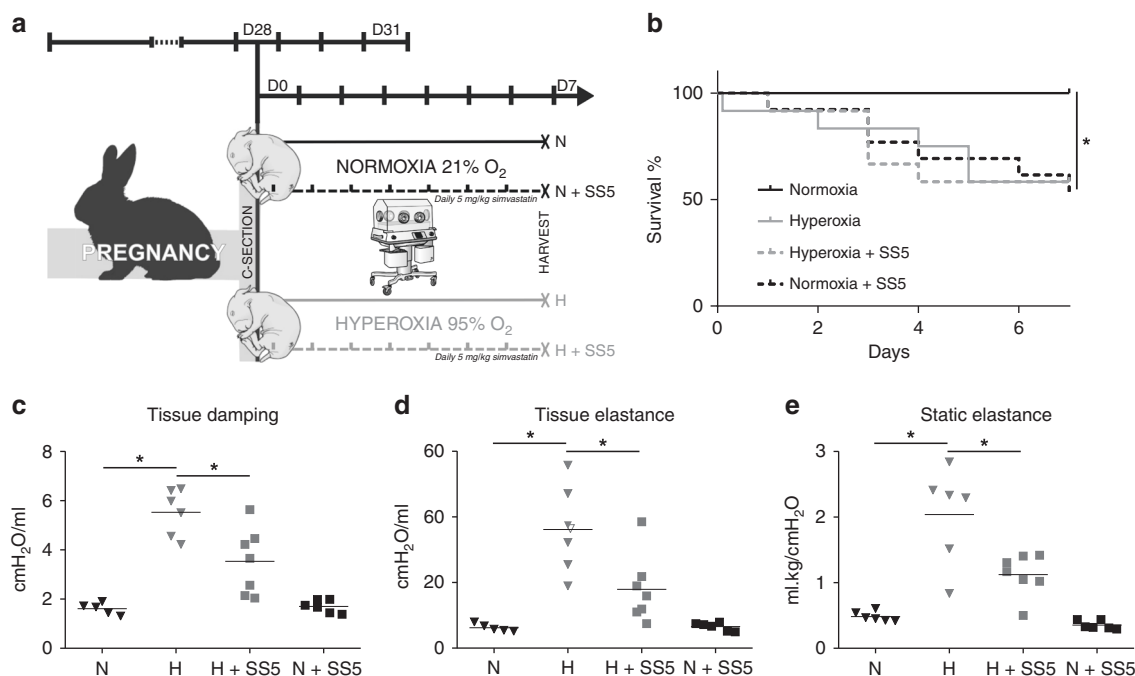
20 mg/kg of simvastatin per day was tested. In this experiment pups were randomized again in 4 groups (N, H, H + SS20, N + SS20) (Supplemental Fig. S6A). Randomization was performed in such a way that pups from the same litter were evenly distributed over the four groups. Treatment allocation was concealed for the investigator performing the randomization. In treated animals, simvastatin (Simvastatine EG; Eurogenerics, Brussels, Belgium) was mixed in the milk and administered once daily through orogastric gavage.

#### Neonatal rabbit care

Rabbit care was performed as previously described.<sup>7</sup> In both conditions pups were housed in an incubator controlling humidity at 50% and temperature at 32 °C. Pups were kept in the incubator for 7 days and only removed during maximum 5 min/day for feeding. Pups were fed twice a day with a 3.5 Fr orogastric tube placed just before feeding. The feeding consisted of a milk replacer (Day One, Protein 30%, Fat 50%; FoxValley; IL, USA) mixed with water according to the manufacturer's instructions. Probiotics, electrolytes and vitamins were added during the first 5 postnatal (PN) days (Bio-Lapis; Probiotics International Ltd.; Somerser, UK) and immunoglobulins during the first 2 PN days (Col-o-Cat, SanoBest; Hertogenbosch, The Netherlands). The amount of feeding steadily increased from 80 to 100 to 150 to finally 200 ml/kg day on PN days 0, 1, 2, and 3 (until 7), respectively. On PN day 2, vitamin K1 was administered intramuscularly (0.25 mg/kg, Konakion pediatrique; Roche, Basel, Switzerland), and from that point until harvest, pups were given a daily intramuscular injection of benzylpenicillin (20,000 IU/kg Penicilline®; Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg, Amukin®; Bristol-Myers-Squibb, Brussels, Belgium). After 7 days pups were harvested for functional and histological analysis.

#### Lung function

The FlexiVent system (FlexiVent 5.2; SCIREQ; Montreal, QC, Canada) was used to perform invasive lung function, as previously



**Fig. 1 Methodology, survival and lung function.** **a** At 28 days of gestation pups are delivered by cesarean section, and randomized to four groups ( $n = 7-13$ ): two groups are raised in normoxia (21% O<sub>2</sub>) and two are raised in hyperoxia (95% O<sub>2</sub>) with in both conditions a control group and a group treated with 5 mg/kg simvastatin per day. All pups were harvested at day 7. **b** Survival is significantly decreased by 5 mg/kg simvastatin in normoxia, but not in hyperoxia. **c-e** Lung function read-outs tissue damping, tissue elastance and static elastance at day 7 show a significant effect of simvastatin in hyperoxia. \* $p < 0.05$ .

described.<sup>7</sup> Pups were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (6 mg/kg), followed by tracheostomy with the insertion of an 18-gauge needle. Pups were ventilated in the supine position at 120 breaths/min and with a tidal volume of 8 ml/kg. The following perturbations were performed to obtain lung function parameters: inspiratory capacity, snapshot single frequency oscillation (dynamic compliance and elastance), prime-8 broadband frequency oscillation (airway resistance, tissue damping and tissue elastance) and a pressure-volume loop (static compliance and elastance). Measures were used if the coefficient of determination >0.95. The mean of three measurements per pup was calculated and used for analysis. Inspiratory capacity and static elastance and compliance were corrected for body weight.

#### Lung histology

After functional analysis deeply anesthetized pups were euthanized by exsanguination. The lungs and trachea were removed "en bloc" after thoracotomy. The right bronchus was ligated. Right lungs were snap-frozen for molecular analysis (see below). A 20G catheter was inserted into the trachea to fix the remaining left lung with 4% paraformaldehyde by a constant hydrostatic pressure of 25 cm H<sub>2</sub>O for 24 h in a purposely designed pump. Samples were subsequently embedded in paraffin, and 5 µm sections were made.

One slide for each lung was stained with hematoxylin and eosin, to assess the structure of the airspaces and lung inflammation. The mean linear intercept (Lm) and mean wall transection length (mean interalveolar septal wall thickness; Lmw) were obtained by point and intersection counting using an overlying grid of 64 points and 16 lines on 20 randomly selected lung fields (500 µm × 500 µm) using Stepanizer software.<sup>14</sup> Calculations were performed as described earlier.<sup>15</sup> To assess lung tissue inflammation, the acute lung injury score was used in 20 randomly selected fields (100 µm × 100 µm) per lung. This score was calculated by semi-quantitative analysis of neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, protein debris in the airspace and alveolar septal thickening as proposed by the American Thoracic Society.<sup>16</sup>

Vascular morphometry was performed on sections stained with Miller's elastic staining. In at least 20 peripheral arteries with an external diameter between 30 and 100 µm, the external and internal diameter (ED; ID) of the media layer on the shortest axis were measured. These parameters were used to calculate medial thickness (MT% = (ED - ID)/2ED × 100%), as previously described.<sup>15</sup>

Additionally, one slide per lung was stained for collagen with Sirius Red. The amount of collagen was estimated by measuring the stained area on 20 randomly selected lung fields (500 µm × 500 µm) relative to the whole field.

Finally, for every lung, two slides were used for endothelial and smooth muscle cell staining. Tissue preparation for immunohistochemistry included deparaffinization, heat retrieval, endogenous blockage of peroxidase (0.5% H<sub>2</sub>O<sub>2</sub> in methanol) and protein blockage. Smooth muscle cells and myofibroblasts were stained with a mouse anti-human α smooth muscle actin (αSMA) antibody at 0.71 µg/ml (M0851, DakoCytomation, Glostrup, Denmark). A secondary goat anti-mouse antibody conjugated with horseradish peroxidase (115-035-044, Jackson ImmunoResearch, Ely, UK) was used with aminoethyl carbazole as chromogen. In order to quantify muscularization of airways, the stained area in the wall of at least ten airways per lung was measured, and normalized to airway perimeter.<sup>17</sup> Furthermore, the αSMA-positive area in the parenchymal tissue was quantified on 20 randomly selected lung fields (250 µm × 250 µm) relative to the whole field and excluding fields with muscularized airways and vessels.

Endothelial cells were stained with a mouse anti-human CD31 antibody at 10.25 µg/ml (M0823, DakoCytomation). A secondary goat anti-mouse antibody (Z0420, DakoCytomation) was used in combination with an alkaline phosphatase anti-alkaline

phosphatase antibody (STAR67, Bio-Rad, Hercules, CA, USA) and nitroblue tetrazolium as a chromogen. During normal lung development, a double capillary layer in newly formed septa matures to a single capillary layer.<sup>18</sup> In order to quantify this on 2D images, at least 20 high power fields (100 µm × 100 µm) with septal tissue were randomly selected from each lung slide. The fraction of fields with a double capillary layer to the total number of fields was calculated as a parameter for microvascular (dis)maturation.

#### Quantification of gene expression by qPCR

RNA from snap-frozen rabbit lungs was extracted using the Tri-Pure Isolation reagent (Roche Diagnostics, Basel, Switzerland). RNA concentration was measured using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was assessed by checking the integrity of the 18S and 28S band on agarose gel electrophoresis. cDNA was synthesized with Taq Man® Reverse Transcription Reagents (Thermo Fisher Scientific). The Platinum® SYBR® Green qPCR Supermix-UDG with ROX (Thermo Fisher Scientific) was used to detect expression of IL8, CCL2, IL1B, VEGFA, VEGFR2, ANGPT2 and SPP1. A geometric mean of housekeeping genes *YWHAZ*, *HPRT* and *ACTB* was used to normalize mRNA levels. An overview of used primers (Integrated DNA Technologies, Haasrode, Belgium) is provided in Supplemental Table S2. Samples were run in triplicate on a StepOne Plus instrument (Thermo Fisher Scientific). Relative quantitation was determined using the comparative Ct method. Statistics were calculated on  $\Delta\Delta C_t$ -values, while fold change (FC) was used for visualization.

#### Statistical analysis

All statistical analyses were done using GraphPad Prism 7.0 software (GraphPad, La Jolla, California, USA). Kaplan–Meier curves with post hoc log-rank testing were used to quantify postnatal survival of rabbit pups. One-way ANOVA was used to compare read-outs between groups. Post hoc Bonferroni-Sidak multiple comparisons were used to compare H to N, H + SS5 to H and N + SS5 to N. Normality was assumed after visual inspection of the data. In the analysis of MT%, one outlier was identified using the Grubbs' test and excluded from the analysis. A *p* value of <0.05 was considered statistically significant. All values are expressed as mean ± standard deviation.

Sample size calculation was performed using GPower 3.1, based on historical data of hyperoxia versus normoxia exposed rabbit pups.<sup>7</sup> We defined tissue damping as the primary outcome measure. To pick-up a 50% correction of the effect of hyperoxia on G, with a power of 80% and an  $\alpha$  of 0.0167 (Bonferroni correction for three comparisons) in a two-tailed *t* test, seven surviving pups in each group were needed.

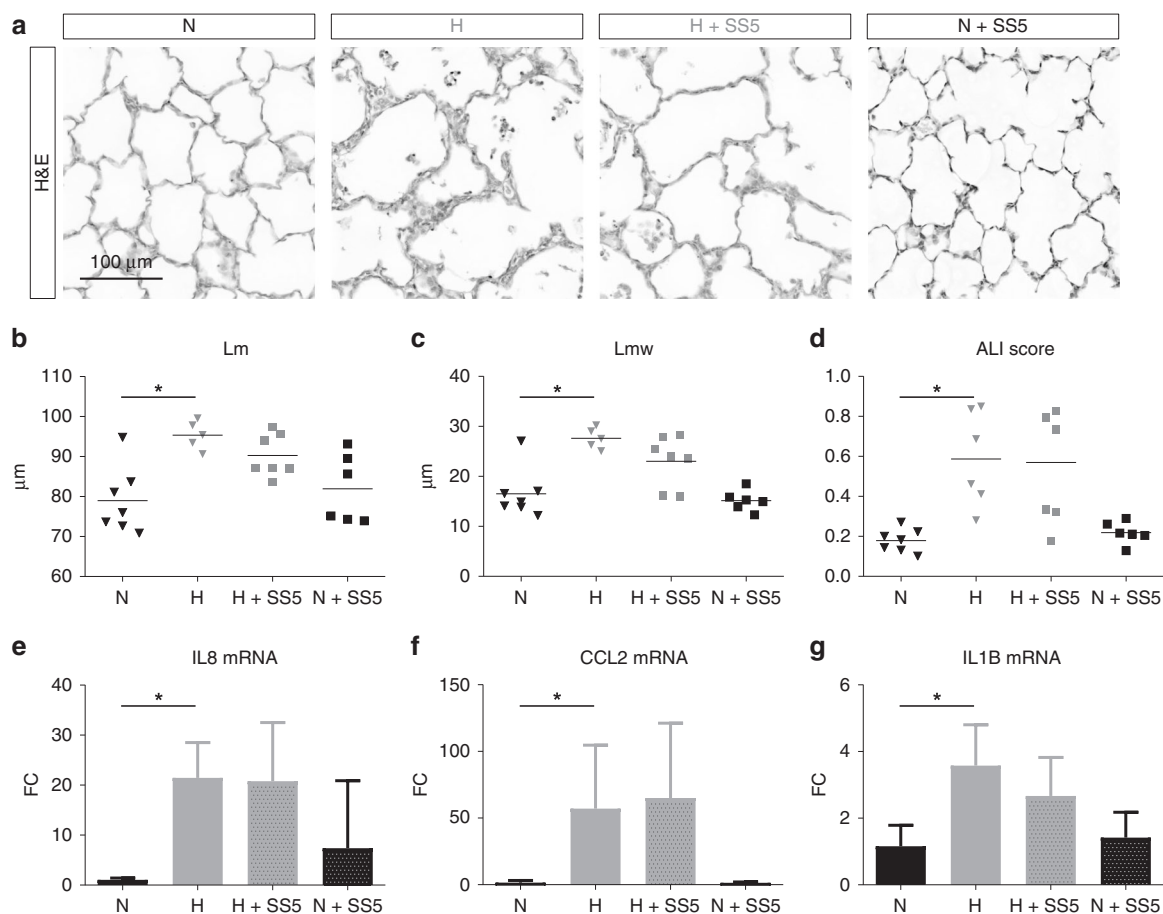
## RESULTS

Simvastatin 5 mg/kg affects survival in normoxia, but not in hyperoxia

A total number of 44 pups (from 8 mothers) were randomized. There were no significant differences in birth weight between the groups (Supplemental Table S3). Of these pups, 29 survived until day 7. There was a trend towards decreased survival in hyperoxia (H) versus normoxia (N; *p* = 0.06). Daily simvastatin administration at 5 mg/kg did not alter survival in H; however, it did reduce survival in N (*p* = 0.04) (Fig. 1b). Weight gain was not significantly different among the groups (Supplemental Table S3).

Simvastatin 5 mg/kg improves lung function in hyperoxia

Tissue damping and tissue elastance were increased in H (versus N; both *p* < 0.0001). Simvastatin (5 mg/kg) however reduced tissue damping and elastance in hyperoxia (H + SS5 versus H; respectively *p* = 0.002 and *p* = 0.004), but not in normoxia (N + SS5 versus N) (Fig. 1c, d).



**Fig. 2 Lung morphology and inflammation.** **a** Representative pictures of HE-stained lung slides depict altered alveolar structure and inflammation in hyperoxia, which is not prevented by 5 mg/kg simvastatin. **b** Lm (mean linear intercept) and **c** Lmw (mean transectional wall length) reflect alveolar structure and are not significantly affected by 5 mg/kg simvastatin. **d** ALI score is a composite score of septal thickening, neutrophil invasion and exudates, protein debris and reflects lung injury or inflammation, and is not affected by 5 mg/kg simvastatin. **e–g** Expression of IL8, CCL2, IL1B as measured by qPCR is increased in hyperoxia, but not affected by 5 mg/kg simvastatin ( $n = 5–7$ ). \* $p < 0.05$ .

Comparably, static elastance was increased in H (versus N;  $p < 0.0001$ ), and this effect was partially prevented by 5 mg/kg simvastatin (H + SS5 versus H;  $p = 0.001$ ). Simvastatin had no significant effect in normoxia on static elastance (Fig. 1e). Furthermore, inspiratory capacity was decreased in H (versus N;  $p < 0.0001$ ). In N + SS5 (versus N), a nonsignificant trend towards increased inspiratory capacity in 5 mg/kg simvastatin-treated pups was observed ( $p = 0.06$ ). This is in line with static compliance being increased in N + SS5 (versus N;  $p = 0.007$ ), while clearly decreased in H (versus N;  $p < 0.0001$ ) (Supplemental Table S4).

Observations on dynamic compliance and elastance in the different subgroups are in parallel with their static counterparts; however, 5 mg/kg simvastatin only affected these dynamic lung function measures in hyperoxia, not in normoxia. Airway resistance was neither affected by hyperoxia, nor 5 mg/kg simvastatin (Supplemental Table S4).

Simvastatin 5 mg/kg did not alter alveolar structure or inflammation

Exposure to hyperoxia resulted in a higher mean linear intercept (Lm; H versus N;  $p = 0.002$ ) and a higher mean transectional wall length (Lmw, H versus N;  $p = 0.0004$ ). Simvastatin (5 mg/kg) did not affect alveolar morphometry significantly (H + SS5 versus H;  $p = 0.53$  and  $p = 0.17$  for Lm and Lmw respectively; Fig. 2a–c). An increased ALI score, suggesting lung inflammation, was observed in H (versus N;  $p = 0.002$ ). Neither in normoxia, nor in hyperoxia, was the ALI score altered in the 5 mg/kg treated pups (Fig. 2a, d).

Similarly, high mRNA-expression of the proinflammatory cytokines IL8, CCL2 and IL1B is observed in H (versus N; respectively  $p < 0.0001$ ,  $p < 0.0001$  and  $p = 0.004$ ). Increased expression of these cytokines is not prevented by 5 mg/kg simvastatin, nor is their expression affected in normoxia (Fig. 2e–g).

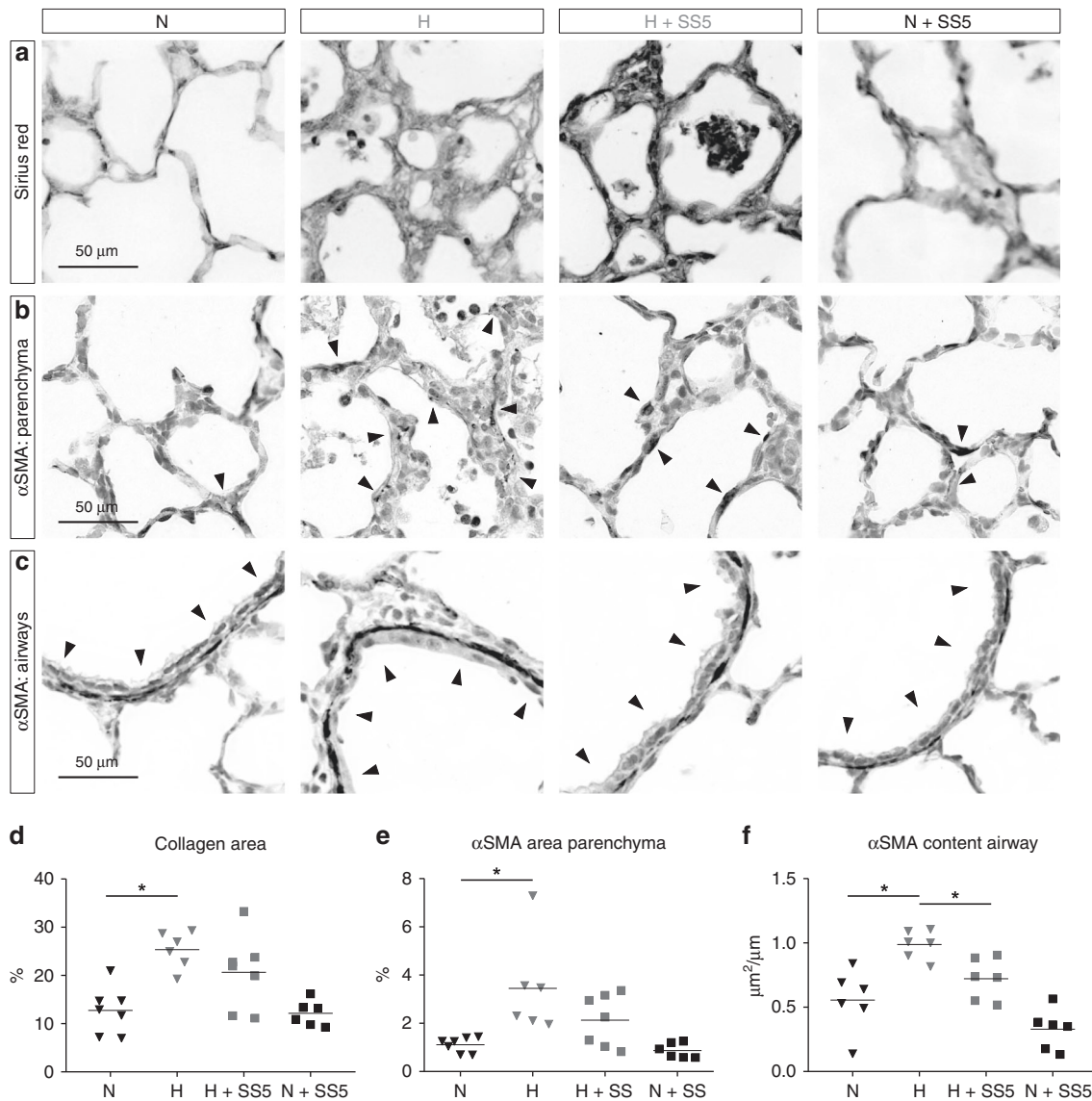
Simvastatin 5 mg/kg decreases the smooth muscle cell content of airway walls in hyperoxia

Hyperoxia exposure resulted in an increased collagen content as quantified by Sirius-staining (H versus N;  $p = 0.0008$ ). In parallel, it also resulted in an increased area stained for  $\alpha$ SMA in the parenchymal tissue, reflecting a higher number of myofibroblasts and smooth muscle cells (H versus N;  $p = 0.003$ ). This evolution to a fibrotic phenotype was not significantly prevented by 5 mg/kg simvastatin (H + SS5 versus H;  $p = 0.13$ ). Simvastatin (5 mg/kg) did not alter collagen or  $\alpha$ SMA content in normoxia (Fig. 3a, b, d, e).

Finally, we observed a thicker ( $\alpha$ SMA-positive) smooth muscle cell layer in airway walls in H (versus N;  $p = 0.001$ ), which was significantly reduced in H + SS5 (versus H;  $p = 0.04$ ). Again, there was no significant effect of simvastatin in normoxia (N + SS5 versus N;  $p = 0.10$ ; Fig. 3c, f).

Simvastatin 5 mg/kg prevents vascular effects of hyperoxia

Hyperoxia increased the thickness of the medial layer of the pulmonary arteries (MT%) significantly (H versus N;  $p = 0.0002$ ). Administration of 5 mg/kg simvastatin resulted in a complete prevention of the effect of hyperoxia (H + SS5 versus H;  $p = 0.004$ ).



**Fig. 3 Collagen and αSMA quantification.** **a** Representative pictures of Sirius Red-stained lung slides showing collagen deposition in red, quantified in pane (**d**). **b** Representative pictures of αSMA-stained lung slides showing brown αSMA-positive areas in septal tissue (myofibroblasts or pericytes, indicated by arrows), quantified in pane (**e**). **c** Representative pictures of αSMA-stained lung slides showing a brown αSMA-positive layer in airway walls, quantified in pane (**f**). **d** Quantification of Sirius Red-positive area demonstrates increased collagen deposition in hyperoxia, not significantly affected by 5 mg/kg simvastatin. **e** Quantification of αSMA-positive area in parenchymal tissue indicates more positivity in hyperoxia, not significantly affected by 5 mg/kg simvastatin. **f** Quantification of αSMA-positivity in airway walls indicates an increased smooth muscle cell layer in hyperoxia, significantly prevented by 5 mg/kg simvastatin. \* $p < 0.05$ .

There was no effect of 5 mg/kg simvastatin in normoxia (Fig. 4a, c). Hyperoxia also affected microvascular maturation, quantified by an increased number of septations with a double capillary layer in H (versus N;  $p = 0.02$ ). Simvastatin (5 mg/kg), however, did not alter pulmonary capillary conformation, in contrast to arterial medial thickness (Fig. 4b, d).

The vascular effects of hyperoxia are associated to a decrease in VEGFA- and VEGFR2-mRNA in lung tissue (H versus N; both  $p < 0.0001$ ). Simvastatin (5 mg/kg) partially prevented this decrease in hyperoxia (H + SS5 versus H;  $p = 0.03$  and  $0.04$  for VEGFA and VEGFR2, respectively). There was again no effect of simvastatin in normoxia (Fig. 4e, f).

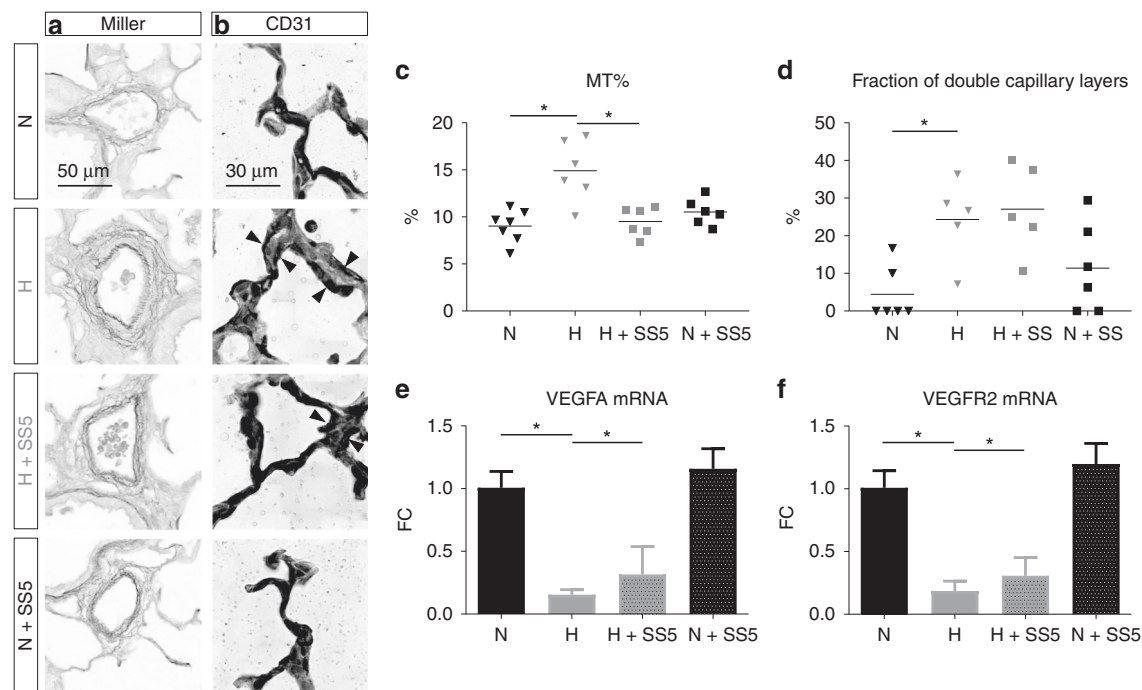
#### Simvastatin 20 mg/kg results in high mortality

A total of 62 pups (from 7 mothers) was included in this experiment. There were no significant differences in birth weight (Supplemental Table S5). Survival was significantly and

importantly decreased by 20 mg/kg of simvastatin in both hyperoxia and normoxia ( $p = 0.006$  and  $p = 0.0003$  respectively, Supplemental Fig. S6B). Due to the high mortality, the outcome results of this high-dose experiment are underpowered. In general, however we see the same trends occurring on lung function and medial thickness in the pups treated with 20 mg/kg (Supplemental Fig. S6C–H). In contrast to the findings in 5 mg/kg, 20 mg/kg simvastatin had a significant effect on acute lung injury scores (reflecting tissue inflammation), and might decrease expression of IL8 and CCL2 mRNA (however not significantly at  $n = 4–7$ ; Supplemental Fig. S6I–K).

#### DISCUSSION

In this study we tested the hypothesis that simvastatin prevents hyperoxia-induced lung injury in preterm rabbits. It is the first to explore statin therapy in a BPD context, but builds further on



**Fig. 4 Vascular morphology and VEGF expression** **a** Representative pictures of lung slides stained with Miller's elastic staining showing the tunica media of lung arteries in between the lamina elastica interna and externa, quantified in pane (c). **b** Representative pictures of CD31-stained lung slides showing brown capillaries in interalveolar septa, double capillary layers are indicated with arrows and quantified in pane (d). **c** Thickness of the tunica media (MT%) of lung arteries increases in hyperoxia and almost completely normalizes with 5 mg/kg simvastatin. **d** Hyperoxia exposure leads to an increased amount of double capillary layers suggesting delayed microvascular maturation, which is not prevented by 5 mg/kg simvastatin. **e, f** Expression of VEGFA, VEGFR2 as measured by qPCR is decreased in hyperoxia, and partially restored by 5 mg/kg simvastatin ( $n = 5-7$ ).  $*p < 0.05$ .

previous reports on the beneficial effects of simvastatin on developmental lung diseases such as neonatal pulmonary hypertension and congenital diaphragmatic hernia.<sup>11,12</sup> We observed a significant improvement of our primary outcome (tissue damping) in the hyperoxia-exposed animals treated with simvastatin. This was associated with similar beneficial effects on elastance and compliance. Improvements in lung function by simvastatin have also been found in other models for lung injury (induced by mechanical ventilation or cigarette smoke<sup>19,20</sup>). Unlike these studies we could not demonstrate a reduction of lung inflammation and epithelial injury which can explain the functional effect at 5 mg/kg. At a higher dose of 20 mg/kg however, simvastatin might also inhibit lung inflammation. The lung function improvements were neither associated to improved alveolar structure, but a trend was noted towards a milder fibrotic phenotype (thinner septations, less collagen and less  $\alpha$ -SMA). This is in line with findings in adult lung fibrosis models, demonstrating antifibrotic effects of simvastatin<sup>21,22</sup> and clinical studies on idiopathic pulmonary fibrosis, suggesting a role for adjuvant statin therapy.<sup>23</sup>

Additionally, there was a significantly thinner smooth muscle cell layer in conductive airways of treated animals. This suggests an effect on airway remodeling, as also demonstrated in experiments with simvastatin in asthma models.<sup>24</sup> Obstructive airway disease remains an important consequence of BPD and this is thus particularly interesting.<sup>1</sup> A limitation of the preterm rabbit model in this regard is the absence of increased airway resistance in (unstimulated) hyperoxia-exposed animals. Future perspectives include the evaluation of airway hyperreactivity with methacholine in the preterm rabbit model, and eventually the effect of simvastatin on this aspect.

Most importantly, we observed a complete normalization of arterial wall hyperplasia (MT%), which is a histologic sign of pulmonary hypertension.<sup>7</sup> Pulmonary vascular disease is an

important characteristic of BPD, with pulmonary hypertension present in 17% of patients and being a strong risk factor for increased mortality.<sup>25,26</sup> In the other direction, pulmonary vascular disease is also crucial for alveolar developmental arrest, which is described as the hallmark of BPD.<sup>27</sup> As developmental lung diseases in general are the result of an interplay between parenchymal and vascular maldevelopment, repurposing of simvastatin could be an interesting strategy for this group of diseases. Similar remodeling effects of simvastatin on the arterial wall have been observed in models for congenital diaphragmatic hernia<sup>11</sup> and persistent pulmonary hypertension of the newborn.<sup>12</sup> In our experiment the decrease in medial thickness by simvastatin was associated with a partial recovery in VEGFA and VEGFR2-mRNA levels, however in the absence of an effect on microvascular maturation. The exact role of VEGFA in arterial wall remodeling still needs to be elicited.

Based on our previously performed transcriptome analysis (Supplemental Fig. S1), we anticipated that simvastatin would work by normalizing gene transcription of key genes, such as IL8, CCL2, IL1B (inflammation), VEGFA, ANGPT2 (vascular growth) and SPP1 (lung development). For all these, we validated with qPCR, the effect of hyperoxia on gene transcription, as measured by mRNA-seq (Figs. 2e-g, 4e, Supplemental Figs. S6J-K, S7<sup>8</sup>). Nevertheless, except for VEGFA, the prediction that simvastatin would prevent the up- or downregulation of these transcripts in hyperoxia was not confirmed. Two possible reasons for this discrepancy can be identified. First, these predictions are based on literature data on various cell types from different species in many settings, using many different doses. They might be invalid in neonatal rabbit lungs tested *in vivo*. Second, mRNA-isolates from whole lung tissue are analyzed, which might mask key dysregulations in specific cell types (e.g. myofibroblasts). Transcriptome analysis (with upstream regulator analysis) is a useful hypothesis-generating method, but these limitations should be taken into account.<sup>28</sup>

The pleiotropic effects of statins have generally been attributed to alteration of posttranslational protein modification.<sup>29</sup> Statins are inhibitors of HMG CoA reductase, the rate-limiting enzyme of the mevalonate pathway. They thereby also decrease the synthesis of isoprenoid intermediates, which are linked to proteins to regulate their function (posttranslational protein prenylation).<sup>30</sup> It has been shown that simvastatin inhibits proliferation, and synthesis of extracellular matrix proteins in airway smooth muscle cells through inhibition of prenylation of RhoA.<sup>31,32</sup> In vascular smooth muscle cells for instance it attenuates proliferation, induces apoptosis, and also alters contractility.<sup>33–35</sup> Lung fibroblast to myofibroblast differentiation is limited by statins (decreased  $\alpha$ SMA-expression, decreased production of collagen) through the same pathway.<sup>36,37</sup> These RhoA-mediated effects might explain the observed phenotype of improved lung function, trends towards a decreased collagen and  $\alpha$ SMA content, with normalization of arterial medial thickness and airway smooth muscle content.

The translational value of our findings is however limited by the survival data in this experiment, especially in the 20 mg/kg, but also in the 5 mg/kg experiments. This contrasts findings in other animal models such as mice and rats.<sup>11,12</sup> In a small explorative analysis of liver, muscle and serum samples of treated rabbit pups we could not identify a clear reason for this mortality. We could see some de- and regenerating muscle fibers in quadriceps muscles of surviving pups treated with simvastatin for 3 days; however, the relevance of this finding is unknown. It is definitely a limitation of our study that we could not identify a clear reason for this increased mortality. The most well-known side effect of simvastatin is rhabdomyolysis, which has also been described in adult rabbits, although at higher doses.<sup>38</sup> Furthermore, rabbits are known to be susceptible to centrilobular hepatic necrosis due to exposure to simvastatin.<sup>39</sup> It is not known whether neonates are more susceptible to these side effects, because not surprisingly, there are no data on the use of simvastatin in human neonates. Anyway doses of 5–20 mg/kg are much higher than what is currently used for cholesterol lowering (10–80 mg for an adult patient). Our findings are relevant step in the translation towards clinical application, but warrant further investigations on the safety profile of simvastatin at these high doses.

The major limitation of our study is the lack of in-depth molecular data (such as Western blot) on the exact working mechanism of simvastatin. This relates to the limited availability of reagents, inherent to the rabbit model. A second limitation is the use of enteral simvastatin, for which there is limited pharmacokinetic data in rabbits. Third, we only assessed pulmonary hypertension by arterial medial thickness. Direct right ventricular pressure measurements or longer term experiments showing right ventricular hypertrophy could strengthen our findings. Finally, unlike during data collection and analysis of the outcome parameters, researchers were not blinded to treatment allocation during rabbit care (because of the different feeding regimen).

In conclusion, our results suggest repurposing of simvastatin could be an advantageous therapeutic strategy for bronchopulmonary dysplasia, and maybe other developmental lung diseases for which pulmonary vascular disease is involved in the pathogenesis. Under the current form however it is not easily translatable. Future perspectives should include the evaluation of more local and targeted (simva)statin therapy, omitting the observed mortality.<sup>40</sup> Furthermore, a better identification of the working mechanism of the pleiotropic effects of simvastatin in this condition could result in a more targeted therapeutic strategy (e.g. direct RhoA-inhibitor with a better safety profile).

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## AUTHOR CONTRIBUTIONS

All authors fulfill the journal's author requirements. T.S. and J.J. conceived, designed and performed the main experiments. T.S. and B.T. had a substantial contribution in acquisition, interpretation and analysis of the main data. A.G., F.L., D.d.W. and N.B. had a substantial contribution in the acquisition and analysis of several explorative outcome measures. T.S., A.G., K.A., J.D. and J.T. drafted and revised the article. All authors approved the final version before submission.

## ADDITIONAL INFORMATION

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