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TOWARDS NEW THERAPIES FOR BRONCHOPULMONARY DYSPLASIA

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Questions of science, science and progress
Do not speak as loud as my heart.
Coldplay (The Scientist)

To Sandra.

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List of abbreviations

^{18}F-FDG	2-deoxy-2-(18F)fluoro-D-glucose
2D	two dimensional
3D	three dimensional
αSMA	α smooth muscle actin
ACE	angiotensin I converting enzyme
ADR	adverse drug reaction
AE	adverse event
ALI	acute lung injury
ANGPT2	angiopoietin 2
BPD	bronchopulmonary dysplasia
BSID	Bailey Scale of Infant Development
CAV1	caveolin1
CCL2	(C-C motif) ligand 2
cDNA	copied deoxyribonucleic acid
COPD	chronic obstructive pulmonary disease
COV	coefficient of variation
COX2	cyclo-oxygenase 2
CPAP	continuous positive airway pressure
CSF3	granulocyte colony stimulating factor
CTCAE	Common Terminology Criteria for Adverse Events
ANGPT2	angiopoietin 2
BPD	bronchopulmonary dysplasia
BSID	Bailey Scale of Infant Development
CAV1	caveolin1
CCL2	(C-C motif) ligand 2
cDNA	copied deoxyribonucleic acid
COPD	chronic obstructive pulmonary disease
COV	coefficient of variation
COX2	cyclo-oxygenase 2
CPAP	continuous positive airway pressure
CSF3	granulocyte colony stimulating factor
CTCAE	Common Terminology Criteria for Adverse Events
CXCL8	(C-X-C motif) ligand 8 or interleukin 8
CYP1A1	cytochrome P450 1A1
DKK1	dickkopf WNT signaling pathway inhibitor 1
ED	external diameter
ELBW	extremely low birth weight
ENaC	epithelial sodium channel

FC	fold change
FDR	false discovery rate
FEV	forced expiratory volume
FIJI	FIJI is just ImageJ
FiO2	fraction of inspired oxygen
GA	gestational age
G-CSF	granulocyte colony stimulating factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HU	Hounsfield Unit
ICC	Intraclass Correlation Coefficient
ID	internal diameter
IFNγ	interferon γ
IGF1	insulin-like growth factor 1
IL1α	interleukin 1α
IL1β	interleukin 1β
IL8	interleukin 8
IMP	investigational medicinal product
INC	International Neonatal Consortium
IPA	Ingenuity Pathway Analysis
IT	intratracheal
IUGR	intrauterine growth retardation
KLF2	Kruppel-like factor 2
LAIF	Lipase Added to Infant Feeding
LLT	lowest level term
Lm	mean linear intercept
Lma	mean linear intercept of the airspaces
Lmw	mean transsectional wall length
MedDRA	Medical Dictionnary for Regulatory Activities
microCT	micro computed tomography
microPET-CT	micro positron emission tomography combined with CT
mRNA	messenger ribonucleic acid
mRNA-seq	mRNA sequencing
MSSO	MedDRA Maintenance and Support Services Organization
MT%	relative (arterial) medial thickness
NAESS	neonatal adverse event severity scale
NCI	National Cancer Institute
nCPAP	nasal continuous positive airway pressure
NEOPROP	Exploratory Propofol Dose Finding Study In Neonates
NFE2L2	nuclear factor erythroid 2-like 2
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NICHD	National Institute of Child Health and Human Development
NICU	neonatal intensive care unit
NIRTURE	Neonatal Insuline Replacement Therapy in Europe
NOS2	nitric oxide synthase 2
PAAT	pulmonary artery acceleration time

PAET	pulmonary artery ejection time
PedsQL	pediatric quality of life scale
PPARγ	peroxisome proliferator-activated receptor γ
PTGS2	cyclo-oxygenase 2 (xxx)
qPCR	quantitative polymerase chain reaction
RAC	radial alveolar count
RDS	respiratory distress syndrome
RhoA	Ras homolog gene family, member A
ROP	Retinopathy of prematurity
ROS	reactive oxygen species
RPKM	reads per kilobase per million reads
S	surface (total alveolar surface)
SAVES-2	spinal adverse event severity system
SOD2	superoxide dismutase 2
SPP1	secreted phosphoprotein 1
SRXN1	sulfiredoxin 1
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TIE2	TEK tyrosine kinase
TNFα	tumor necrosis factor-alfa
TXNRD1	thioredoxin reductase 1
VEGF	vascular endothelial growth factor
VEGFA	vascular endothelial growth factor A
VEGFR2	VEGF receptor 2
VLBW	very low birth weight
VVOC	Vlaamse vereniging voor ouders van couveusekinderen
V_{vsep}	volume density of alveolar septa
wt	weight
YWHAZ	14-3-3 protein zeta/delta
VLBW	very low birth weight



De Formato Foetu – Spigelius (1624):
one of the first known descriptions of fetal and neonatal anatomy

Introduction

Chapter adapted from:
Salaets T, Gie A, Tack B, Deprest J, Toelen J. Modelling Bronchopulmonary Dysplasia in Animals: Arguments for the Preterm Rabbit Model. Curr Pharm Des. 2017;23(38):5887-5901. (1)

New therapies for bronchopulmonary dysplasia are needed

Fifty years ago Northway was the first to describe fibrotic changes in the lungs of preterm infants who could only be kept alive with aggressive mechanical ventilation and supraphysiological oxygen administration (2). He referred to this disease as bronchopulmonary dysplasia (BPD). Even though advances in perinatal medicine have increased the survival of ever more premature infants, these were unable to reduce the incidence of long-term respiratory morbidity. At present 45% of survivors of extremely preterm birth (<28 weeks of gestation) still develop BPD (3). The most recent consensus definition is based on the need for supplementary oxygen or respiratory support on 36 weeks postmenstrual age (4). Despite doubts regarding the predictive value of any of these definitions, BPD continues to be associated with disabling consequences throughout life. It is linked with respiratory morbidity (lung function abnormalities, increased episodes of wheezing and frequent hospital admissions), pulmonary hypertension (which is associated with a mortality rate up to 38% (5, 6)), and even abnormal neurological development (7, 8). Also from a health economics perspective, BPD remains an important problem (9). When you count the economic effects over a lifetime of six to seven decades, even a small impact on an individual's health can severely impact his or her contribution for or dependency on society. As the current clinical care fails to prevent BPD, the development of effective preventive and therapeutic strategies remains imperative.

Current preventive strategies for BPD focus on reducing lung injury, by using less invasive support measures (such as CPAP)(10) and titrating oxygen exposure to saturations of 91-95%(11). In current clinical care there is less attention for pharmacological prevention of BPD. Jensen et al. have reviewed the evidence of the current pharmacological strategies in 2015 (12). There is high-quality evidence that vitamin A (13) and caffeine (14) reduce BPD, but their effect size is rather limited (number needed to treat of 11,5 and 9,5 respectively). Systemic corticosteroids also lower the incidence of BPD, but serious concerns exist on their safety profile. Recently however, a number of clinical trials have shown success with local delivery (15, 16) and low dose regimens (17, 18) of steroids. However, the uncertainty about their safety remains as for instance inhaled budesonide resulted in a trend towards increased mortality (15). The most promising form of local corticosteroids seems to be intratracheal administration of a surfactant plus budesonide mixture(16).

Only a few novel medicinal products are being investigated. Firstly, stem cells are one of them. The preclinical data on different types of stem cells for BPD are promising (19), as are the data on exosomes (20). In South-Korea, a clinical trial in which mesenchymal stem cells are administered intratracheally is currently ongoing (21). Many questions however remain on their safety profile, production (batch to batch variation, …) and the need for immunosuppression (22). Second, in a trial where recombinant insulin-like growth factor 1 (IGF1) was given to preterm neonates with the primary aim to reduce the incidence of retinopathy of prematurity the researchers noticed a significant reduction in BPD (Ley et al., abstract presented at the European Academy of Pediatric Societies 2016 congress). Since then, research interest in IGF1 for neonatal respiratory purposes has certainly increased, however without confirmatory trials until now. Besides those 2 targets, no other therapy candidates are close to clinical translation.

Appropriate animal models for BPD are needed

In order to develop innovative therapeutic strategies to improve the chronic respiratory outcome of survivors of preterm birth, the knowledge on BPD pathophysiology needs to increase. On this point the limitations of exploratory research on humans become clear. Research on biomaterial of human cases is limited to what can be easily obtained: mostly serum (23, 24) or tracheal aspirates, broncho-alveolar lavage fluid or exhaled air (25-27). While studies on lung tissue of patients with BPD would be able to provide important information, they are understandably rare (especially in the post surfactant era) and mostly represent the lethal end of the disease spectrum (28-31). Even though they are interesting for biomarker identification (32) and to generate new hypotheses, studies on human material often do not prove causality. Therefore, researchers use animal models as they provide the ability to use a plethora of experimental techniques to measure, control and influence relevant factors. A good animal model can increase the understanding of pathophysiology of a disease and can enable the development of novel therapeutic strategies.

However, the translation of therapeutic findings in animal models have been proven difficult in the past (33). There is a need to improve our BPD models, mimicking both the pathophysiology and phenotype of the disease.

Pathophysiology of BPD

BPD is a unique disease as it is not defined by its pathophysiology but by its management. It is no wonder that this invokes increasing critique in this age of 'omics' and systems biology (34). The phenotype of BPD is very heterogeneous and reflects the complex interplay of individually varying insults on the developing lung, which goes through a critical adaptation process (35). Additionally the phenotype and pathophysiology of BPD has changed significantly over time. Where the so-called 'old BPD' was a fibrotic disease, occurring in moderately preterm infants exposed to aggressive respiratory support, 'new BPD' is described as a developmental arrest, that occurs in extremely preterm infants benefiting from antenatal steroids, surfactant and minimal invasive supportive measures (36). This spectrum continues to evolve, in parallel with recent advances in perinatal care. In the following paragraphs we will, based on recent data, review the factors that are involved in the etiology of BPD, as encountered by neonatologists today. These factors could be used as insults to create experimental BPD in animal models.

Prematurity

The primary and essential factor in the development of human BPD is premature birth. The incidence of BPD depends strongly on gestational age and birth weight. A recent follow-up study revealed an incidence of about 75% in a population of periviable infants (survivors of birth at 22-24 weeks of gestation) (37). Another dataset revealed an incidence of moderate to severe BPD in 69% of infants born in the 24th week of gestation, while it only occurred in 24% of the infants born in the 28th week (3). In general, the incidence of BPD increases with decreasing gestational age and birth weight (36, 38).

Babies at the highest risk for BPD (22-28 weeks of gestation) are born in the late canalicular to early saccular stage of lung development (Figure 1.1) (39). While in healthy pregnancies the saccular stage proceeds into alveolarization in utero, both pre- and postnatal factors disturb this developmental program in preterm birth. It thus seems sensible to favor an animal model in which birth occurs in the same late canalicular to early saccular stage of lung development.

Besides structural prematurity of the lung, it is logical to also take functional prematurity into account. For example, the surfactant system develops during the saccular stage of lung development, lowering alveolar surface

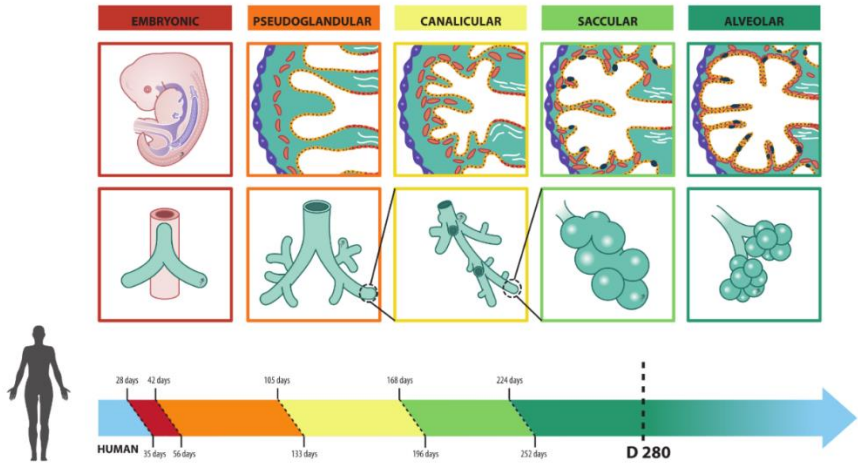


Figure 1.1. Stages of lung development in humans. *In the embryonic phase budding of the primitive lung occurs from the foregut. This buds expands to a bronchial tree in the pseudoglandular phase. In the canalicular phase the primitive lung parenchyma branches out of the bronchioles, evolving to saccular structures in the saccular phase, divided by primary septa. In the alveolar phase secondary septation occurs increasing the surface available for gas exchange. Humans start alveolarization before term birth around day 280. Babies at risk of BPD (22-28 weeks of gestation) are born in the late canalicular to early saccular stage.*

tension and thus facilitating expansion of the lung at birth. Preterm neonates are thus at birth at least partially deficient for surfactant, and therefore often exhibit signs of respiratory distress or failure (so called respiratory distress syndrome (RDS)) (40). Below we will also discuss the antioxidant defense enzymes, which mature in parallel with the surfactant system, as do probably many other pathways, to prepare a fetus for its pulmonary adaptation at term birth. The lack of this preparation in the preterm neonate, requires endogenous adaptive strategies and medical interventions (antenatal steroids, surfactant administration and respiratory support), which allow for survival by facilitating the transition to postnatal life. However this process alters the normal development of the lung at longer term. An interesting concept is to look at BPD as the adaptation of a functionally preterm lung to the new reality of postnatal life (35). This adaptation seems especially crucial in new BPD, where additional iatrogenic insults are often non-aggressive and tapered to individual needs, but many preterm birth survivors still develop BPD. We therefore hypothesize that the preterm animal models, as they allow to study the long term respiratory consequences of preterm birth, have an important translational advantage.

Insults to the developing lung

Superimposed on this adaptation of the preterm lung to postnatal life, a variety of endogenous and exogenous factors interferes with lung development through injury and consecutive repair (summarized in Figure 1.2) (35). A complete review of BPD related noxiae is beyond the scope of this text and we will limit ourselves to the major contributors.

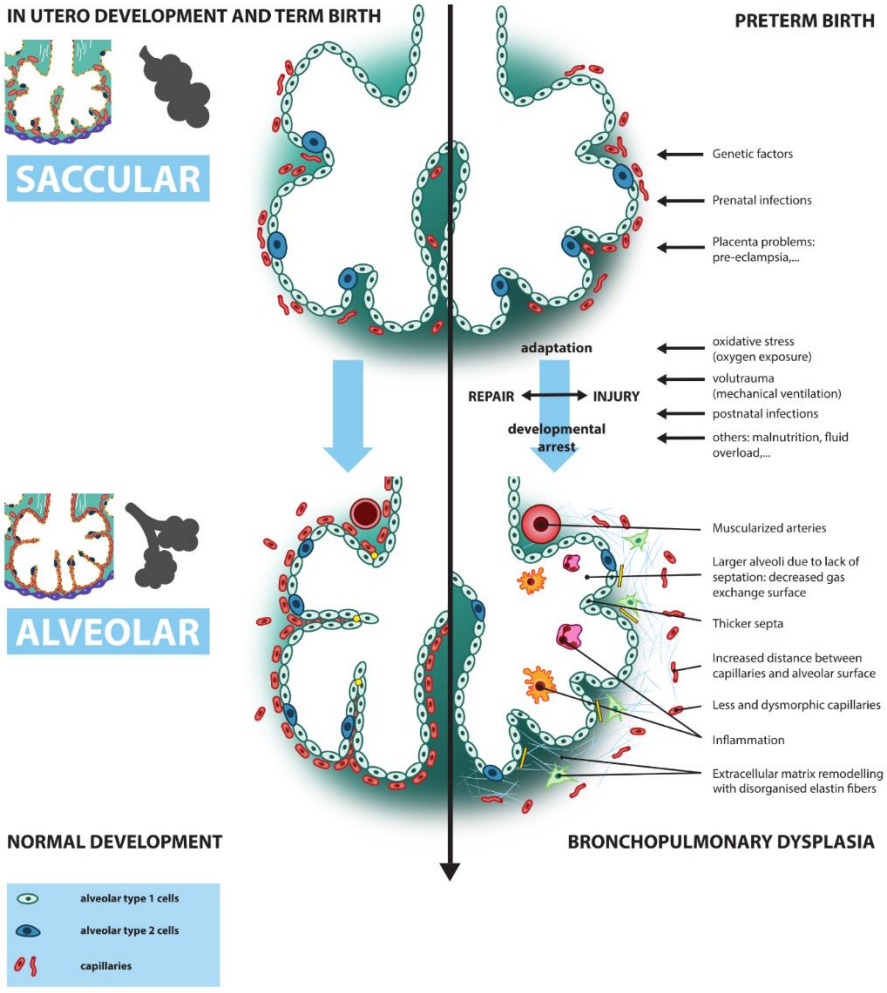


Figure 1.2: Pathophysiology of human BPD. *Schematic overview of the factors involved in chronic lung disease after preterm birth and schematic representation of BPD histology.*

Oxygen

Signs of protein and lipid peroxidation have been noted in exhaled gases and broncho-alveolar lavage fluid from the lungs of ventilated preterm neonates,

with changes being most pronounced in those developing BPD (41-43). This form of injury can in the preterm neonate be seen as the result of an imbalance between antioxidant defense mechanisms on the one hand and on the other hand reactive oxygen species (ROS) overproduction due to hyperoxia, mechanical ventilation and infection.

During prenatal life the oxygen exposure in the lungs is low. Oxygenated blood enters the fetal circulation through the umbilical vein delivering a partial oxygen pressure of about 20 mmHg to the fetal lung through the vasculature. After birth, the lungs are filled with air, resulting in alveolar partial oxygen pressures of 100 mmHg even in normal room air (a factor 5 increase in oxygen exposure). Term neonates seem to respond to this marked increase with an upregulation of antioxidants and oxygen scavenging enzymes, especially in conditions with high oxidative stress (44, 45). Animal data suggest that these high enzyme levels are another part of the preparatory process during the final part of gestation (46). Preterm birth will thus untimely expose unprepared lungs to room air, which already contains a supraphysiological concentration of oxygen. As preterm neonates appear to have weaker antioxidant defense mechanisms than term babies (47-49), this might lead to oxidative damage even in the absence of inspired oxygen fractions (FiO_2) above 21%.

Raising the fraction of inspired oxygen (FiO_2) above 21% room air - often crucial for maintaining viable arterial oxygen pressures - might further increase the amount of ROS and subsequent oxidative damage in the developing lung. Studies published in the early 2000's, have revealed an excess of BPD in patients where supplementary oxygen was administered to target oxygen saturations above 95% (50-52). This has led to the current practice to target lower oxygen saturation levels (90-94%). More recent trials have gone even further in lowering saturation targets to 85-89%, thereby significantly decreasing the FiO2 to which babies were exposed. This resulted in increased mortality, and a decreased rate of retinopathy of prematurity (ROP), but interestingly no further decrease in BPD prevalence was noted in comparison to the higher target group (90-94%) (53, 54). These findings pare down the role of oxygen supplementation as the most important contributor to the pathophysiology of contemporary BPD, where extreme saturation targets of >95% are no longer standard of care.

This is in contrast to many neonatal animal models, in which solely the exposure to excessive FiO2 has been used to create a damage reminiscent of human BPD, making use of different timings and intensities (55-58). Recently

a robust study compared different oxygen exposure protocols in neonatal mice, highlighting advantages and disadvantages of each protocol (59). Generally, in these models the exposure to very high oxygen concentrations results in comparable oxidative damage and subsequent interference with lung maturation, as seen in infants developing BPD (41, 43, 60, 61). However, due to the lack of prematurity and other factors contributing in oxidative damage (for instance ventilation or infection), the mechanisms leading to this phenotype in term hyperoxic animal models might be different.

Ventilation

Another intervention, which might add damage to the already abnormal adaptation of the preterm lung to postnatal life, is mechanical ventilation. In a large preterm birth cohort of infants born between 25 and 28 weeks of gestation, 83% required some form of mechanical ventilation. Even when continuous positive airway pressure (CPAP) was the initial treatment modality, 43% of the patients required switch-over to mechanical ventilation at some point (10). Mechanical ventilation thus remains a very frequent intervention, and ventilator induced lung injury is therefore thought to be an important contributor to BPD (62).

The injury induced by mechanical ventilation is probably caused by strain and distention, more than by pressure itself. Volutrauma thus seems a more correct denominator than barotrauma (63, 64). In preterm infants the presence of marked differences in ventilation within the lung, adds on even more strain through atelectrauma and unequal distribution of distention (62, 63). Overall, excessive distention of the parenchymal tissue leads to disruption of the epithelium and increased permeability of the alveolo-capillary membrane, exudation of proteins, release of cytokines and enzymes, and subsequently an inflammatory reaction (65, 66). Even in the absence of increased FiO2 the stretch of lung tissue might also lead to the formation of ROS (67, 68).

It is also established that the avoidance of mechanical ventilation in preterm infants reduces BPD prevalence, albeit only modestly. A meta-analysis of 4 clinical trials comparing the use of nasal continuous positive airway pressure (nCPAP) or intubation as initial respiratory strategy revealed that one additional infant could survive to 36 weeks without BPD for every 25 neonates intended to be treated with nCPAP instead of intubation and mechanical ventilation (69). An explanation for the low effect size, might be the high rate of nCPAP-failures in the very immature lung and the need for cross-over to conventional ventilation. The modest effect of nCPAP is in contrast to earlier

observational data on the role of mechanical ventilation, as lung immaturity is an important confounding factor here (70). One might thus hypothesize that being born with very immature lungs, necessitating mechanical ventilation, is more essential in the pathophysiology of BPD than mechanical ventilation itself. This point is also supported by data which show that also preterm neonates who never required mechanical ventilation, still develop BPD (18,5% of neonates born between 25 and 28 weeks of gestation in group of nCPAP successes) (10).

In baboons and lambs, researchers have been able to combine extreme lung immaturity with mechanical ventilation to create experimental BPD (71, 72). Both invasive and non-invasive strategies of respiratory management have been used successfully in these larger animal models (73, 74), accurately mimicking current practice in neonatology. Term small animals have also been ventilated conventionally for shorter periods of time to study the effects of volutrauma on the developing lung, however translation to the modern human disease - in an era of increasingly immature babies and less invasive supportive measures - is obviously more difficult (75, 76).

Other postnatal factors

Besides the exposure to supplemental oxygen and mechanical ventilation, many other postnatal factors add to the developmental arrest occurring when preterm lungs are exposed to a postnatal environment. Postnatal infections for instance (pneumonia, sepsis) will increase inflammatory damage in the lung, and determine the long term respiratory outcome (77, 78). There also seems to be a relationship between malnutrition and BPD (79) or fluid overload and BPD (80). Although controversial some authors also suggest a link between patent ductus arteriosus and BPD (81). These clinically relevant risk factors have occasionally been studied in animal models as well (82-85).

Extremely low birth weight (ELBW) infants (at risk of BPD) are currently almost routinely exposed to surfactant and caffeine treatment, and most of them have had a course of antenatal steroids as well, since these interventions are all part of the current standard of care. It is likely that the widespread availability of these interventions has changed the phenotype of BPD, in terms of survival of more preterm children and less-invasive supportive requirements (“new BPD” (34, 36)), but maybe also in terms of altered physiology (86-88). In the context of drug development, these current pharmacological therapies, might also affect the pharmacokinetics and –dynamics of an investigated drug. The addition of these standard of care

interventions could thus be advantageous or are at least relevant for a preclinical BPD model.

Prenatal factors

There is increasing interest in prenatal biomarkers for the early prediction of BPD (32), as prenatal factors determine the lung (im)maturity at birth, and subsequently the long term respiratory outcome. Previously, it has been suggested that the absence of pregnancy related complications in many animal models, explains the difficult translation of findings to human BPD (33).

Prenatal infection or chorioamnionitis is a frequent cause of preterm birth and seems to be associated to BPD (89). In utero inflammation might interfere with normal lung development, however its direct effect, independent of gestational age remains unclear (78). In animal models the effect of prenatal inflammation on lung development has been studied by injecting E. Coli lipopolysaccharides or Ureaplasma organisms in the amniotic sac (90, 91).

When normal *functioning of the placenta* is disturbed, growth and development of the fetal lung will be affected. Associations between pre-eclampsia, intrauterine growth retardation (IUGR) and BPD have been described (89, 92). An interesting finding is also the association between placental vascularity and pulmonary vascular disease in BPD (93). In animal models, this state of decreased angiogenesis has been mimicked by intra-amniotic injections with an antagonist of vascular endothelial growth factor (VEGF) (94).

There has been increasing interest in *maternal smoking*, as a risk factor for the development of BPD. Smoking during pregnancy interacts with BPD in a complex manner, as it is both associated to preterm delivery and impaired lung development (95). In a Canadian cohort a significant increase in BPD was noted in preterm babies of which mothers smoked during pregnancy (96). Maternal smoking has also been used as an insult in animal models, to create a BPD-like phenotype (97, 98).

The *genetic constitution* might also predispose to more immature and underdeveloped lungs at the start of life (e.g. polymorphisms in surfactant proteins or VEGF), but might also increase the susceptibility to postnatal damage (e.g. polymorphisms in scavengers of ROS) (99). On the other hand the genetic constitution might also determine the response to an (experimental) treatment. It seems therefore important that a translational drug evaluation occurs in an animal model reflecting the genetic

heterogeneity of human newborns, which is not the case in inbred strains. On the other hand, for basic explorative research, the genetic homogeneity can be an advantage as it increases reproducibility. The importance of the genetic background is also nicely illustrated by the variation in responses of adult mice from different strains to hyperoxia (100).

Phenotype of BPD

In this section we will describe the histological, functional and vascular manifestations of the human disease, and the options to study this in animal models.

Histology

The histological hallmark of BPD is impaired alveolar and vascular development, with a variable degree of extracellular matrix remodeling. Being the only extensive histological evaluation in the post surfactant era (yet before antenatal steroids were introduced), Husain et al. found inhibition of acinar development (lower radial alveolar count (RAC) and increased mean linear intercept of the airspaces (Lma)) in 22 BPD autopsy specimens, compared to 15 lungs of age-matched controls (101). The question remains whether findings in autopsies can represent the structural changes in BPD survivors. Nevertheless, a study of lung biopsy specimens from surviving BPD infants (without surfactant or antenatal steroids) showed comparable enlarged air spaces with simplified alveoli and variable alveolar wall cellularity or fibrosis (31). Since those studies have been published, care in neonatology has evolved significantly, changing the phenotype of BPD. For understandable reasons there are no recent reports on biopsy specimens of BPD infants which benefited from antenatal steroids, non-invasive ventilation strategies and other current practices. We could only identify a few more recent autopsy series reports, all of them with a very specific scope (mainly vascular manifestations of BPD) (29, 30, 102, 103). Despite the gaps in knowledge on contemporary human BPD histology, animal models are still generally accepted if they display an alveolar developmental arrest, with increased septal thickness, as described in the aforementioned historical case series.

Impaired alveolarization can be quantified in experimental animals by measuring alveolar size, surface area and number, with appropriate unbiased stereological techniques after correct processing of the tissue (104). The most reported measure is probably Lma, reflecting the mean length of a random line between to gas exchange surfaces. Increased Lma as shown by Husain et al. in BPD autopsy specimens has been reproduced in many animal models

(59, 105-108). Lmw on the other hand (transsectional wall length) reflects the thickness of the alveolar septations, on a random line. More theoretical background on these stereological read-outs can be found in chapter 2. Other none stereological measurements can complete the picture: RAC (referring to complexity of peripheral airspaces), septal crest counting (referring to the number of secondary alveolar septations) and acute lung injury score (assessing lung inflammation and injury) (109-111). Manual counting of morphometric parameters is a time consuming effort, and is prone to bias, certainly if not properly blinded. Computer-aided morphometry can make analysis less labor intensive and biased, however some evaluations might still require a trained eye (112, 113). Alternatively, direct 3D assessment of alveolar architecture by means of microCT and subsequent automated analysis will gain importance in the future (114, 115).

Another interesting finding in the limited histological data, is the disorganization of the elastin fiber network (116). Quantitatively more elastin is present, but the fibers occur more tortuous and less organized (117). This is particularly interesting because of elastin's key role in the process of secondary septation (118, 119), as a dysfunctional elastin matrix might explain the alveolar developmental arrest described above. Furthermore, because of elastin's function in tethering of the airways (120), this finding could also clarify the lung function abnormalities mentioned below.

Lung function

Pulmonary function testing has been performed in short- and long-term studies of BPD, applying a variety of techniques (121). The major finding in these studies is that BPD patients depict signs of obstructive airway disease. Classical spirometry showed decreased forced expiratory volumes (FEV) and increased responsiveness to bronchodilation in older children surviving BPD (122). In neonates developing BPD, more complex techniques have to be used, however the same conclusions are reached: at both single breath occlusion and forced oscillation increased airway resistance is noted (123, 124). Additionally higher functional residual capacities and residual volumes are measured with body plethysmography and helium dilution, suggesting hyperinflation and air trapping (121, 125, 126). These findings suggest an important factor of small airway pathology in BPD, which is often neglected in research.

Besides airway disease, preterm (ventilated) infants developing BPD also have lower lung compliance (123, 125), indicating increased stiffness of the lung in the acute phase of their disease. A final functional characteristic of the BPD

lung is a decreased gas exchange efficiency. Diffusion capacity measurements reveal a reduction of both pulmonary membrane diffusing capacity and pulmonary capillary blood volume (127, 128), which supports the paradigms of impaired alveolarization (see above) and vascular maldevelopment (see below).

Evaluation of lung function in experimental animal BPD, revealing changes in line with observations in humans, can thus improve the strength of the model. Measuring lung functions in animals can be challenging, and there are often parallels between the techniques used for non-cooperative neonates and laboratory animals (129). Invasive forced ventilatory maneuvers (pressure-volume loops and recruitment maneuvers) are commonly used to look for decreased lung capacities and compliance in BPD models (130, 131), however do not provide information on airway obstruction. In our experiments we report on inspiratory capacity (IC) and static compliance and elastance (Cst and Est). Forced oscillation is therefore often added, to estimate airway (and tissue) resistance on non-breathing animals (130, 131). In our studies we use both single frequency (Snapshot) and broadband (Prime 8) forced oscillation. Snapshot provides dynamic compliance and elastance (Cdyn and Edyn) together with resistance of the whole respiratory system (R). Prime 8 provides information on tissue mechanics: tissue damping (G) and tissue elastance (H) reflects the absorption and dissipation of energy in the alveolar tissue while airway resistance (Rn) represents the resistance of the central or conducting airways.

An alternative is to add adapted harnesses or negative pressure chambers, to measure forced expiratory volume and flow (132), however this technique has, by our knowledge not been applied in neonatal animals until now. We intend to use this technique in the future, however did not perform it in the context of this thesis. Both techniques can also be performed after methacholine instillation, to assess the hyperreactivity of the airways (131). A final addition can be gas dilution and diffusion techniques: by using those researchers have revealed changes in diffusion or residual capacities in line with human observations (108, 133). On the other hand, there are non-invasive measurements of lung function of which plethysmography has been used in many animal models of BPD (134-136).

Pulmonary vascular disease

About 14% of BPD-infants has pulmonary hypertension, as diagnosed on ultrasound at 36 weeks of post menstrual age (5), however they are thought to be only the worst end of a spectrum. Probably all BPD infants have

pulmonary vascular disease in more subtle forms (5, 137), reflecting the close interaction between acinar and vascular development (105, 138, 139). Clinical echocardiographic data demonstrate a high incidence of both early and late pulmonary hypertension in BPD and an association with BPD severity (5, 140). In addition, Thibeault et al. studied arterial histopathological changes in autopsy specimens from infants with severe BPD and found at the level of terminal bronchioles an increased intra-acinar arterial wall thickness due to muscularization (103). Autopsy and biopsy specimens from BPD infants also demonstrated a dysmorphic microvascular pattern with variable abnormalities in the pulmonary capillaries (102, 141).

It thus seems essential that an animal model for BPD also exhibits pulmonary vascular disease (functional or structural signs of pulmonary hypertension and/or a dysmorphic capillary bed). A higher medial thickness index and a decreased peripheral vascular density have been described consistently in BPD-phenotypes in mice (142-144), rats (105, 145), rabbits (146), lambs (147) and baboons (107). In this thesis, the main read-out used for pulmonary artery remodeling is medial thickness (MT%), reflecting the thickness of the tunica media of the arterial wall, in relation to the size of the artery (148). Also in animal models pulmonary hypertension can be evaluated by echocardiography. Right ventricular free wall thickness, pulmonary artery acceleration time (PAAT) and pulmonary artery acceleration time/pulmonary artery ejection time ratio (PAAT/PAET) have been used as surrogates for pulmonary artery pressure in small BPD animal models (142, 143, 149, 150). Good correlation between these surrogates and invasive measures by catheterization have been reported (146, 151). Finally, also microCT angiograms after the (lethal) injection of barium in the pulmonary artery have provided interesting data on the 3D structure of the pulmonary arterial tree in experimental BPD (105, 142, 146).

Overview of the available animal models

Multiple animal models have been developed to investigate the pathophysiology of BPD. Each animal model has a unique set of translational and practical advantages and limitations which should be considered carefully in the context of preclinical research on BPD.

Small animal models: mice and rats

Mice and rats have often been used for BPD research and have well established models that use term neonates, in whom damage is induced by hyperoxia, hypoxia, prenatal inflammation, transgenesis and even short term

ventilation (55, 56) (Table 1). The animals are small (birth weight mice: 0.5–1.5 g and rats: 5-6 g), cheap to house and have a short gestation (21 days for mice, 22 days for rats) with a large litter size making experiments relatively easy to perform and plan. The major advantage however of the mouse models (and to a lesser extent rats) is the availability of reagents and technology for extensive pathway analysis: e.g. (conditional) gene knock-out strains; validated antibodies for flow cytometry, western blot or immunohistochemistry; transcriptomics, proteomics and advanced microscopic techniques (152, 153). At term, both mice and rat are in the saccular phase of lung development, which is morphologically similar to human premature infants at 28-30 weeks gestation (Figure 1.3). This means that they are good models to study alveolarization, but not necessarily the transition of the canalicular to saccular stage as occurring in human preterm babies developing BPD (Figure 1.3). Additionally these pups do not encounter the gas exchange limitations seen in premature infants. Rat and mice seem to

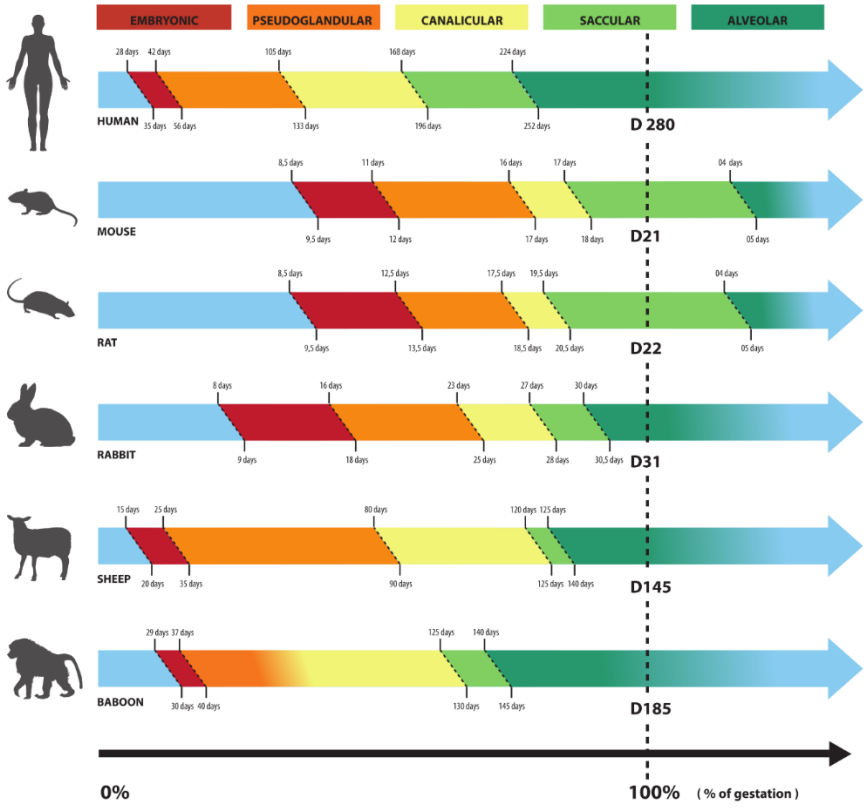


Figure 1.3. Comparative mammalian lung development. *The figure shows the timelines of the 5 phases of lung development in humans and experimental animals frequently used to mimic BPD. Adapted from (55, 56, 71, 155-158)*

have developed adaptive mechanisms to function normally, with structurally preterm lungs (e.g. they express sufficient levels of surfactant and antioxidant defense enzymes (46, 154)). Truly preterm mice and rat models have not been described so far. Moreover the small size of the pups limits the possibility of performing long term monitored mechanical ventilation experiments.

Overall the term mice and rat provide good models for basic explorative research and pathway analysis of specific mechanisms involved in the pathogenesis of human BPD. Resulting insights are essential in the conception of innovative treatment ideas (a good example is the research on the relationship between angiogenesis and alveolarization in hyperoxia (105)). However, the lack of prematurity, and the need for insults exceeding what is common practice in neonatal medicine, questions the translatability of findings in mice and rats to current human BPD.

Large animal models: baboons and lambs

Large animal models in the field of BPD research are the premature lamb and the premature baboon model. The lung development of lambs and especially baboons reflects human lung development closely, as they start alveolarization before term birth, and the length of the developmental stages is proportionate to the length in humans (Figure 1.3). The animals can be delivered prematurely and have respiratory failure as seen in human preterms, necessitating mechanical ventilation or non-invasive support measures (73, 74). Standard of care interventions (exogenous surfactant, antenatal steroids, titrated oxygen supplementation and caffeine) have been added to the models. Their size (mean 382 g for preterm baboons at day 125, mean 3 kg for preterm lambs at day 131), also allows for intensive monitoring along with repeated blood sampling and catheterization, simulating nicely the clinical neonatal intensive care setting (71, 72). These models thus reproduce the human disease and context very closely. An additional advantage is that, similar to human BPD, signs of obstructive airway disease have been well described. The ability to closely mimic the airway pathology in BPD, can be explained by the similarities between human and large animal airway anatomy (the presence of respiratory bronchioles, the presence of cartilage in the intrapulmonary airways and symmetrical splitting of airways) (71, 72, 159). On the other hand the intensity of care and the high cost involved are major disadvantages. The scientific community and the public might also have ethical constraints with large animal experiments, especially with non-human primates, however these considerations are also relevant when using smaller animals. Furthermore the availability of reagents is rather limited, when

compared to research in the murine models. A final disadvantage is translational, as most models are created by cesarean section in a formerly healthy pregnancy. This does not parallel most human preterm births, where prenatal pathology leads to preterm birth, and predisposes the lung towards BPD. In both baboons and sheep the influence of perinatal infection or IUGR in the etiology of BPD has also been studied (91, 160, 161) (Table 1), and one could argument to include these prenatal factors in preclinical studies evaluating therapeutic options for BPD.

Many slightly different variations on these models have been published. The preterm baboon model has closely followed the evolving clinical practice in neonatology, with both a model for old BPD (moderately preterm animals delivered at day 140, supported with aggressive mechanical ventilation) and new BPD (severely preterm animals delivered at day 125, supported with surfactant administration and non-invasive ventilation). Preterm (ventilated) lambs between 125 and 132 days of gestation, have been used by many groups for a wide range purposes: to study the effects of short term in utero ventilation (162), to study respiratory stability during different ventilation modalities (163) and recently also for trials with an ex vivo uterine environment (164). Fewer groups however have in sheep focused on the effect of interventions on longer term BPD related outcomes, mainly in models born around day 125 (72). It is important to note that there is only a narrow time window to mimic the human setting of birth in the early saccular stage of lung development, as sheep have a relatively short saccular stage, and around day 130 alveolarization has already started in many of them (Figure 1.3).

Medium sized animal models: rabbits

Rabbits are medium sized animals that have the main advantage that their lung development closely mirrors that of humans. In contrast to smaller rodents, alveolarization starts in-utero in rabbits (Figure 1.3). They also have large litter sizes, relatively short gestations (31 days), are easy to house and have a size that allows for certain technical manipulations (30-60g) (57). Many different insults have been used to mimic BPD in term rabbit models, including different hyperoxia protocols (165-167), short term ventilation (168) and chorioamnionitis (169). However, term rabbit pups are in the alveolar phase of lung development, and thus lack both structural and functional prematurity (Table 1). The major asset of the rabbit over the smaller rodent models, is the possibility to induce prematurity (106, 130). Below we will discuss the preterm rabbit model in more detail.

	Factors used to mimic human BPD
Mice	- Hyperoxia - Short term and intermittent mechanical ventilation - Pre- and postnatal hypoxia - Prenatal inflammation: LPS - Transgenic mice - Pre-eclampsia: sFlt1
Rats	- Hyperoxia - Short term and intermittent mechanical ventilation - Pre- and postnatal inflammation: LPS
Rabbits	- Prematurity - Hyperoxia - Malnutrition - Prenatal inflammation: E. Coli - Short term mechanical ventilation
Sheep	Combination of: - Prematurity - Long term mechanical ventilation or non-invasive ventilatory support - Oxygen (titrated) - Prenatal inflammation: LPS - Postnatal inflammation: intratracheal LPS - Malnutrition - IUGR - Antenatal steroids, postnatal surfactant and caffeine
Baboons	Combination of: - Prematurity - Long term mechanical ventilation or non-invasive ventilatory support - Oxygen (titrated) - Prenatal inflammation: Ureaplasma species - Antenatal steroids, postnatal surfactant

Table 1: Animal models for BPD. *Non-exhaustive summary of the factors used in the different animal models to mimic BPD pathophysiology. References: (55-57, 71, 72, 75, 82, 94, 160, 161, 170, 171)*

The case for the preterm rabbit model

The stage for the development of a preterm rabbit model for BPD has been set in the 1980s. At that time researchers could show a doubling in fetal lung antioxidant enzyme levels during the last 3-5 days of normal term gestation in rabbits (172), as well as in other laboratory animal species (46). This physiological increase was hypothesized to prepare the fetuses for the

upsurge in oxygen pressure related with a normal fetal to postnatal life transition at term birth. Consequently, they could demonstrate that preterm rabbit pups (day 29) had more severe lung injury in response to 48-72h of hyperoxia (FiO_2>90%) than term pups. Preterm pups were, besides their uncompleted fetal maturation, also unable to upregulate their antioxidant enzymes in defense to a hyperoxia insult (173). These findings illustrate that prematurity is a major contributor to neonatal lung disease, and emphasize the functional prematurity of the preterm rabbit models. Additionally, the fetal increase in antioxidant levels in rabbits coincides with a steep maturation of the surfactant system and a swift development of the fetal lung from late canalicular, over saccular, to the alveolar stage (Figure 1.3) (155, 174). Preterm rabbits are thus born in a comparable stage of lung development as human preterms developing BPD, and have a comparable immaturity of the antioxidant and surfactant systems.

Additional injury to the developing lung of the preterm rabbit, has mainly been administered under the form of hyperoxia. Initial studies with the preterm rabbit model, were limited to short term exposures (FiO_2 90-100%, during 1-4 days), mainly using pups after 29 days of gestation (173, 175, 176). These short-term experiments, focused on acute consequences of hyperoxia (lipid peroxidation, pulmonary edema and inflammation), and did not evaluate the effects on architectural changes in the lung related to lung development. The first longer term experiment was published by Mascaretti et al. in 2009. In pups delivered at 28 days of gestation and exposed to FiO_2>95% for 11 days, they could demonstrate an arrested lung development, however survival was extremely low (11% in hyperoxia, 31% in normoxia) (177). In later work they showed that the developmental arrest is preserved in less preterm pups (29 days of gestation), exposed to lower oxygen concentrations (FiO2 80%), however mortality remained high (106). Furthermore the additional effect of postnatal malnutrition on the pulmonary phenotype of the model was explored (84).

Our group developed a comparable preterm rabbit model for BPD (Figure 1.4) (130). Preterm rabbit pups (New Zealand White and Dendermonde hybrids) are delivered by cesarean section after 28 days of gestation. Pups are hand raised in either hyperoxia (FiO2>95%) or normoxia (FiO2 21%) in an incubator at a constant temperature (32°C) and humidity (75%). Standardized gavage-feeding is administered to all pups, eliminating the variability associated with maternal feeding: a milk formula adapted for neonatal rabbits (30% protein, 50% fat), supplemented with colostrum and probiotics is administered twice

daily. Pups are also injected prophylactically with vitamin K and antibiotics. After 7 days all outcome measures are obtained and lungs are harvested.

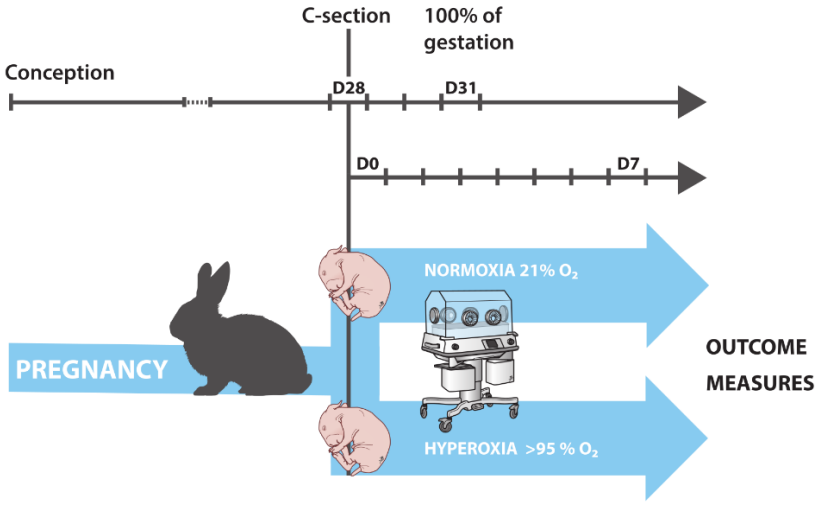


Figure 1.4: Methodology of the preterm rabbit model. *Schematic representation of the preterm rabbit model as commonly used in our lab and described in (130)*

Hyperoxia exposed preterm rabbit pups exhibit morphological, functional and vascular manifestations comparable to human BPD. Morphologically signs of alveolar simplification and developmental arrest are present: increased alveolar size (Lm) and increased thickness of the alveolar septa. Furthermore increased amounts of collagen, but not elastin, and increased numbers of proliferating cells were noted when compared to normoxia preterm controls (130, 177). Signs of increased inflammation were also present (178). Functionally the lungs of the hyperoxia exposed pups had a lower total lung capacity, decreased compliance and increased tissue resistance (tissue damping). Signs of obstructive airway disease, as seen in humans, were not observed (130), however no provocation studies have been undertaken to study bronchial hyperreactivity. At the vascular level increased arterial wall thickness has been observed, together with signs of pulmonary hypertension (decreased PAAT/PAET ratio) at cardiac echo Doppler (146).

The preterm rabbit model combines some crucial advantages of both the large and small animal models. First, its major asset is without doubt that it consists of premature animals (both structurally and functionally), that need to go through a precocious adaptation process to postnatal life, even without the addition of extra insults. The preterm pups at day 28 depict signs of

respiratory distress at birth, however can survive without surfactant administration or mechanical ventilation. Second, unlike other preterm animal models they are easy to house and handle, have large litter sizes, short gestations and relatively low cost, facilitating many practical aspects of research. Third, the size of the pups allows for certain technical manipulations (tracheostomy for ventilation and surfactant administration, cardiac ultrasound, gavage feeding). Long term ventilation has not been described, yet many investigators have evaluated the effect of exogenous surfactant administration on ventilation of preterm pups delivered after 27 days of gestation (179, 180).

Obviously working with preterm rabbit also has disadvantages. The paucity of antibodies and absence of genetic knock-outs limits in depth pathway analysis in rabbits, though measuring messenger RNA (mRNA) expression (qPCR, RNA sequencing) can compensate partially (130). Furthermore, the high mortality is often considered as an issue, but workable survival rates of 56% in hyperoxia and 83% in normoxia at day 7 have been reported (130). As often only surviving pups can be included for outcome measures, it is important to note that this high mortality might bias study results. Another drawback of the preterm rabbit model however is that mortality might fluctuate because of maternal and seasonal influences that are often difficult to control for (181, 182). On the other hand, the mortality can also reflect a translational advantage, as it illustrates the functional prematurity of the rabbit pups. Finally, the lack of prenatal pathology leading to prematurity in a cesarean section based model (130), can also be considered as a translational deficit.

Based on the arguments mentioned above, we consider the preterm rabbit model a valuable model for preclinical drug research in BPD. Until now, the effect of caffeine and omeprazole on the phenotype have been assessed in our model (181, 183). An important consideration for drug research are the pharmacokinetics and pharmacodynamics of the tested pharmaceuticals. When considering a preclinical study, it should be evaluated if the translation of the findings is not hindered by important differences in absorption, distribution, metabolism or excretion between the animal model and the clinical target population. Of course this depends on the specific characteristics of every individual drug, but in general the rabbit bears some interesting similarities to the human. In terms of absorption, it is mainly the variety in administration methods that mimics clinical practice. In the smaller neonatal rodents the intraperitoneal route is often used as the only option for systemic drug delivery, however many factors may determine (and limit) subsequent absorption (184). The size of the rabbit pups allows, besides

intraperitoneal administration, for more translatable methods: either subcutaneous, intramuscular, intravenous, intratracheal or enteral administration by gavage.

In terms of metabolism and excretion, it is important to notice that there are significant developmental differences between the adult and (preterm) neonate (185). As in humans, both phase I (e.g. cytochrome P450 monooxygenases) and II (e.g. glucuronidation enzymes) hepatic drug metabolism mature over the lifespan of a rabbit (186-188). Equally, renal clearance of neonatal rabbits is very low, and increases with aging (189). For instance, for caffeine, this results in an accumulation of the drug and its metabolites in neonatal rabbits, as it is also the case in preterm infants (190). Equally, the main metabolic pathway of caffeine is the same in rabbits as in humans (N-7 demethylation) (191). In studies testing prenatal drug therapies, a final aspect of pharmacokinetics that should be considered is placental transfer of the drug. The structure of the placental barrier of the rabbit (hemodichorial) is closer to the human placenta (hemomonochorial in the third trimester), than the placenta of sheep (epitheliochorial) and rats or mice (hemotrichorial) (192), making the rabbit a popular model for fetal toxicity studies (193). Recent data also suggest functional similarities in placental drug transporter spectra between humans and rabbits (194). Transplacental treatment strategies in the preterm rabbit model have been carried out with rosiglitazone and omeprazole (181, 182).

Difficulties in drug development for BPD

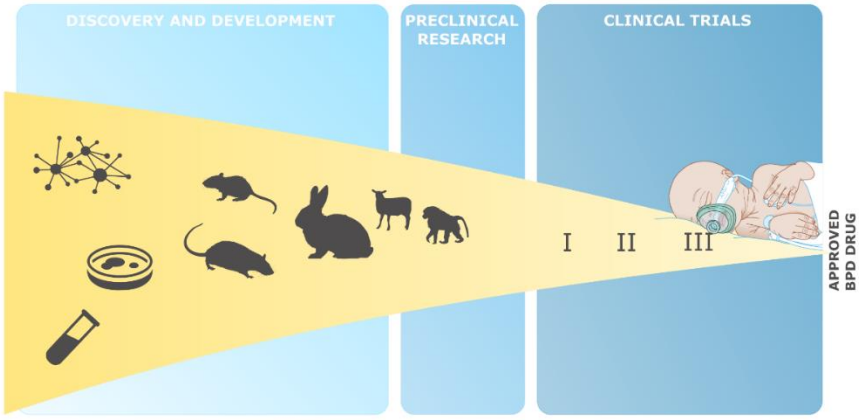


Figure 1.5. Drug development pathway for BPD. *Many molecules are screened in a first discovery and development-phase. Only a limited number proceeds to more formal preclinical research and later clinical trials. Every phase of drug development requires its own methods.*

Developing safe and effective drugs is an expensive and time consuming process (Figure 1.5). Drug development starts with the discovery of new targets by research on disease models or clinical samples. For each drug that is ultimately approved, several thousand molecules have been screened and tested in the initial discovery and development phase. Only a limited number eventually proceed to more formal preclinical testing according to good laboratory and clinical practices. For both drug discovery and preclinical research appropriate models are required that reflect the phenotype and physiology of the disease. Consequently the most promising molecules undergo several phases of clinical trials before being approved (with an estimated success rate of 5%) (195).

Difficulties in preclinical research

The drug development process has so far not been particularly successful for drugs aiming to treat or prevent BPD. As stated above, only a limited number of novel drugs are in the pipeline (e.g. local corticosteroids (15, 16)) and many of the investigated treatments, were unsuccessful in clinical trials (12, 33). The difficult translation of findings from bench to bedside, could be explained by the fact that none of the above described models perfectly mimics the human setting. The development of BPD in a patient can be caused by a myriad of pre- and postnatal factors next to prematurity, while animal models only include 1 or 2 stable insults. A second contributing factor to the limited applicability of these preclinical findings is the research methodology itself. A systematic review of animal research in BPD demonstrated that only occasionally the necessary scientific methods to avoid bias in these animal studies are applied (e.g. randomization, blinding, …). Even though these methods are the cornerstone of clinical research that lead to evidence based medicine, they are yet to be implemented in preclinical (and especially translational) research (19). Finally one of the main read-outs in BPD research, the 'mean linear intercept of the airspaces' (Lma; a measurement that reflects alveolar simplification) is often obtained after a quantification process that is prone to bias (104). Robust and reproducible research methods are necessary to investigate novel BPD therapies.

Difficulties in neonatal clinical research

Newborns are not just small adults, making drug development for them even more complex. A major difference is for instance the ontogeny of both pharmacokinetic and –dynamic processes, and the enormous impact of long term effects. Furthermore, newborns in clinical trials encounter many adverse events, which are not always recognized and reported (196). Due to

the lack of standardized adverse event terminology this safety information is difficult to use. Finally, ethical issues arise when clinical trials are performed in vulnerable neonates, as parents are asked for consent during a very emotional period in their life. In this context only the most promising candidate drugs –as tested in relevant preclinical models- should be taken towards neonatal applications.



Der Scheintod Neugeborener – Schultze (1871):
The Schultze method for resuscitation of newborn babies aimed to fiercely stimulate non-breathing babies after birth.

Objectives

The overall aim was to search for novel treatment strategies for BPD and improve the research methods necessary to develop new therapies along different stages of the drug development pathway. In Figure 2, we placed the chapters of this thesis along the drug development pathway that was already visualized in Figure 1.5.

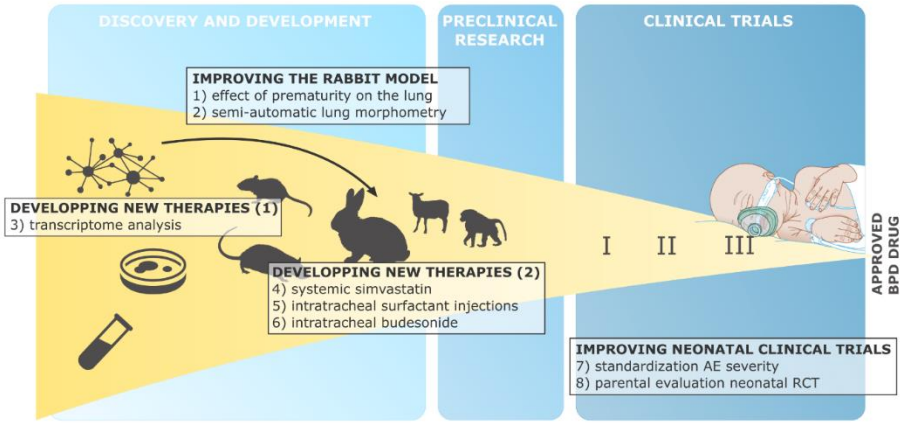


Figure 2. *Different chapters of this thesis along the drug development pathway for BPD.*

In a first group of chapters we focused on improving the methods for drug discovery and development and preclinical research.

Improving the preterm rabbit model for BPD	Aim: To further characterize the effect of hyperoxia and prematurity on the lungs rabbit pups in order to develop a robust platform for early and preclinical BPD research. *Chapter 1: Preterm birth impairs postnatal lung development in the neonatal rabbit model* *Chapter 2: A semi-automated method for unbiased alveolar morphometry: validation in a bronchopulmonary dysplasia model*

In a second group of chapters we used the preterm rabbit model to develop (novel) therapeutic strategies for BPD. We first focused on target identification by transcriptome analysis and then continued with studies to assess efficacy of therapies aimed at 2 of the identified targets (simvastatin and budesonide). Surfactant was considered as a drug carrier.

Developing novel therapies for BPD	Aim: To identify novel treatment strategies for BPD and assess their safety and efficacy in a preterm rabbit model. *Chapter 3: Transcriptome analysis of the preterm rabbit lung after seven days of hyperoxic exposure* *Chapter 4: Simvastatin attenuates lung functional and vascular effects of hyperoxia in preterm rabbits* *Chapter 5: Local pulmonary surfactant delivery in the preterm rabbit: feasibility and efficacy of daily intratracheal injections* *Chapter 6: A single dose of budesonide added to surfactant prevents hyperoxia induced lung injury in preterm rabbits*

In a third and final group of chapters, we focused on improving the methods for clinical trials in newborns.

Improving clinical trials in newborns	Aim: To study ethical and regulatory hurdles and facilitate clinical research in neonates *Chapter 7: Development of a neonatal adverse event severity scale through a Delphi consensus approach* *Chapter 8: Parental perspectives long term after neonatal clinical trial participation.*



Tarnier's incubators in the Maternite´ Hospital, Paris (1884):
The first incubators increased survival of preterm babies by providing warmth.

Chapter 1

Preterm birth impairs postnatal lung development in the neonatal rabbit model

Salaets T., Aertgeerts M., Gie A., Vignero J., de Winter D., Regin Y., Jimenez J., Vande Velde G., Allegaert K., Deprest J., Toelen J.
Under revision in Respiratory Research

Abstract

Introduction - BPD continues to cause important respiratory morbidity throughout life, and new therapies are needed. The common denominator of all BPD cases is preterm birth, however most preclinical research in this area focusses on the effect of hyperoxia or mechanical ventilation. In this study we investigated if and how prematurity affects lung structure and function in neonatal rabbits.

Methods - Pups were delivered on either day 28 or day 31. For each gestational age a group of pups was harvested immediately after birth for lung morphometry and qPCR for surfactant protein B and C. All other pups were hand raised and harvested on day 4 for the term pups and day 7 for the preterm pups (same corrected age) for lung morphometry and lung function testing. A subset of pups underwent micro computed tomography (microCT) and dark field imaging on day 0, 2 and 4 for terms and on day 0, 3, 5 and 7 for preterms.

Results – Preterm pups assessed at birth depicted a more rudimentary lung structure (larger alveoli and thicker septations) and a lower expression of surfactant proteins in comparison to term pups. MicroCT and dark field imaging revealed delayed lung aeration in preterm pups, in comparison to term pups. Preterm birth led to smaller pups, with smaller lungs with a lower alveolar surface area on day 7/day 4. Furthermore, preterm birth affected lung function with increased tissue damping, tissue elastance and resistance and decreased dynamic compliance.

Conclusions – Preterm birth affects lung structure and function at birth, but also has persistent effects on lung development. This supports the use of a preterm animal model, such as the preterm rabbit, for preclinical research on BPD. Future research that focuses on the identification of pathways involved in in-utero lung development and disrupted by pre-term birth, could lead to novel therapeutic strategies for BPD.

Introduction

Changing practices in neonatology have altered the phenotype of BPD. Antenatal steroids and surfactant supplementation have improved survival of more preterm infants with more immature lungs. This is associated with an increasing incidence of BPD, as defined by oxygen dependency at 36 weeks of life (3). The adoption of non-invasive ventilation and lower oxygen saturation

targets on the other hand have blunted postnatal lung injury. As a result BPD shifted from a fibrotic disease (2) to a disease characterized by alveolar developmental arrest (36).

Despite this evolution to a “new” type of BPD, respiratory disease remains an important long term consequence of preterm birth. For instance, increased rates of respiratory symptoms (e.g. coughing, wheezing, respiratory infections, respiratory drug use) have been reported in survivors of preterm birth (197, 198). Lung function in survivors of extreme preterm birth is significantly different from term controls at long term, even in the absence of BPD or oxygen dependency (199, 200)**.** Most importantly, a model based on perinatal characteristics such as gestational age, birth weight, sex, race, familial history and intubation at birth is a better predictor for respiratory outcomes than the presence of BPD (as defined by the need for supplementary oxygen) (197). Further efforts to improve the respiratory outcomes of former preterm neonates thus remain necessary, regardless of oxygen dependency.

Postnatal care (e.g. hyperoxia or mechanical ventilation) can influence respiratory outcome, but the common factor contributing to all BPD cases is prematurity (1, 38). Nevertheless, most preclinical research on BPD is performed in term neonatal rodents exposed to excessive hyperoxia or aggressive mechanical ventilation, in absence of any prematurity (55, 56). The translatability of these findings to human “new” BPD can be questioned. Large animal models like preterm baboons and lambs on the other hand can be used to mimic intensive neonatal care with invasive ventilation or even non-invasive pressure support, but impose significant costs (71, 72). The preterm rabbit model could be an intermediate that combines prematurity and the possibility of perinatal manipulations with relative low cost (1, 57). Surprisingly, little animal experimental data is available on the persistent effects of a preterm transition to postnatal life, in the absence of any other noxiae, on lung structure and function. Investigating the effect of preterm birth on lung development in animal models however could lead to novel therapeutic strategies for “new” BPD.

In this study we are the first to investigate the effect of prematurity on lung structure and function in neonatal rabbits.

Methods

Animal protocol and care

The animalium of KU Leuven provided pregnant rabbits (New Zealand White and Dendermonde hybrid). All experiments were approved by the Ethics Committee for Animal Experimentation (P060/2016 and P080/2017) and were performed according to local guidelines on animal welfare. Does were housed in individual cages with free access to food and water in a controlled 12h/12h light-dark cycle. For Cesarean section does were sedated with intramuscular ketamin 35 mg/kg (Nimatek®; Eurovet Animal Health BV, Bladel, The Netherlands) and xylazin 6 mg/kg (XYL-M®; VMD, Arendonk, Belgium). Subsequently they were euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (IV bolus of 1 ml T61®; Intervet Belgium, Mechelen, Belgium). Immediately after death, the abdomen was opened and the uterus exposed to extract all pups through hysterotomy. After birth, pups were dried, stimulated and placed in an incubator (Dräger Incubator 7310; Dräger, Lübeck, Germany) at 32°C, 50% relative humidity and 21% of oxygen. Pups were fed twice daily via an orogastric tube with a milk replacer (Day One®, Protein 30%, Fat 50%; FoxValley, Illinois, US) with additional probiotics, electrolytes and vitamins (Bio-Lapis®; Probiotics International Ltd, Somerset, UK) and immunoglobulins during the first 2 days (Col-o-Cat®, SanoBest, Hertogenbosch, Netherlands). The volume of feeds is increased from a total of 80ml/kg body weight/day on day 0 of life to 200ml/kg/day on days 3-7. On day 2, vitamin K1 is administered intramuscularly (0.25 mg/kg, Konakion pediatrique®; Roche, Basel, Switzerland), and from day 2 onwards, pups are given a daily intramuscular injection of benzylpenicillin (20,000 IU/kg Penicilline®; Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg day, Amukin®; Bristol-Myers-Squibb). MicroCT and dark field imaging was performed under isoflurane anesthesia (2% isoflurane in 2l/min of pure oxygen). Lung functions were terminal experiments performed in deeply anesthetized postnatal rabbit pups (ketamine 35mg/kg and xylazin 6mg/kg IM). Anesthetized animals were euthanized by exsanguination for dissection and lung harvest.

Study groups

The study groups and timeline are illustrated in Figure 3.1. Pregnant dams were randomized to either a Cesarean section on day 31 (term, alveolar stage of lung development) or day 28 (preterm, saccular stage). Of each group, randomly selected pups were harvested before their first breath: they constitute the fetal groups 28F (preterm, day 28) and 31F (term, day 31). In

fetal pups left lungs were harvested for histology, and right lungs snap frozen for mRNA-extraction and qPCR. The other pups were stimulated to breath by tactile stimulation. Pups surviving the first adaptation hour after birth were hand reared until day 7 (preterm pups) and day 4 (term pups; same corrected age): they constitute the postnatal groups P (preterm) and T (term). A subset of the postnatal pups underwent in vivo microCT and dark field imaging on day 0, day 3, day 5 and day 7 in P and on day 0, day 2 and day 4 in T (same corrected ages). On day 7/day 4, all postnatal pups underwent lung function testing before left lung harvest for histology.

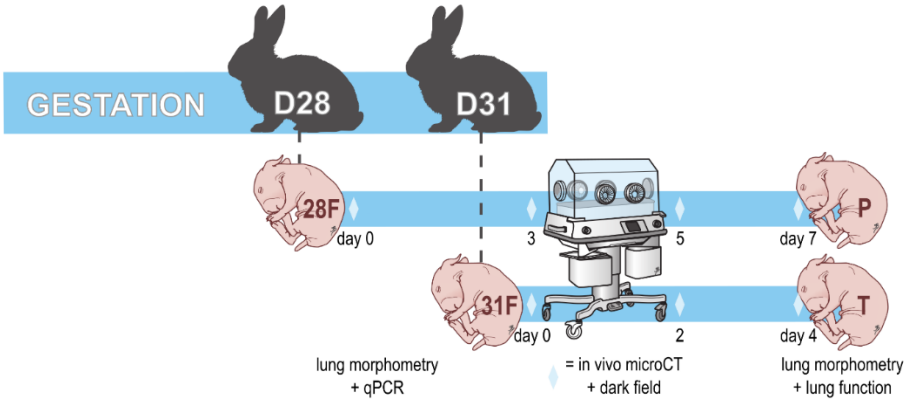


Figure 3.1. *Pups were delivered on either day 28 or day 31. For each gestational age pups were harvested (n=10-15) immediately after birth, before the first breath: 28F and 31F. All other pups (n=15-25) were hand reared and harvested on day 4 for the term pups (T) and day 7 for the preterm pups (P; same corrected age). A subset of pups underwent microCT and dark field imaging on day 0, 2 and 4 for terms and on day 0, 3, 5 and 7 for preterms.*

Alveolar and vascular morphometry

Left lungs were harvested for histology, and therefore pressure fixed on 25cmH_2O of paraformaldehyde for 24h. Volume of fixed left lungs was measured by water immersion (Scherle's principle). Lungs were embedded in paraffine, and for every lung a central sagittal 5µm slide was made for hematoxylin-eosin staining. Stained sections were scanned with a slide scanner (Axio Scan® Slide Scanner, Zen Zeiss, Oberkochen, Germany). Alveolar morphometry measurements are semi-automatically performed with a self-designed FIJI-plugin on 20 randomly selected fields per lung. Based on the counts mean linear intercept (Lm, estimating alveolar size) and mean transsectional wall length (Lmw, estimating septal wall thickness), and total alveolar surface (S, by taking the reference left lung volume in to account) were calculated (104, 148).

Another central 5µm slide was stained with Miller's elastic staining. For at least 15 arteries of 30-100µm in diameter, the internal and external diameter of the medial-layer was determined on the shortest axis. Average relative thickness of the medial layer (MT%) was calculated per animal as described before (148).

mRNA expression of surfactant proteins

Right lungs were snap frozen for qPCR of surfactant protein B and C mRNA expression. First, mRNA was extracted from the tissue using the Tri-Pure® Isolation reagent (Roche Diagnostics, Germany). RNA concentration was measured using the Nanodrop 1000® spectrophotometer (Thermo Scientific) and RNA integrity was assessed by checking the integrity of the 18S and 28S band by agarose gel electrophoresis. cDNA was synthesized with Taq Man® Reverse Transcription Reagents (ThermoFisher Scientific). The Platinum SYBR® Green qPCR Supermix-UDG with ROX (ThermoFisher Scientific) was used to detect expression of surfactant protein B and C. YWHAZ, HPRT and actin B were used as stable housekeeping genes to normalize mRNA levels. An overview of used primers, obtained from Integrated DNA Technologies (Haasrode, Belgium), can be found in Supplementary Table S3.1. Samples were run in triplicate on a StepOne Plus® instrument (ThermoFisher Scientific). Relative quantitation was determined using the comparative Ct method. Statistics are calculated in based on ΔΔCt-values, while fold change (FC) is used for visualization.

Lung function testing

FlexiVent 5.2 (SCIREQ, Montreal, Canada) is used to perform pulmonary function tests on deeply anesthetized postnatal pups. The trachea of the pup is surgically exposed and an 18G metal cannula is inserted in the trachea and connected to the FlexiVent ventilator (120 breaths/min ventilation; tidal volume of 8ml/kg). A pressure volume perturbation is performed to measure inspiratory capacity and static compliance. Snapshot is a single-wave forced oscillation perturbation used to assess dynamic compliance, elastance and resistance from a single compartment model. Finally, Prime-8 is a broadband forced oscillation technique and is used to assess tissue damping (frequency independent parenchymal energy dissipation) and tissue elastance (energy conservation of lung tissue). Three measures for each perturbation are obtained per pup and the mean value is used in the analysis. Measures for compliance, elastance and inspiratory capacity were corrected for weight. After lung function, deeply anesthetized animals are euthanized by exsanguination.

MicroCT

Rabbit pups were fixated on a Styrofoam bed in the supine position in a low-dose small animal microCT scanner (SkyScan 1278, Bruker micro-CT, Kontich, Belgium). The following acquisition parameters were used: 1 mm Al filter, 50 kVp X-ray source voltage, 918 µA source current, 55 ms exposure time, 9 projection images per 0.9° rotation step over a total angle of 180°. Respiratory gating was performed retrospectively by matching projection images to the signal of a camera registering breathing movements. A 3D datasets with a 50 µm isotropic reconstructed voxel size was created. We used software provided by the manufacturer (TSort, NRecon, DataViewer and CTan) to gate, reconstruct, visualize and analyze microCT data (201). End-expiratory data is used for analysis.

Lung volume was manually delineated on all slices of the reconstructed microCT dataset, avoiding the inclusion of the heart and mediastinal structures, by a researcher (MA) blinded for group allocation. Total (end-expiratory) lung volume and mean lung density were calculated. Additionally, by applying a fixed density threshold (85 on a grayscale from 0 to 255), aerated lung tissue was segmented from the non-aerated lung tissue. Aerated and non-aerated lung volume was calculated as a percentage of the total lung volume. To calibrate mean lung density for Hounsfield units (HU), a phantom (air-filled tube of 1.5 mL inside a water-filled tube of 50 mL) was scanned (202). Only the outcomes of the pups that survived until day 7 were used for the analysis (n=7-9).

Dark field imaging

Dark field imaging is a form of phase-contrast x-ray imaging, and was performed on a Talbot-Lau interferometer with the pup held in a straight position on a Plexiglas mold. The interferometer operated at 40 kVp with a 2 µm G2 grating period and a system visibility of 22%. The radiation dose per round was 0.51 mGy (203, 204). From the acquisitions, dark field images were reconstructed through a MATLAB-algorithm. The intensity of the dark field signal in 4 quadrants of every lung image was scored on a scale from 0 (no signal indicating little aeration) to 8 (strongest signal indicating complete aeration) by 3 independent observers who were blinded for the group allocation. The average score of the 4 quadrants was considered representative for the animal, and the average of the 3 observers is reported.

Statistical analysis

GraphPad Prism® 7.0 software (GraphPad, La Jolla, California, USA) was used for statistical analysis. Normality was assumed after visual inspection of the

data. Statistical outliers were removed from the analysis if a Grubbs test was positive. All values are expressed as mean ± standard deviation. Unpaired t-testing with Welch correction for unequal variance was used to compare outcome measures in 28F to 31F and P to T. A mixed-effects model was used to compare the longitudinal outcomes between the different time points. Bonferroni-Sidak correction for multiple comparisons was used to compare groups on individual time points. Kaplan–Meier curves with post hoc log-rank testing were used to quantify survival of rabbit pups from fetal day 28 until postnatal day 7 (preterm) or 4 (term), assuming that intrauterine deaths in the term mothers occurred before day 28 (n=3). A p-value of <0.05 was considered statistically significant.

Results

Fetal rabbits on day 28 exhibit functional and structural immaturity

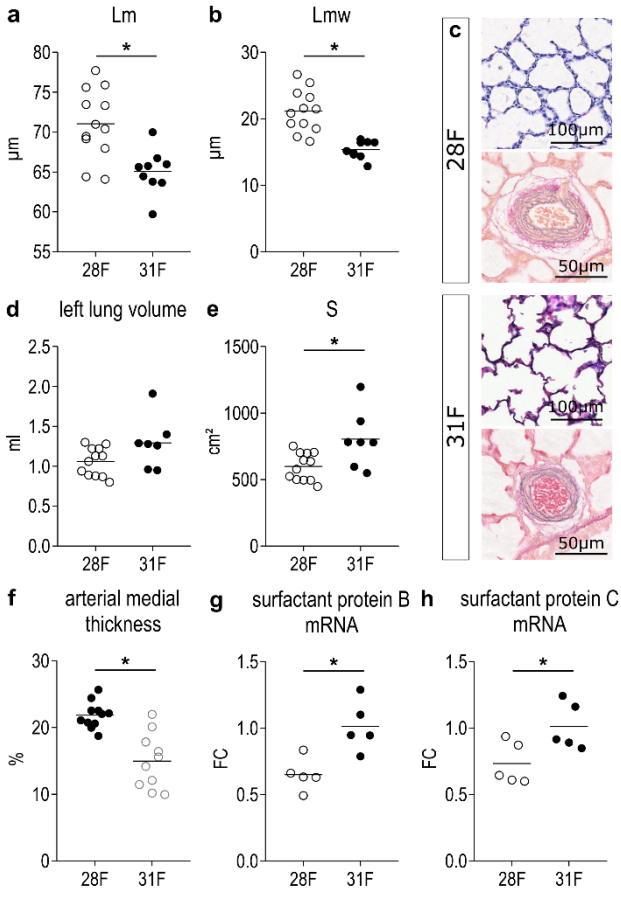


Figure 3.2. *(A) Lm and (B) Lmw are measurements of alveolar size and septal thickness respectively and are both increased in preterm pups at birth. (C) Representative pictures of lung slides stained with HE and Miller indicate more rounded and less complex airspaces and more muscularized arteries in preterm pups at birth. (D) Left lung volume, as measured by water displacement, is not significantly different, however (E) total alveolar surface is significantly decreased in preterm pups. (F) Arterial medial thickness is higher in preterm pups at birth. (G-H) Surfactant protein B and C mRNA expression is significantly lower in preterm pups before the first breath. *p<0.05*

Fifteen preterm and 10 term fetal animals were harvested before the first breath, from respectively 5 and 3 mothers. Birth weight was significantly lower in 28F than in 31F (p=0.0109; Supplementary Table S3.2). Lungs of preterm rabbits had a significantly higher Lm (p=0.0012) and an increased Lmw (p<0.0001) compared with term fetuses (Figure 3.2A-B). The representative figures depict the more rounded and less complex airspaces, with a higher cellularity in 28F (Figure 3.2C). Left lung volume was not significantly lower in 28F (p=0.2927), but due to the simplified lung structure total alveolar surface (S) is significantly decreased (p=0.0484; Figure 3.2D-E). However, the difference in alveolar surface is proportional to the difference in birth weight (p=0.5162 if alveolar surface is normalized to weight). Additionally, arterial medial thickness was higher in 28F compared to 31F (p=0.0004; Figure 3.2F); representative pictures in Figure 3.2C. Finally, preterm fetuses also demonstrate lower mRNA-expression of surfactant protein B and C than term fetuses (p=0.0055 and p=0.0282 respectively; Figure 3.2G-H).

Preterm rabbit pups have higher mortality and lower weight than term controls

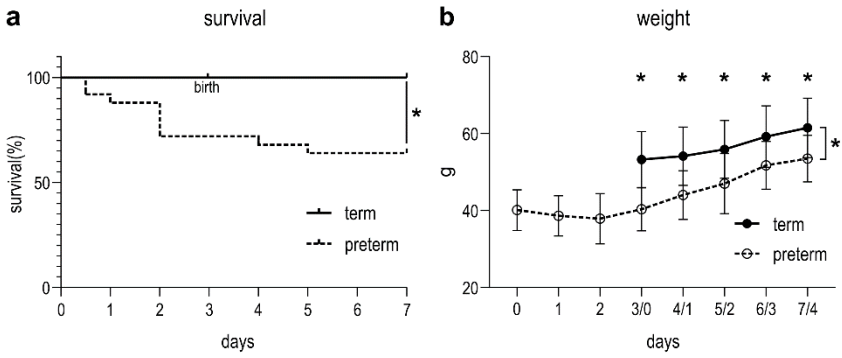


Figure 3.3. *(A) Survival was significantly lower in pups born preterm (60%; 15/25) then in animals born at term (100%; 15/15). (B) At every time point, preterm pups were significantly smaller than term controls. *p<0.05*

Four mothers were randomized to term and 8 to preterm delivery. In the first hour after birth, 100% (47/47) of term pups and 55.8% (43/77) of preterm pups survived the adaptation to postnatal life. Fifteen term and 25 preterm randomly selected pups were included in the experiment. Survival was significantly lower in pups born preterm (60%; 15/25) then in animals born at term (100%; 15/15; p=0.0105; Figure 3.3A). Over time, weight evolved significantly different between preterm and term animals (p=0.0005). On day 7/day 4 (day of harvest), preterm pups were significantly smaller than term counterparts (p=0.0190; Figure 3.3B; Supplementary Table S3.2).

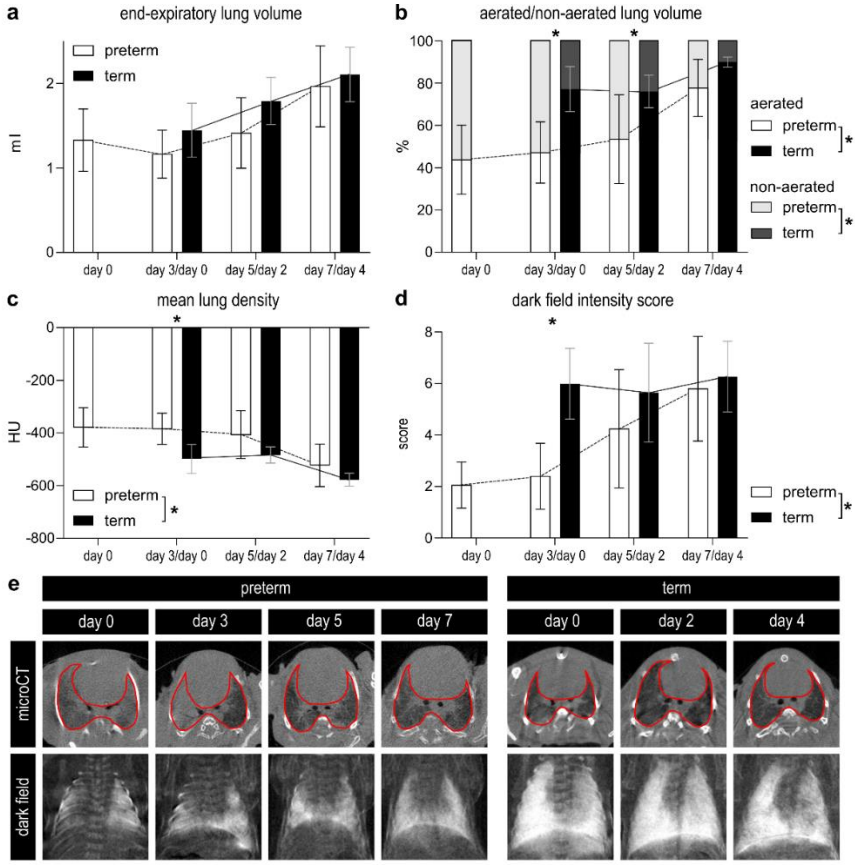


Figure 3.4. *(A) Total lung volume assessed by microCT increases over time in both term and preterm pups (n=7-9). (B) Preterm pups have a lower proportion of aerated lung volume then term pups on day 3/day 0 and on day 5/day 2 (n=7-9; assessed by microCT). (C) Preterm pups have lower mean lung density then term pups on day 3/day 0 (n=7-9; assessed by microCT; expressed in Hounsfield units (HU)). (D) Dark field score is significantly lower in term animals versus preterm animals on day 3/day 0. (E) Representative pictures of transversal sections of microCT volumes and dark field images in preterm and term animals on all time points.*
**p<0.05*

In vivo microCT-derived end-expiratory lung volume grew significantly over time in both the preterm and term animals (p<0.0001). Lung volume was not significantly different in both groups (p=0.1297; Figure 3.4A). Additionally, the proportion of aerated lung volume changed over time in both P and T (p<0.0001). Lungs of pups in P had a significantly lower proportion of aerated lung volume (p=0.0001), with significant posthoc differences on day 3/day 0 and day 5/day 2, and a strong trend towards less aerated volume on 7/day 4 (p=0.0022, p=0.0282 and p=0.0504 respectively). Conversely, non-aerated

lung volume decreased over time and was significantly larger in P (Figure 3.4B). Consequently, also the mean lung voxel density decreased with time ($p<0.0001$), with a generally significantly higher density in P compared to T ($p=0.0008$). Posthoc testing only revealed a significant difference on day 3/day 0 ($p=0.0085$; Figure 3.4C). At visual analysis, lungs of preterm pups, especially in the early time points but also later, were characterized by atelectatic lung regions, diffuse ground glass areas and sometimes air bronchograms. Representative images of the transversal microCT-sections can be found in Figure 3.4E.

In line with the microCT-results also the dark field intensity scores, reflecting lung aeration, increased over time in the preterm group ($p=0.0016$), but not in the term animals ($p=0.6723$) that obtained high scores already immediately after birth. Dark field intensity was overall higher in T in comparison to P ($p=0.013$), however at post hoc comparisons the difference was only significant on day 3/day 0 ($p<0.0001$; Figure 3.4D). Representative dark field images can be found in Figure 3.4E.

Preterm birth has persistent effects on lung structure and function

Seven days after birth, Lm and Lmw were not significantly different in preterm pups (P), versus term pups at the same corrected age (T; Figure 3.5A-B and Figure 3.5D). Because ex vivo left lung volumes were significantly smaller in P than T ($p=0.0042$; Figure 3.5C), total alveolar surface area (S) was decreased in P versus T ($p=0.0210$; Figure 3.5E). The difference in alveolar surface area was proportional to the difference in body weights of the groups ($p=0.3231$ if alveolar surface is normalized to weight). Arterial medial wall thickness was not significantly different between P and T ($p=0.5386$; Figure 3.5D and Figure 3.5F). Tissue mechanics were altered in preterm animals, with increased tissue damping and tissue elastance in P ($p=0.0104$ and $p=0.0240$ respectively; Figure 3.5G-H). Airway resistance tended to be higher in P ($p=0.1008$; Supplementary Table S3.3). At single wave oscillation, dynamic compliance corrected for weight was increased in P ($p=0.0079$; Figure 3.5I). Consequently, dynamic elastance corrected for weight was decreased in P ($p=0.0159$; Supplementary Table S3.3). Resistance was also significantly higher in P ($p=0.0140$; Figure 3.5J). In pressure-volume perturbations, static compliance and elastance, and inspiratory capacity (all corrected for weight), were not significantly different (Supplementary Table S3.3).

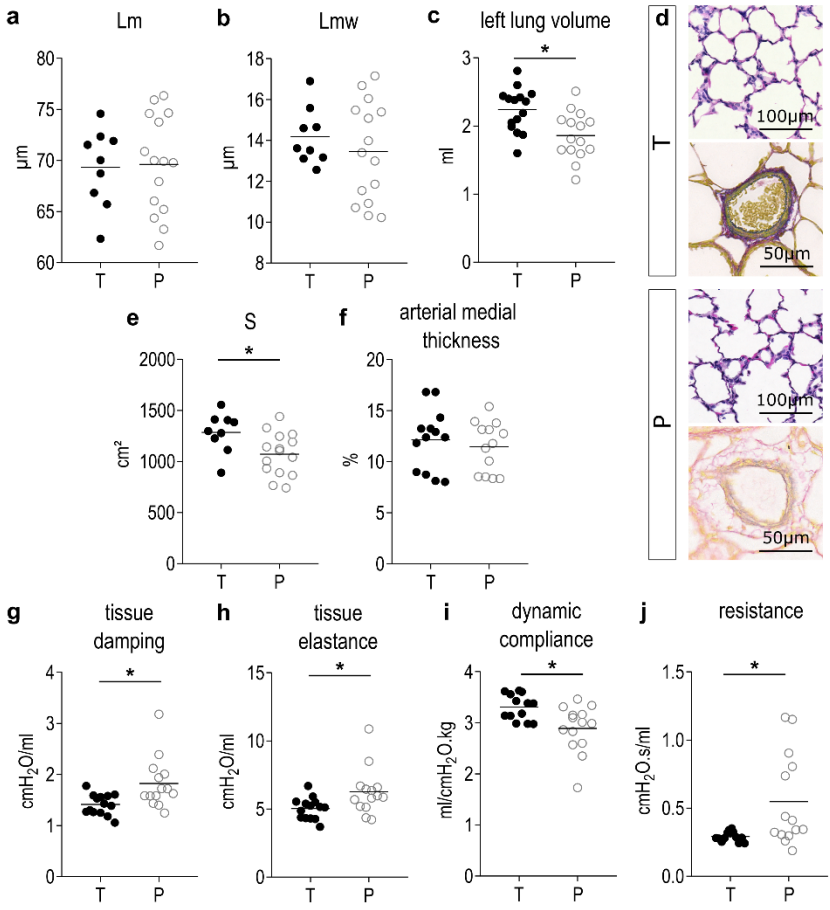


Figure 3.5. *(A) Lm and (B) Lmw are not affected by preterm birth. (C) Left lung volume is smaller in preterm animals. (D) Representative pictures of lung slides stained with HE indicate comparable alveolar structure in both groups. (E) Total alveolar surface is smaller in preterm animals compared to term controls. (F) Arterial medial thickness is not affected by preterm birth. (G-J) Lung function read-outs tissue damping, tissue elastance, dynamic compliance and resistance are significantly affected by preterm birth. *p<0.05*

Discussion

In this study we investigated the effect of preterm birth on lung development in preterm rabbits. In preterm fetal rabbits, harvested at birth, we demonstrated structural immaturity. Thicker septa with a higher cellularity and less complex airspaces indicate that preterm pups at day 28 of gestation are in the saccular stage of lung development. This observation is in line with early descriptive work on lung development in rabbits (155). Furthermore preterm fetuses exhibited thicker arterial walls, indicating higher pulmonary

vascular resistance. We also observed lower surfactant mRNA expression compared with term animals, indicating functional prematurity. Additionally we noted significantly decreased lung aeration on microCT and dark field imaging, in comparison to term pups. Together with earlier described maturational lung function changes in preterm rabbits at birth (205), this suggests preterm rabbits exhibit a phenotype comparable to respiratory distress syndrome (RDS). In the absence of respiratory support this resulted in increased work of breathing and apnea, with a mortality of 44,2% in the first hour of life. This proves the structural and functional immaturity of preterm rabbits born on day 28, which also constitutes the limit of viability in the rabbit. All animals born at day 27 die in the first hour after birth.

The primary objective of this study was to assess whether birth with immature lungs affects further lung development. By performing in vivo microCT and dark field imaging we established that preterm pups demonstrate delayed lung aeration, requiring at least 7 days before reaching recruitment comparable to term pups. Despite birth in a saccular stage of lung development, after 7 days the alveolar structure of pups born preterm was comparable to that of term pups on day 4, indicating continued postnatal lung development. However, because lungs of (smaller) preterm pups were smaller in size, they had lower total alveolar surface area. It is relevant to note here that preterm pups did not catch-up with the weight of the term pups, within the time course of the experiments, and that the difference in alveolar surface area was proportionate to the difference in weight. There were no differences in arterial wall thickness. Finally, despite the catch-up of structural lung development (relative to body weight), lung function on corrected day 4 was significantly affected with altered tissue mechanics, dynamic compliance and elastance.

Only a few research groups so far have investigated the effect of a precocious adaptation of immature lungs to postnatal life on further lung development. Experiments in preterm sheep and baboons indicate that preterm birth together with mechanical ventilation results in an alveolar developmental arrest (71, 72). However, when ventilator induced lung injury was reduced by the use of non-invasive respiratory support, the difference between preterm and term lungs disappeared (163, 206). In contrast, our data suggests that even in the absence of respiratory support and prenatal injury (e.g. chorio-amnionitis), preterm birth affects lung development in rabbits. Many studies have looked at different etiological factors involved in the pathogenesis of BPD, such as hyperoxia of ventilator induced lung injury, but to our

knowledge, this is the first study that focuses on the effect of preterm birth alone on lung development.

We postulate three possible explanations for the persistent effects of preterm birth on lung function and structure. Even in the absence of hyperoxia, oxidative stress might play an important role in arresting lung development after preterm birth. Room air contains a supraphysiological oxygen pressure in comparison to the uterine environment, and preterm lungs have weaker anti-oxidant defense mechanisms (172, 173). Recently it has also been shown that even room air affects the behavior of fetal lung mesenchymal stem cells (207). Another possible explanation is related to nurture and growth. With twice daily gavage feedings using a specific rabbit formula, we were not able to match in utero growth. This is similar to human neonatal care. Digestive immaturity, a deficit in specific nutritional components and the absence of placental or maternal growth factors might delay body growth and lung development (208). Experimental therapy with recombinant IGF1 is also based on the latter hypothesis (209). Finally, altered lung function might also be explained by atelectasis or delayed lung fluid clearance in preterm lungs, as suggested by the microCT imaging.

Our study has several limitations. First, the time frame of the study is limited. In the absence of a mother it is challenging to make rabbits pups thrive longer than 1 week. It is possible that lung development of preterm pups would catch-up if studied over a longer period of time. Second, the rabbit pups in this study were, despite proven structural and functional immaturity, born only 3 days (or 10% of gestational age) before term. Decreasing the gestational age of the animals might increase the effect on lung development. The high perinatal mortality at day 28 and our unpublished experiences with pups born at day 27, suggest however that day 28 is the limit of viability, in the absence of respiratory support measures. It should be noted that this mortality in the preterm group induces a selection bias, with only data of the stronger survivors included in the outcome parameters. Any study investigating the effect of preterm birth will have to balance maximal immaturity, with an acceptable survival rate. Adding surfactant administration or respiratory support measures might increase survival and enhance translatability, but can mask the effect of preterm birth alone. Third, the model lacks prenatal insults. On day 28 we deliver healthy preterm rabbits, while in humans prematurity rarely comes alone. Pre-eclampsia or chorioamnionitis do not only predispose for preterm birth, but also affect lung development in utero as part of a multiple hit concept (94, 210). The lack of a prenatal insult however could also be a strength, as this allows us to study

the individual effect of prematurity. Finally, in this study we focus on lung function and alveolar and arterial structure. Exploring airway or microvascular read-outs for instance, might lead to additional understanding of the effect of preterm birth (211-213).

As stated in the introduction, the phenotype of BPD in clinics has changed significantly. Furthermore, recent data suggest that long term respiratory outcome is affected in survivors of extreme preterm birth, regardless of a formal BPD diagnosis (197-199). Even late preterm birth has been shown to affect lung function later in life (214, 215). In general, pre- and postnatal factors (e.g. infection, oxygen, volutrauma) can contribute to the development of post-prematurity respiratory disease or BPD, however the one common denominator is preterm birth itself (1, 38). The findings in this study support the idea that preterm birth alone, even in the absence of any other pre- and postnatal injury, affects lung development.

Translation of findings from BPD animal models to new BPD in clinics has been difficult in the past (33). A possible explanation can be the lack of prematurity in commonly used rodent models for BPD (55, 56). The knowledge from this study asks for a paradigm shift in BPD research, focusing less on hyperoxia and ventilator induced injury, and more on the effect of prematurity. Large animal models such as preterm lambs and baboons mimic the structural and functional immaturity of an extremely preterm neonate, however practical and ethical constraints limit extensive use (71, 72). The preterm rabbit model could be an elegant compromise between the presence of prematurity and ease of experimentation (1, 57). The data in this report prove the functional and structural immaturity of lungs of rabbits born on day 28 of gestation, and thereby support the use of this model for the study of preterm lung injury and BPD.

Future research initiatives should focus on disentangling the physiological processes and molecular pathways involved in the effect of preterm birth on lung development (e.g. transcriptome analysis in preterm versus term lungs). Additionally, more research is needed to identify the growth factors and processes that are essential for normal in utero lung development in the second and third trimester. Insight in these mechanisms could lead to novel therapeutic strategies to improve the respiratory outcomes of survivors of preterm birth.

Appendix

Supplementary Table S3.1. Primer sequences used for qPCR in this study.

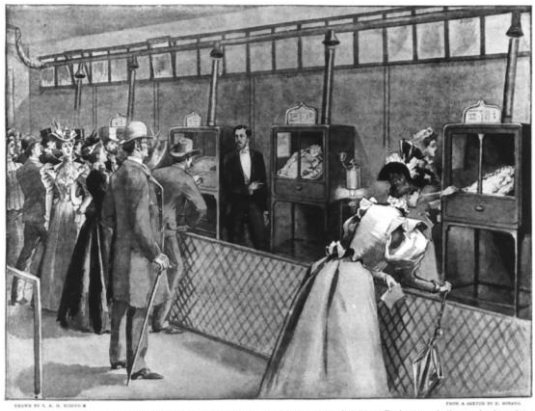
Supplementary Table S3.1. Primer sequences.	
surfactant protein B FW	ACTGTCTACAGGAAGTCTG
surfactant protein B RV	GAAGTAGACGTCAAGCACG
surfactant protein C FW	CAAAGAGGCCTTGATGGAGA
surfactant protein C RV	ATCTCTAGGACCATCTCGGT
YWHAZ FW	TGATTAGTGATGATGAACCG
YWHAZ RV	CACACAGAGGGCTACAATG
HPRT FW	GGTCTGGCCCTTAACTTCTCTGTGTTCTA
HPRT RV	GCGTGCTGTCTTTGTATGATTCTTCACTT
actin B FW	CAAGCGTGGCATCCTGAC
actin B RV	CTCGTTGTAGAAGGTGTGGTG

Supplementary Table S3.2. Weight and survival data of the pups used in this study

Supplementary Table S3.2. Baseline characteristics.				
	Preterm fetal	**Term fetal**	**Term**	**Preterm**
	28F	**31F**	**T**	**P**
	mean ± sd	mean ± sd	mean ± sd	mean ± sd
Day 0				
Number of pups (n)	15	10	15	25
Number of mothers (n)	5	3	4	8
Birth weight (g)	36.9±4.6	47.9±10.7	53.2±7.3	40.1±5.3
Day 7				
Survival (n, %)	-	-	15 (100%)	15 (60%)
Harvest weight (g)	-	-	61.5±7.7	53.5±6.0

Supplementary Table S3.3. Overview of lung function data

Supplementary Table S3.3. Lung function data.			
	Term **T** mean ± sd	**Preterm** **P** mean ± sd	*p-value*
Static measures			
Inspiratory capacity (ml/kg)	62.7±5.2	61.2±7.9	*0.5486*
Static compliance (ml/cmH_2O.kg)	2.2±0.5	2.5±0.7	*0.2488*
Static elastance (cmH_2O.kg/ml)	0.48±0.10	0.42±0.12	*0.3236*
Forced oscillation			
Tissue damping (cmH_2O/ml)	1.4±0.2	1.8±0.5	*0.0104*
Tissue elastance (cmH_2O/ml)	5.0±0.8	6.3±1.7	*0.0240*
Airway resistance (cmH_2O.s/ml)	0.13±0.04	0.21±0.17	*0.1008*
Single frequency oscillation			
Dynamic compliance (ml/cmH_2O.kg)	3.3±0.3	2.9±0.5	*0.0079*
Dynamic elastance (cmH_2O.kg/ml)	0.30±0.03	0.36±0.08	*0.0159*
Resistance (cmH2O.s/mL)	0.29±0.04	0.55±0.34	*0.0140*



"AN ARTIFICIAL FOSTER-MOTHER": BABY INCUBATORS IN THE BERLIN EXHIBITION

Lion incubators at the Berlin exposition, Berlin (1896):
This technology was adverted for as an Artificial Foster Mother. Until the 1940's preterm babies in incubators were regularly exhibited at fairs and luna parks.

Chapter 2

A semi-automated method for unbiased alveolar morphometry: validation in a bronchopulmonary dysplasia model

Salaets T., Tack B., Gie A., Pavie B., Sindhwani N., Allegaert K., Deprest J., Toelen J.
Manuscript in preparation

Abstract

Introduction - Reproducible and unbiased methods to quantify alveolar structure are important for research on many lung diseases. However, manually measuring alveolar morphometry is time consuming and inter-observer variability is high. The objective of this work was to develop and validate a fast, reproducible and accurate (semi-)automatic alternative.

Methods – A FIJI-macro was designed that automatically segments lung images to binary masks, and counts the number of test points falling on tissue and the number of intercepts of the air-tissue interface with a set of test lines. Manual selection remains necessary for the recognition of non-parenchymal tissue (conductive airways, arteries, veins) and alveolar exsudates. Volume density of alveolar septa ($V_{V_{sep}}$) and mean linear intercept of the airspaces (Lma) as measured by the macro were compared to theoretical values for 11 artificial test images and to manually counted values for 17 lungs slides using linear regression and Bland-Altman plots. Inter-observer agreement between 3 observers, measuring 8 lungs both manually and automatically, was assessed using intraclass correlation coefficients (ICC).

Results – $V_{V_{sep}}$ and Lma measured by the macro closely approached theoretical values for artificial test images (R^2 of 0.9750 and 0.9573 and bias of 0.34% and 8.7%). The macro overestimated $V_{V_{sep}}$ and underestimated Lma for lungs in comparison to manually counted values (R^2 of 0.8262 and 0.8288 and bias of -6.0% and 12.1%). Visually, semi-automatical segmentation was accurate. Most importantly, manually counted $V_{V_{sep}}$ and Lma had moderate to good inter-observer agreement (ICC 0.859 and 0.643), but agreements were excellent for semi-automatically counted values (ICC 0.956 and 0.900).

Conclusion - This semi-automatic method provides rapid, accurate and highly reproducible alveolar morphometry results. Future efforts should focus on refining methods for automatic detection of non-parenchymal tissue or exsudates, and for assessment of lung structure on 3D reconstructions of lungs scanned with microCT.

Introduction

During normal in utero lung development, large saccular airway structures with thick walls, evolve in to smaller thin-walled alveoli through a process called secondary septation (216). This process increases the total amount of surface available for gas exchange, and continues, however at a slower pace

until adolescence (217). During aging, alveolar size increases and surface area decreases again by coalescence (218). Several diseases are known to affect alveolar structure. Often however, there is only limited information on human lung tissue. BPD is characterized by larger alveoli, with thick septa (219). Emphysema results in larger alveoli and a decreased total alveolar surface area (220), while fibrosis locally increases septal wall thickness (221). The alveolar structural changes are often considered as primary read-outs in preclinical research on these disease patterns. Reproducible and unbiased methods to quantify alveolar structure are of uttermost importance for the quality of this research.

Stereology is a set of probabilistic methods used to define the physical properties of an irregular 3D structure based on 2D transsections. It has been used in lungs to determine the volume of tissue and air, alveolar surface area and even number of alveoli, in a way that is unbiased by assumptions on the shape of the airspaces (104). The most commonly used read-out is probably Lma, reflecting the volume to surface-ratio of the airspaces. Proper determination of Lma includes manual point and intercept counting on a high number of test points and lines (222). Manual counting is however very time consuming and our experience suggests important inter-observer variability.

Methods to automate or facilitate counting for alveolar morphometry (sometimes compatible with a stereological approach) have been developed. STEPanizer© is a free tool that loads test systems on digital images, but still involves manual counting (223). More recently a system for automated high –throughput analysis of whole lung slides was described, however it requires specific software (113). Plug-ins or macros for freely available software to count individual fields have also been developed (112, 224, 225). The common disadvantage of all these automated counting methods is, that they do not discriminate large airways and blood vessels from lung parenchyma, and exsudates or debris from septal tissue. The inability to filter out non-parenchyma or recognize inflammatory exsudates might importantly bias morphometric results.

The objective of this work was to develop and validate a fast, reproducible and accurate method for lung morphometry measurements that is compatible with a stereological approach.

Methods

Macro

For this purpose a FIJI-macro (226) was designed that automatically counts the number of test points falling on reference tissue (parenchymal lung tissue, excluding conductive airways and vessels; P_{ref}) or septal tissue (in contrast to air; P_{sep}), and the number of intercepts of the air-tissue interface (I) with a test line system. This macro automates the process of manual counting. The detection of non-reference tissue such as conductive airways, arteries, veins, pleura is based on manual selection. In contrast, the detection of septal tissue was based on automatic thresholding, however with additional manual cleaning of exsudates in the alveolar spaces. The user is asked to manually define the threshold between tissue and air, only on the first image of a batch (under the assumption that all images in a batch are stained in the same batch and acquired with the same microscope settings). The macro is developed for batches of lung fields of 500x500µm, in PNG-format.

The image processing and analysis sequence that is automatically followed for all PNG-images in a folder, is briefly described below and summarized in Figure 4.1:

- Resize the image to 680x680 pixels (pixel resolution of 0,735µm) to match the test system.
- Manually select the non-parenchymal tissue (conductive airways, arteries and veins) using a freehand selection tool (Figure 4.1A).
- Delete the points matching the non-parenchymal tissue on a quadratic test system of 64 points on a central counting area of 567x567µm. Count the remaining points in the grid: P_{ref} (Figure 1B).
- Process the image to an 8-bit image and subsequently to a binary mask of tissue versus air by applying the user-defined threshold (Figure 4.1C).
- Automatically detect exsudates, as unconnected particles of 0 to 1000 pixels that do not overlap with the border of the image and remove them from the mask (Figure 4.1D).
- Visually remove the detected exsudates from a duplicated original image. Manually select the exsudates that were not automatically detected (too large or in contact with septal tissue) and remove them from the mask (Figure 4.1E).

- Clean the mask, by filling white particles of 1 to 200 pixels within the septal tissue, by removing outliers and by a dilate-erode sequence (Figure 4.1F).
- Calculate a binary image containing all pixels that are black in both the clean mask and the test grid without the non-parenchymal points. Count the number of 1-pixel particles: P_{sep} (Figure 4.1G).
- Find the edges of the mask and create an image with only the edges (Figure 4.1H).
- Calculate a binary image containing all pixels that are black in both the edge image and an image of 8 horizontal (Figure 1I) and then 8 vertical lines (Figure 4.1J) of 567 pixels (unit length d = 104µm or 141.75 pixels). Count the number of particles of both the horizontal and vertical image: I.

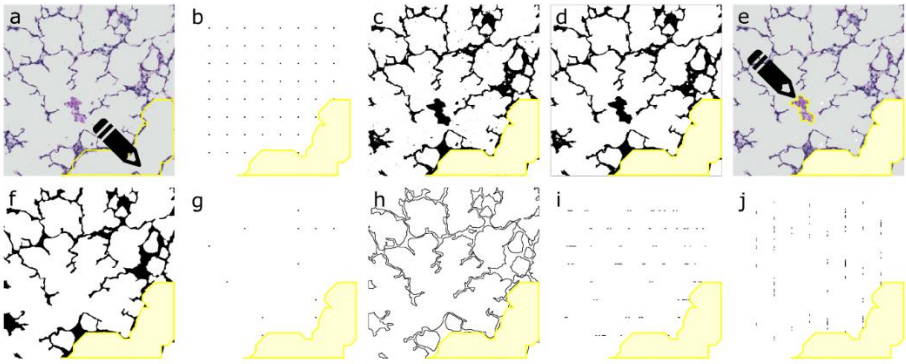


Figure 4.1. *Summary of image processing and analysis from lung field to P_{ref}, P_{sep} and I*. (A) Non-parenchymal tissue (in this case conductive airway) is selected manually. (B) P_{ref} is counted as the number of points of a 64 point test grid, outside the selection of non-parenchymal tissue. (C) Image is thresholded to binary mask. (D) Automatically all small and unconnected exsudates are removed. (E) Larger and connected exsudates require manual selection. (F) Mask is cleaned and smoothened. (G) P_{sep} is counted as the number of points of the test grid that are common to the cleaned mask. (H) The edges of the cleaned mask are aligned. (I-J) I is counted as the number of intercepts of a test system of 8 horizontal and 8 vertical lines with the edge-image. *P_{ref} = reference points, P_{sep} = septal points, I = intercepts.*

The macro generates one .csv file containing P_{ref}, P_{sep} and I for every lung field. It also automatically saves intermediate images and selections for an eventual visual check. The macro (morphometry_v2.1.ijm) will available for download with the paper, together with a user manual and second macro that can be used for checking the accuracy of the algorithm in batch (checkmorphometry_v2.1.ijm).

Artificial test images

To test and validate the counting procedure in the macro, a set of artificial binary images was made in FIJI. All images were 2040x2040 pixels and were composed of wallpaper-like repetitions of a combination of geometric figures for which surface and perimeter can be calculated. Within each square-shaped repetition the geometric figures (in white) were randomly organized against a black background. Based on the surface and perimeter of the geometric figures, a theoretical "septal volume" density ($V_{V_{sep}}$) and mean linear intercept of the geometric "airspaces" (Lma) could be calculated.

$$V_{V_{sep}} = \frac{2040^2 - \sum surface_{geometric\ figures}}{2040^2}$$

$$Lma = \frac{4 * (1 - V_{V_{sep}})}{\sum perimeter_{geometric\ figures}}$$

Twenty (partially overlapping) fields of 680x680 pixels were randomly extracted using the results of a random number generator as coordinates. An example is shown in Figure 4.2A. These 20 fields were automatically counted using a slightly modified version of the macro that omitted the steps converting a color image to a cleaned binary mask.

Lung images

Lung slides were obtained from previous experiments in the preterm rabbit model for BPD that has been described previously (130, 146). Briefly, pregnant rabbit dams underwent cesarean section on day 28 of gestation, (normal gestation is 31 days). Preterm rabbit pups were raised in an incubator with controlled temperature and humidity, in either normoxia (21% O_2) or hyperoxia (95% O_2) conditions. Pups were hand-fed, twice daily, by gavage, using a milk replacer appropriate for rabbits. Colostrum and probiotics were added to the feeding. Vitamin K was supplemented intramuscularly, and antibiotics were administered to prevent infections. On day 7 pups were euthanized. Lung slides were obtained from experiments approved by the Ethical Committee for Animal Research at KULeuven as P059/2016.

Lungs of preterm rabbits on day 7 were harvested "en-bloc" after thoracotomy, with the trachea and carina intact. Right bronchus was ligated and right lungs removed and snap frozen for eventual molecular analysis. A 20G catheter was inserted and fixed in the trachea, through which the left lung was connected to a pump filled with paraformaldehyde 4%, delivering a constant hydrostatic pressure of 25cmH_2O. For 24h, the left lung was kept in

a bath for immersion fixation, while connected to the system for pressure-fixation. Afterwards lungs are rinsed in phosphate buffered saline, dehydrated in ethanol and embedded in paraffin as a whole. Sagittal 5µm thick microtome sections were made and stained with hematoxylin and eosin.

Lung slides were scanned with a whole slide scanner (Axio Scan® Slide Scanner, Zen Zeiss, Oberkochen, Germany), generating 1 CZI-file for each whole lung slide. A customized Groovy Script for FIJI randomly sampled 20 fields of 500x500µm within the edges of the lung tissue, and exported them as PNG-images. The Groovy Script for random sampling will be available together with the paper (extract_some_tiles_from_tissue_czi_v02.groovy). An example is shown in Figure 4.3A.

Manual counting

The results of semi-automatic counting with the FIJI-macro were compared to manual counting, the current gold standard. The same 20 lung fields were converted to JPG-images for counting with STEPanizer (223). A quadratic test system of 64 points and 8 horizontal and 8 vertical lines with a total length of 6670µm (d = 104 µm) was used, with an equal length and position as the test system in the macro (227). The points falling on non-parenchymal tissue ($P_{non-par}$), P_{sep} and I were manually counted.

Morphometric read-outs

Volume density of alveolar septa ($V_{V_{sep}}$) and mean linear intercept of the airspaces (Lma) of each test image or lung slide was calculated using the following formulas, according to the guidelines for quantitative assessment of lung structure of the American Thoracic Society (104):

$$P_{ref} = 64 - P_{non-par}$$

$$V_{V_{sep}} = \frac{\sum P_{sep}}{\sum P_{ref}}$$

$$Lma = 2 * d * \frac{\sum P_{ref} - \sum P_{sep}}{\sum I}$$

Other read-outs such as mean transsectional wall length (Lmw), Lm (Lm = Lma + Lmw) (148) and the surface area density of the air spaces ($S_{V_{air}}$) (104, 227) can be calculated based on P_{ref}, P_{sep} and I. When the total lung volume is available, volume and surface density can be converted to absolute volume (V_{sep}) and surface (S) respectively. For the purpose of validation of the counting macro, we only focused on $V_{V_{sep}}$ as a read-out based on point

counting alone, and Lma as a read-out based on both point and intercept counting.

Validation

For validation, we first tested the macro on a set of 11 artificial test images, and compared the automatically calculated $V_{V_{sep}}$ and Lma to the theoretical values. On lung images, $V_{V_{sep}}$ and Lma were determined both manually and semi-automatically by a single observer for a set of fields from 17 lungs and by 3 independent observers for a set of 8 lungs. Semi-automatic results of 17 lungs were compared to manual results for a single observer (TS). The results of the 3 observers (TS, BT and AG) were used for assessment of inter-observer agreement (reproducibility). TS, BT and AG are trained in pediatrics, with a research interest in lung development. Erik Verbeken, an expert lung pathologist, confirmed the manual counting methods.

Results were compared to gold standard (theoretical or manual values) by means of linear regression and Bland-Altman plots using GraphPad Prism® 7.0 software (GraphPad, La Jolla, California, USA). We report R^2-values, a best-fit equation for the regression line and relative bias (with confidence interval) derived from the Bland-Altman plots. We calculated ICC (two way random effects model for absolute agreement) using SPSS® version 25 (IBM, Armonk, New York, USA) for inter-observer agreement between the 3 observers (228).

Results

We observed a very strong correlation between the results of the macro and the theoretical results of the artificial test images. For $V_{V_{sep}}$ R^2 was 0.9750 and the trend line was almost on the identity line (Y = 0.9824*X + 0.006889; Figure 4.2B). Bias was 0.35% (CI -7.5 - 8.2%; Figure 4.2D). For Lma R^2 was 0.9573 and the trend line was close to identity (Y = 1.000*X – 5.230; Figure 4.2C). The Bland-Altman plot confirmed an underestimation by the macro with a bias of 8.7% (CI -17.2 – 34.7%; Figure 4.2E).

For the lung images, the correlation between manual counting and the macro was slightly less strong (Figure 4.3), despite the use of exactly the same fields and exactly the same test system for both manual and semi-automatic counting. R^2 was 0.8262 for $V_{V_{sep}}$ with semi-automatically obtained results that somewhat overestimated the manual values (Y = 1.137*X - 0.01488; Figure 4.3B). Overestimation was confirmed by a bias of -6.0% (CI -18.9 – 6.9%; Figure 4.3D). Almost always, counting with the macro resulted in a

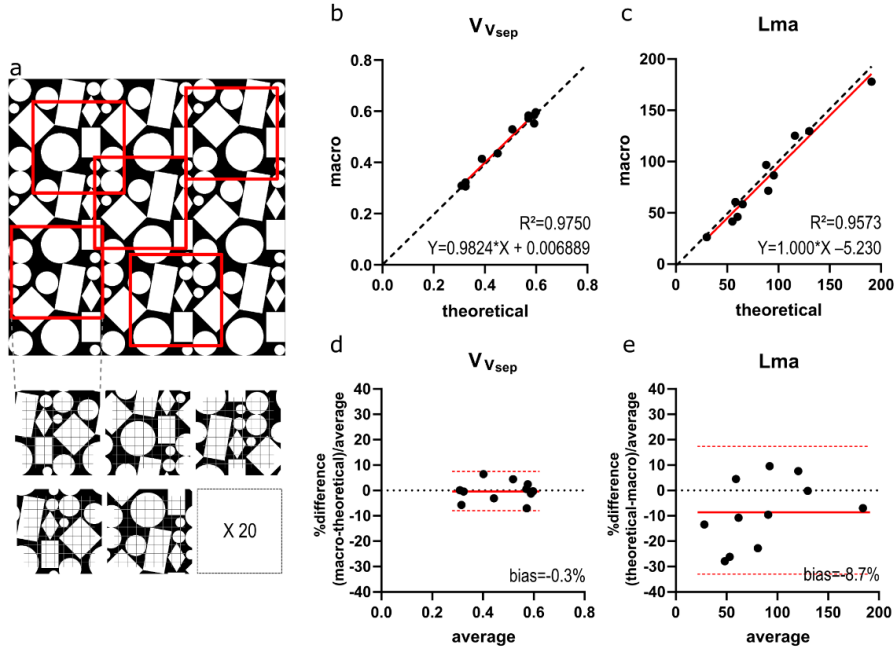


Figure 4.2. *(A) Illustration of an artificial test image with sampling strategy. (B-C) Macro results for $V_{V_{sep}}$* and Lma† plotted against the theoretical values. Regression line is plotted in red, dashed line represents identity (y=x). (D-E) Bland-Altman plots for $V_{V_{sep}}$* and Lma†, plotting the relative difference [(theoretical – macro)/average] to the average result. Red line indicates bias, with confidence interval (dashed red line). *volume density of alveolar septa ($V_{V_{sep}}$); †mean linear intercept of the airspaces (Lma)*

higher number of septal points (P_{sep}) in comparison to manually counting. For Lma R^2 was 0.8288, but the trend line indicated a considerable underestimation of Lma (Y = 0.5494*X + 21.89; Figure 4.3C) in comparison to the manual gold standard. The Bland-Altman plot in Figure 4.3E confirms underestimation of Lma with a bias of 12.1% (CI 0.6 – 23.5%). As an explanation we noted a consistently higher number of intercepts (I) counted with the macro versus manual counting. In general, overlaying the cleaned edge image over the original lung field reveals accurate automatic segmentation of the septal tissue (Figure 4.4).

Evaluation of inter-observer agreement of the manual results demonstrates an ICC of 0.859 (good agreement) for $V_{V_{sep}}$ and 0.643 (moderate agreement) for Lma. ICC increased when the semi-automatic results were used, indicating improved reproducibility. For $V_{V_{sep}}$ ICC was 0.956, while for Lma ICC was 0.900 (both excellent agreement).

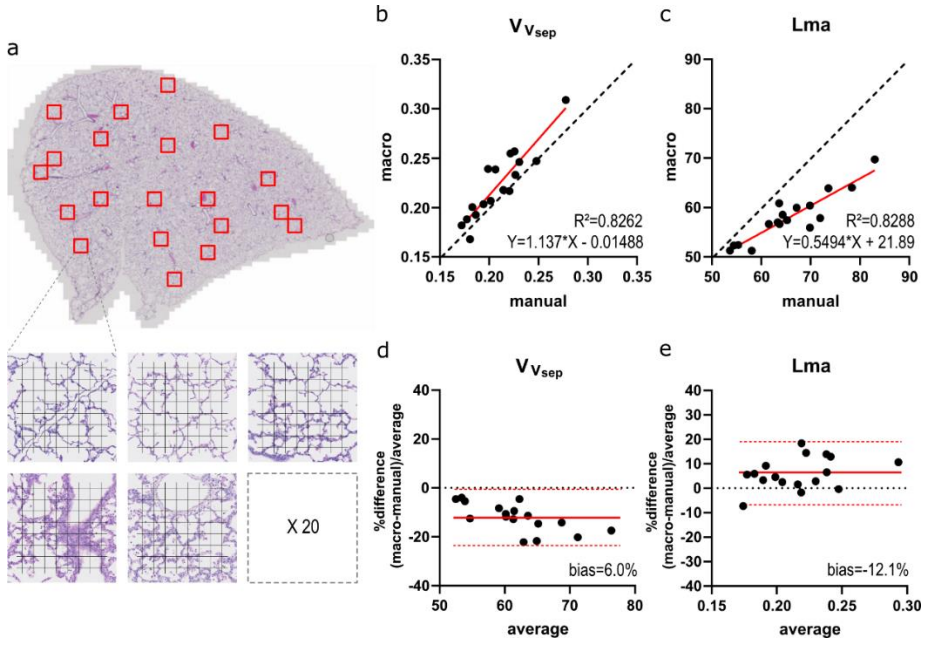


Figure 4.3. *(A) Illustration of a lung slide with sampling strategy. (B-C) Macro results for $V_{V_{sep}}$* and Lma† plotted against the manual values. Regression line is plotted in red, dashed line represents identity (y=x). (D-E) Bland-Altman plots for $V_{V_{sep}}$* and Lma†, plotting the relative difference [(manual – macro)/average] to the average result. Red line indicates bias, with confidence interval (dashed red line). *volume density of alveolar septa ($V_{V_{sep}}$); †mean linear intercept of the airspaces (Lma)*

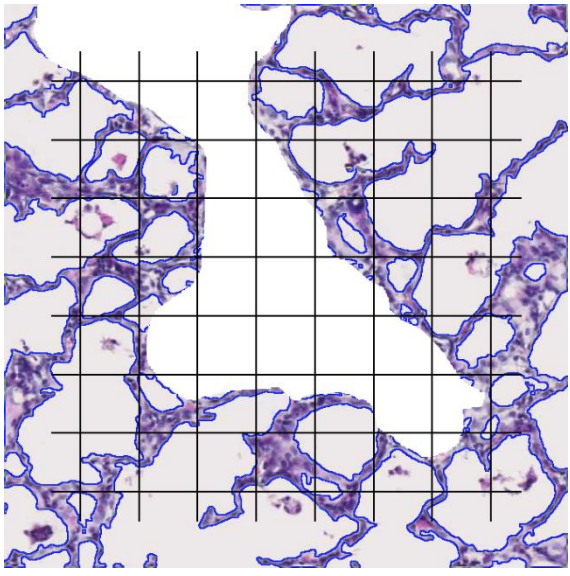


Figure 4.4. *Illustration of a semi-automatically segmented lung image, with overlaid test system. Central white zone hides a manually selected vascular structure. Crossing points not falling on the vascular structure are counted as P_{ref}, crossing points falling on septal tissue are counted as P_{sep} and intersections of the test lines with the blue line indicating the edge of the septal tissue are counted as I.*

Discussion

The objective of this paper was to develop and validate a semi-automatic counting method for lung morphometry. First, we demonstrated in a series of artificial test images, that the use of the macro, results in values that closely approach the true geometric characteristics of the images. We hypothesize that agreement is not perfect because of a potential sampling error, using a quadratic test system on a quadratic repetition of irregularly placed geometric objects. The “find edges”-function, might also decrease the inner size of the geometric objects by 1 pixel at each edge. Both effects are expected to be irrelevant for irregular and non-binary lung images.

Second, we showed on lungs that semi-automatic counting approached the results of manual counting, however R^2 was considerably lower than for the artificial test images. Manual counting is considered as the gold standard, however it is unclear here which methods captures best the true morphometric characteristics of the tissue. In general, a visual check reveals appropriate segmentation with the semi-automatic method. Our results also suggest at least that the semi-automatic method has a higher resolution, leading to a higher count of P_{sep} and I (e.g. the irregularity of the alveolar surface might result in 2 distinct intercepts with a 1-pixel thick test line, where the human eye only sees the alveolar surface touching the test line).

Third, we also observe a relevant inter-observer variability of the manual results. Semi-automatic counting on the other hand resulted in highly reproducible results. We cannot conclude that semi-automatic counting is more accurate than manual counting since the latter is still the gold standard, but it is clear that results are more reproducible. It is possible that the macro makes a systematic error in estimating the true morphometric characteristics of the tissue, while manual counting results in a random error, hence its high inter-observer variability. We conclude that this macro provides a valid alternative to manual counting of morphometry that is at least more reproducible than manual counting.

Another major advantage of semi-automatic counting is efficiency. We estimate that the time necessary to count is reduced by at least a factor 3, in comparison to the described manual method. The time cost of counting however is, next to the capability of the observer to remain undistracted during this cumbersome and repetitive task, strongly dependent on the amount of inflammatory exsudates and non-parenchymal areas on the fields, since these require manual selection. Previously described automatic

counting methods did not exclude non-parenchymal areas or did not discriminate between septal tissue and exsudates (112, 113, 225). This can bias the results of these methods. If fields with many vascular structures or alveolar exsudates are included, P_{sep} and I will probably be overestimated. If these fields are avoided, sampling will be biased towards more peripheral or less diseased areas (less inflammation), while truly random sampling is the cornerstone of a stereological approach. Despite some attempts with WEKA, a trainable machine learning algorithm (229), we were not able to develop an automatic method that could reliably distinguish conductive airways, arteries or veins from alveolar parenchyma, or exsudates from alveolar septa. Further improvements in segmentation tools based on machine learning, might lead to a completely automated determination of lung morphometry. At the moment however, the human eye remains essential for these tasks. We believe the described macro provides the fastest possible solution that takes this in to account.

We also want to stress the accessibility of this method. The macro will be available for download as a supplement to this paper and runs on FIJI (FIJI is just ImageJ), open access software that is widely used for image analysis in biomedical sciences (226). The software can be downloaded from https://imagej.net/Fiji/Downloads.

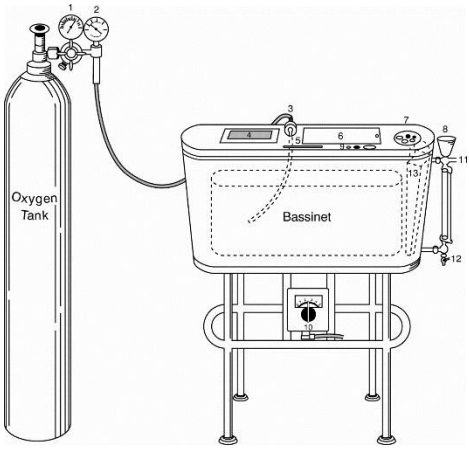
Overall, it is important to note that counting itself is only a minor aspect of determining the structural characteristics of the lung. Because it is of no relevance for the validation of the counting method, we did not focus in this paper on lung processing and sampling. We acknowledge however that it contributes to a great extent to the accuracy of the morphometric read-outs (104). Fixation under a standard pressure is key to standardize the level of inflation of the lungs after harvest. Ideally, a strong fixative such as glutaraldehyde is needed to preserve the lung for structural assessment. However, as we wanted to maintain the immunogenicity of the proteins in the lungs for eventual immunohistochemistry, we used paraformaldehyde. Furthermore, we also did not assess shrinkage of the lung throughout further processing (dehydration and embedding).

It is essential that every piece of the lung has an equal chance of getting counted. Many sampling strategies have therefore been proposed, involving sectioning and rotating blocks of lung tissue (104). As the lungs of preterm rabbits are rather small we chose to sample 1 complete central sagittal section on 1 slide, encompassing both the anterior and posterior lobe. From this 1 transection, 20 fields were sampled randomly for counting.

Unpublished data in our model suggest that within the same lung any sagittal section, except for the most peripheral mm at both sides, results in comparable values for $V_{V_{sep}}$ and Lma, suggesting that our sampling approach is sufficient in this model. Finally, only the pixels of the fields falling on the test system are sampled or counted. As a general rule, the accuracy of the measurement increases if the proportion of the tissue that is sampled (sampling density) increases (104). Using the described approach (total of 1280 test points, with an expected $V_{V_{sep}}$ around 0.20) we expect a relative standard error of the mean of 5% (230). If a higher accuracy is required the number of fields per lung can easily be increased.

Computerized approaches offer the possibility to increase the sample density, at a lower time cost, and thereby improve the accuracy of the result. Some authors have developed methods to quantify structural characteristics on a complete lung slide, not requiring sampling of a set of fields (113). However, the time cost of manually cleaning these whole lung slides from non-parenchymal areas and exsudates, outbalances the accuracy gained by a sample density of 100%. Furthermore it requires specific software and hardware with a high computational speed. Finally, the advances in virtual histology should be noted. Using imaging techniques such as microCT, 3D reconstructions and segmentations have been made on which structural characteristics of pulmonary acini can be measured in 3D, instead of estimated from 2D images (114, 115, 231).

In conclusion, we developed and validated a semi-automatic method to count alveolar morphometry, compatible with a stereological approach. This semi-automatic method provides highly reproducible results in what is currently the fastest possible way, taking the need for manual selection of non-parenchymal areas and exsudates in to account. We think the use of this method would enhance the quality of the morphometric results in research on BPD, emphysema or fibrosis. Future efforts should go to the development of methods for automatic detection of non-parenchymal areas and exsudates and for assessment of structure on 3D reconstructions of lungs scanned with microCT.



A prototype of the Hess incubator, Chicago (1934):
The Hess incubator was the first incubator to include an oxygen source and made oxygen supplementation common practice in the care for preterm babies.

Chapter 3

Transcriptome analysis of the preterm rabbit lung after seven days of hyperoxic exposure

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*(*shared first)*
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Abstract

The neonatal management of preterm born infants often results in damage to the developing lung and subsequent morbidity, referred to as BPD. Animal models may help in understanding the molecular processes involved in this condition and define therapeutic targets. Our goal was to identify molecular pathways using the earlier described preterm rabbit model of hyperoxia induced lung-injury. Transcriptome analysis by mRNA-sequencing was performed on lungs from preterm rabbit pups born at day 28 of gestation (term: 31 days) and kept in hyperoxia (95% O_2) for 7 days. Controls were preterm pups kept in normoxia. Transcriptomic data were analyzed using Array Studio and Ingenuity Pathway Analysis (IPA), in order to identify the central molecules responsible for the observed transcriptional changes. We detected 2217 significantly dysregulated transcripts following hyperoxia, of which 90% could be identified. Major pathophysiological dysregulations were found in inflammation, lung development, vascular development and reactive oxygen species metabolism. To conclude, amongst the many dysregulated transcripts, major changes were found in the inflammatory, oxidative stress and lung developmental pathways. This information may be used for the generation of new treatment hypotheses for hyperoxia-induced lung injury and BPD.

Introduction

Preterm birth leads to a dysregulated development in many organs that are not yet adapted to postnatal life. In the pulmonary system, this often results in BPD, a complex disease in which multiple factors interact. Premature lungs (most frequently in the saccular stage of lung development) are exposed to hyperoxic and hyperbaric conditions during ventilation and administration of supplementary oxygen. This process is often amplified by pre- or postnatal infections, fluid imbalance, malnutrition, genetic predispositions, etc. Persistent inflammation overwhelms natural tissue repair and leads to an arrest in alveolar development and vascular development (232). The resulting lung parenchyma is composed of rudimentary alveoli, interstitial thickening and a dysmorphic capillary configuration (219). These morphological changes can be categorized as a developmental arrest.

The aforementioned risk factors are well-known and the application of less invasive ventilation strategies and permissive hypoxemia have proven their efficacy. They became the cornerstones of current neonatal management (69,

233). Despite this, BPD continues to be a frequent complication of premature birth. About 15–25% of very low birth weight (VLBW) infants develop BPD, and rates in extremely low birth weight (ELBW) infants are even higher (38). Moreover, BPD remains an important risk factor for lung disease later in life (234).

At the molecular level BPD is still poorly studied compared to other pathologies. Several individual molecules assessed in broncho-alveolar fluid have been proposed to play a key role in BPD like interleukin-8 or matrix metalloproteinase-3 (26, 235). Most studies however were hypothesis driven or they examined certain pathways in lung disease and repair.

Animal models are needed to study disease mechanisms and to evaluate new preventive or therapeutic strategies for BPD. Most research into BPD has been performed in hyperoxia-exposed rodent models (236-238). Unfortunately, their lung development differs from humans as birth occurs in the early saccular stage of lung development. In rodents, alveolarization only starts several days postnatally while humans start alveolarizing in utero. This entails that not all the relevant findings can be extrapolated to the human context. As such, there is an advantage to study the hyperoxia induced lung injury in animal models that mimic human development more closely. The rabbit is considered a large animal model that has favorable characteristics for this research question. In contrast to rodents, rabbits indeed start alveolarizing prior to birth, as do pigs, sheep, primates and humans (155). Moreover, rabbits are easy to handle and house, have a large litter size and allow technical manipulation of the fetus at relevant developmental stages which makes them ideal for the study of effects of perinatal interventions. Therefore we used the rabbit as a model for the study of hyperoxia-induced lung injury in the preterm born pup (130).

Rather than focus on single putative molecule or pathways, we herein used a more complete ‘systems biology’-approach (239), that allows the exploration of larger patterns and networks. Herein we analyze transcriptome data using software (IPA) that combines expression data with current generic knowledge of molecular interactions. Our goal was to obtain more complete insights in the pathophysiology and generate new theoretical therapeutic strategies for preterm hyperoxia induced lung injury.

Methods

Animal model

We earlier described in detail the preterm rabbit model for hyperoxia-induced lung injury (130). Briefly, time mated pregnant does underwent cesarean section at 28 days (term = 31 days) of gestation (early saccular lung developmental phase). The pups were randomly divided into two groups: (1) the normoxia group, where pups were housed in 21% oxygen and (2) the hyperoxia group, where pups were nursed in hyperoxia (≥ 95% oxygen); both for seven days. The model has been earlier described in detail elsewhere (84, 177). Briefly, immediately after delivery, pups were placed in an incubator at 32°C, fed twice daily via an orogastric tube and received prophylactic antibiotics and vitamin K. All animals were treated according to current guidelines of animal well-being, and the experiments were approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine of the Katholieke Universiteit Leuven (Leuven, Belgium).

Harvesting of specimens

At day 7 of life, pups were harvested for histological and transcriptome analysis. After euthanizing the pups with a mixture of embutramide 200mg, mebezonium 50mg and tetracain hydrochloride 5mg (intracardiac injection of 0.1mL T61, Intervet Belgium NV, Mechelen, Belgium), thoracotomy was performed and both lungs and trachea were removed “en bloc”. The left bronchus was ligated and the left lung was snap-frozen, while the right lung was processed for histological analysis (see below). Six pups per group were used for histological analysis. Out of the snap frozen samples, four left lungs from the normoxia group, and four from the hyperoxia group were randomly selected for transcriptome analysis.

Lung injury score

A 20G catheter was inserted in the trachea where after the right lung was fixed with 4% paraformaldehyde by immersion and perfusion under a constant hydrostatic pressure of 25cm H2O for 24 hours before embedding. Paraffin sections were stained with hematoxylin and eosin (HE) for further histological analysis. Lung injury score was assessed on 20 random high-power fields (400x total magnification) for each pup. The selection of random fields was obtained by successive random displacements (each at least one high power field in length) from the initial position provided that at least 50% of each field was occupied by lung alveoli. A three-tiered scheme was used to quantify each of five histological parameters: presence of neutrophils in

alveolar and interstitial space, hyaline membranes, debris filling the airspaces and septal thickening (110). The data were analyzed statistically using student-T test, significance was set with a p-value < 0.05.

RNA isolation and sequencing

RNA isolation was performed on snap frozen lung tissue using the RNeasy mini kit (Qiagen). Tissue lysis and homogenization was performed in 1200µL Buffer RLT using the TissueLyser system (Qiagen). Following tissue disruption and homogenization, samples were centrifuged for 3 min at 14000rpm in a benchtop micro centrifuge. Lysate was transferred to fresh tubes and an equal volume of 70% ethanol was added. 600µL of sample was added twice to a spin column, with 2 RNeasy spin columns used per sample. Following wash steps RNA was eluted in 50µL RNAse-free H2O. Total RNA quantification was performed using the Nanodrop 1000 spectrophotometer (Thermo Scientific). RNA integrity was assessed using the RNA 6000 Nano Kit and the Bioanalyser (Agilent Technologies) according to the manufacturer's recommendations. Between 1 and 2µg of total RNA was used as input material for sequencing library preparation which was performed with the TruSeq RNA Library Preparation Kit (Illumina) according to the manufacturer's protocol. Fragmentation was performed for 6 minutes. 8 PCR cycles were used for PCR enrichment step. Samples were indexed to allow for multiplexing. Sequencing libraries were quantified using the Qubit fluorometer (Life Technologies). Library quality and size range was assessed using the Bioanalyser (Agilent Technologies) with the DNA 1000 Kit (Agilent Technologies) according to the manufacturer's recommendations. Individual libraries were diluted to a final concentration of 2nM and pooled for sequencing. Pooled libraries were then sequenced in a single lane of an Illumina HiSeq2000 flow cell generating single end 50bp reads.

Data analysis

Expression values were calculated per gene and normalized to reads per kilobase per million reads (RPKM) values as described by Mortazavi (240). An RPKM value of >1 was applied as cut-off to distinguish between 'noisy' or 'leaky' transcription and 'true' mRNA expression levels for comparison between samples. For comparison between both groups, FC were calculated, indicating the ratio of expression. We applied a cut-off of >2 or <-2, in order to decrease the number of transcripts in our analysis. False discovery rates (FDR) were calculated as a measure for statistical significance of the difference in expression of a gene between the two groups. We considered a transcript change significant if FDR was <0.01.

Further analysis was performed using the Ingenuity Pathway Analysis (IPA) software. The IPA 'Upstream Regulator Analysis' predicts upstream regulators by combining the directional expression changes from our mRNA-sequencing, and knowledge from prior experimental reports on causal effects between molecules (endogenous and exogenous), compiled in the IPA Knowledge Base. Upstream Regulator Analysis calculates a z-score based on the edge of dysregulation of all the downstream molecules and the uniformity of the existing evidence about the upstream-downstream relation, for every upstream regulator known to have a causal effect on at least 4 dysregulated transcripts. Z-scores of <-2 and >2 respectively indicate a significant inhibition and activation state of the upstream regulator, regardless of the actual expression level of these molecules (241). The Network Generation Algorithm links molecules based on experimentally observed interactions, and orders these molecule based on their interconnectedness. In general, the more interactions with other network members, the more central a molecule will be in a network.

Results

Lung injury score

Histological assessment of both normoxia as well as hyperoxia exposed lungs reveals a clear increase in neutrophil infiltration and alveolar wall thickening (Figure 5.1A-B). Generating a combined lung injury score revealed a significant difference between both groups (Figure 5.1C) with an increase in lung injury observed in animals held in hyperoxia ($p<0.01$).

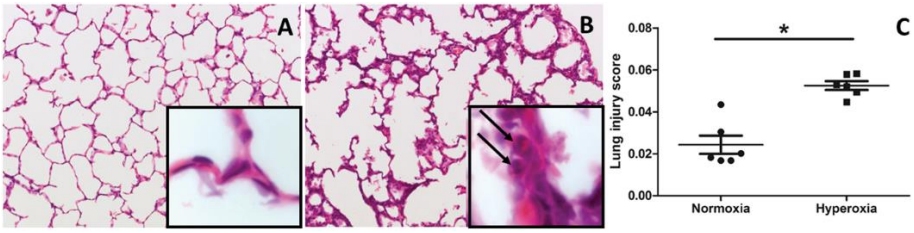

Figure 5.1. HE staining and lung injury score. *A-B: HE staining of a pressure fixed lung of pups held in normoxia (A) or hyperoxia (B) for 7 days of life. In the magnification square, neutrophils are marked by the arrows. C: lung injury score assessing 5 different parameters: neutrophils in alveolar airspace, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspace, alveolar septal thickening.*

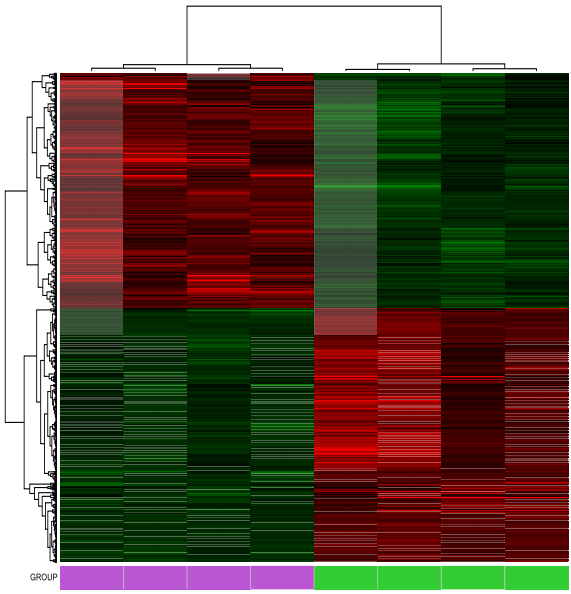

Figure 5.2. Heat map of differentially expressed genes. *Animals held in normoxia (to the left, purple) or in hyperoxia (to the right, green) with FC+-2 and FDR <0.01. Color intensity displayed in the heat map are the Log2 transformed RPKM gene expression value. These are normalized to relative low (green) and high (red) signal intensities shown in the heat map key.*

We identified 2217 transcripts that were dysregulated (FDR < 0.01) in hyperoxia by at least a factor 2 (FC>2 or FC<-2). A heat map of these genes visualizes a clear distinction between hyperoxia and normoxia. (Figure 5.2). We were able to link IPA-gene names to 1989 of the 2217 dysregulated transcripts. This group was used for further analysis in IPA. 1023 of these transcripts are upregulated in hyperoxia, with fold changes up to +235.786. The other 966 are downregulated, as far down as -38.476. The ten most upregulated molecules in hyperoxia are shown in Table 5.1, the ten most downregulated in Table 5.2. A complete list of these 1989 dysregulated molecules can be found in Supplementary Table S5.1.

Rank	IPA name	Full name	FC	FDR
1	RLN1	Relaxin 1	235.786	$1.04x10^{-5}$
2	MT-ND4L	NADH dehydrogenase subunit 4L	153.771	$6.80x10^{-3}$
3	CCL2	Chemokine (C-C motif) ligand 2	148.355	$1.20x10^{-5}$
4	MMP3	Matrix metalloproteinase 3	100.723	$7.57x10^{-6}$
5	BIRC 3	Baculoviral IAP repeat containing 3	53.728	$2.48x10^{-5}$
6	PTX3	Pentraxin 3, long	43.956	$4.00x10^{-4}$
7	SRXN1	Sulfiredoxin 1	42.437	$2.00x10^{-5}$
8	CXCL8	Chemokine (C-X-C motif) ligand 8	37.831	$2.63x10^{-5}$
9	DKK1	Dickkopf WNT signaling pathway inhibitor 1	34.030	$7.57x10^{-5}$
10	LIF	Leukemia inhibitory factor 1	33.378	$6.00x10^{-4}$

doi:10.1371/journal.pone.0136569.t001

Table 5.1. Upregulated molecules. *Ten most upregulated molecules in our dataset; FC: fold change, FDR: false discovery rate.*

Rank	IPA abbreviation	Name	FC	FDR
1	VIPR1	Vasoactive intestinal peptide receptor 1	-38.476	$2.69x10^{-5}$
2	C2orf40	Chromosome 2 open reading frame 40	-32.645	$1.00x10^{-4}$
3	ACE	Angiotensin I converting enzyme	-26.315	$8.19x10^{-6}$
4	CD300LG	CD300 molecule-like family, member g	-22.293	$1.39x10^{-5}$
5	SCBG3A1	Secretoglobulin, family 3A, member 1	-21.677	$8.40x10^{-3}$
6	S100A1	S100 calcium binding protein A1	-19.873	$3.59x10^{-5}$
7	CYP1A1	Cytochrome 450, family 1, subfamily A, polypeptide 1	-17.727	$2.00x10^{-4}$
8	COLEC10	Collectin subfamily member 10 (C-type lectin)	-15.835	$2.00x10^{-4}$
9	CA4	Carbonic anhydrase IV	-14.724	$5.00x10^{-4}$
10	APLNR	Apelin receptor	-14.407	$4.38x10^{-5}$

doi:10.1371/journal.pone.0136569.t002

Table 5.2. Down regulated molecules. *Ten most down regulated molecules in our dataset; FC: fold change, FDR: false discovery rate.*

Pathway analysis of differentially expressed genes

Running the Upstream Regulator Analysis, we identified 58 inhibited and 160 activated significant upstream regulators. Both endogenous and exogenous molecules are included in this analysis. All significant upstream regulators which are classified as transcription factors are displayed in Table 5.3. All other upstream regulator types can be found in Supplementary Table S5.2.

IPA ID	Entrez Gene Name	FC	Z-score	p-value of overlap	# of DTM
Activated upstream transcription factors					
NFE2L2	nuclear factor, erythroid 2-like 2		4.796	$3.80x10^{-13}$	76
CREB1	cAMP responsive element binding protein 1		3.661	$5.10x10^{-8}$	79
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A		2.998	$1.25x10^{-5}$	49
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1		2.952	$2.15x10^{-3}$	29
CDKN2A	cyclin-dependent kinase inhibitor 2A		2.808	$1.67x10^{-5}$	33
ATF4	Activating transcription factor 4	2.637	2.490	$1.59x10^{-14}$	38
SMAD2	SMAD family member 2		2.425	1.00	6
KDM5B	lysine (K)-specific demethylase 5B		2.400	$4.19x10^{-4}$	21
MYC	v-myc avian myelocytomatosis viral oncogene homolog	3.143	2.359	$1.59x10^{-12}$	136
HMGB1	high mobility group box 1		2.303	$6.46x10^{-2}$	10
MAFK	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K		2.170	$1.00x10^{-2}$	5
EHF	ets homologous factor		2.121	$1.35x10^{-1}$	9
MAFG	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G		2.000	$7.58x10^{-3}$	5
MAFF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F		2.000	$9.41x10^{-3}$	4
Inhibited upstream transcription factors					
TWIST1	twist family bHLH transcription factor 1		-3.182	$8.83x10^{-8}$	32
GATA2	GATA binding protein 2		-2.724	$3.03x10^{-6}$	19
KLF2	Kruppel-like factor 2		-2.396	$1.42x10^{-6}$	28
PRDM1	PR domain containing 1, with ZNF domain		-2.255	$5.26x10^{-3}$	17
NEUROG3	neurogenin 3		-2.219	1.00	5
HOXA7	homeobox A7		-2.200	$8.52x10^{-2}$	5
HOXA9	homeobox A9		-2.183	$1.74x10^{-1}$	18
PLAG1	pleiomorphic adenoma gene 1		-2.164	$4.62x10^{-3}$	11
GLI2	GLI family zinc finger 2		-2.159	$6.27x10^{-2}$	11
NEUROG1	neurogenin 1		-2.111	$1.63x10^{-2}$	11

DTM: dysregulated target molecules

doi:10.1371/journal.pone.0136569.t003

Table 5.3. Upstream regulators. *Transcription regulators, recognized to be upstream regulators (z-score +-2), with actual fold change, z-score, p-value of overlap and number of downstream effectors.*

Significantly dysregulated molecules were sorted by their function or involvement in pathophysiological processes. An overview of the most relevant dysregulated molecules is provided in Table 5.4.

IPA ID	Full Name	FC	FDR
Inflammation			
IL1A	Interleukin 1, alpha	6.864	$3.60x10^{-3}$
IL1B	Interleukin 1, beta	5.092	$3.70x10^{-3}$
PTGS2	Prostaglandin-endoperoxide synthase 2	23.625	$7.00x10^{-4}$
NOS2	Nitric oxide synthase 2, inducible	-8.495	$1.90x10^{-5}$
CSF3	Colony stimulating factor 3 (granulocyte)	17.090	$2.30x10^{-3}$
CCL2	Chemokine (C-C motif) ligand 2	148.355	$1.20x10^{-5}$
CXCL8	Chemokine (C-X-C motif) ligand 8	37.831	$2.63x10^{-5}$
Lung development			
PPARG	Peroxisome proliferator-activated receptor gamma	4.268	$2.90x10^{-3}$
SPP1	Secreted phosphoprotein 1	28.159	$3.30x10^{-3}$
CAV1	Caveolin 1, caveolae protein, 22kDa	-6.282	$3.10x10^{-5}$
DKK1	Dickkopf WNT signaling pathway inhibitor 1	34.030	$7.57x10^{-5}$
ACE	Angiotensin I converting enzyme	-26.315	$8.19x10^{-6}$
Vasculogenesis			
VEGFA	Vascular endothelial growth factor A	-5.539	$1.00x10^{-4}$
TEK	TEK tyrosine kinase, endothelial	-7.844	$1.90x10^{-5}$
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	-6.540	$1.82x10^{-5}$
ANGPT2	Angiopoetin 2	12.089	$7.00x10^{-4}$
ROS metabolism			
SOD2	Superoxide dismutase 2, mitochondrial	2.349	$8.90x10^{-3}$
TXNRD1	Thioredoxin reductase 1	7.262	$1.20x10^{-3}$
SRXN1	Sulfiredoxin 1	42.437	$2.00x10^{-5}$
CYP1A1	Cytochrome 450, family 1, subfamily A, polypeptide 1	-17.727	$2.00x10^{-4}$

doi:10.1371/journal.pone.0136569.t004

Table 5.4. Dysregulated genes of special interest. *Dysregulated genes of special interest for hyperoxia-induced lung injury; in order of appearance; FC: fold change, FDR: false discovery rate.*

Inflammation

Although the inflammatory mediators are numerous, several appear to be active molecules, such as interleukin 1α (IL1α, FC 6.864, FDR 3.6x10-3), interleukin 1β (IL1β, FC 5.092, FDR 3.7x10-3), cyclo-oxygenase 2 (PTGS2 or COX2, FC 23.625, FDR 7.00x10-4), nitric oxide synthase 2 (NOS2, FC -8.495, FDR 1.90x10-5), granulocyte colony stimulating factor (G-CSF or CSF3, FC 17.090, FDR 2.30x10-3) and chemokines (C-C motif) ligand 2 (CCL2, FC 148.355, FDR 1.20x10-5) and (C-X-C motif) ligand 8 (CXCL8 or IL8, FC 37.831, FDR 2.63x10-5).(Figure 5.3) Upstream analysis highlights tumor necrosis factor-alfa (TNFα) as a major regulator (z-score 4.465). Downstream effectors of the TNFα-pathway include inflammatory mediators (IL1α, IL1β) but also molecules involved in vascular development (vascular endothelial growth factor A (VEGFA), angiopoietin 2 (ANGPT2)), lung development (secreted phosphoprotein 1 (SPP1), caveolin 1 (CAV1), dickkopf WNT signaling pathway inhibitor 1 (DKK1), peroxisome proliferator-activated receptor γ (PPARγ)) and ROS metabolism (cytochrome P450 1A1 (CYP1A1), superoxide dismutase 2 (SOD2)) (Supplementary Table S5.3). Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB, z-score 2.952), the intracellular transcriptional effector of TNFα is also recognized as an activated upstream regulator. Other significantly dysregulated inflammatory upstream regulators are interferon γ (IFNγ) and several other interleukins (1α, 1β, 2, 3, 4, 5, 6, 12β,

17α, 17F, 18) albeit with lower z-scores (2.756, 3.269, 4.525, 2.004, 3.235, 2.247, 2.131, 2.369, 2.049, 2.658, 2.574, 2.348 resp.). The central molecules involved in the inflammatory pathway have been displayed in Figure 5.3.

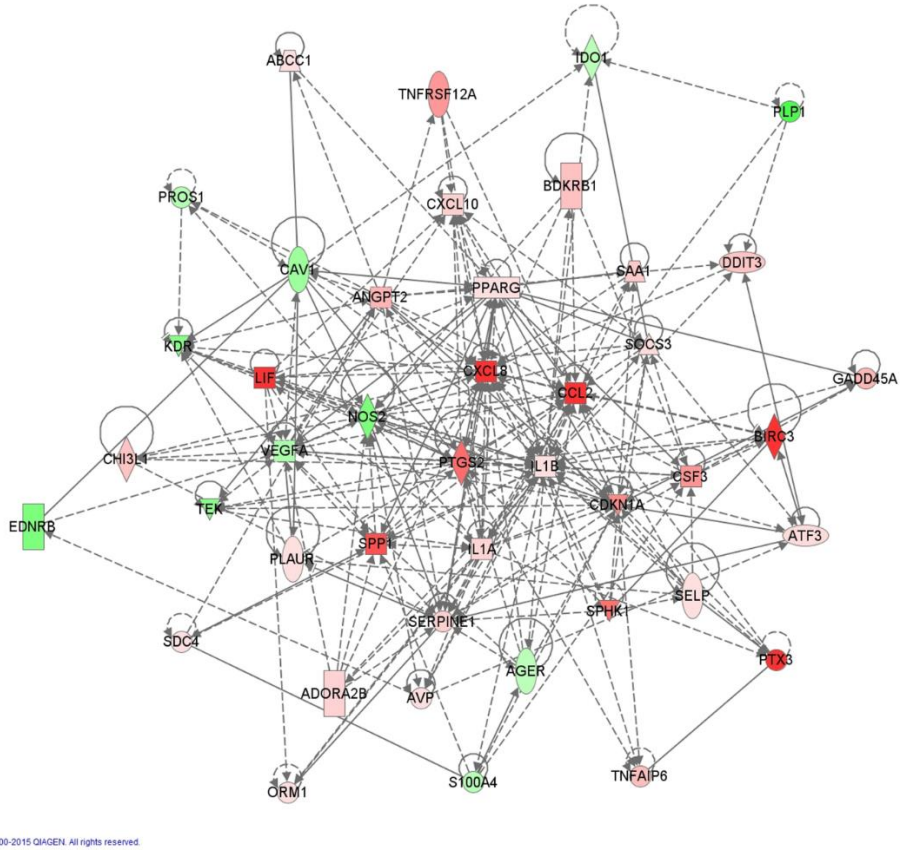

Figure 5.3. Network of dysregulated molecules involved in IPA-function: 'Inflammatory Response'. *The following molecules play a central role: IL1α (FC 6.864), IL1β (FC 5.092), PTSG2 (FC 23.625), NOS2 (FC -8.495), CXCL8 (FC 37.831) and CCL2 (FC 148.355). In order to increase readability, only molecules with a FC of +/- 4 and 3 or more connections are shown in this figure.*

Lung development

Molecules involved in lung development which are dysregulated in our model are shown in Figure 5.4. Central molecules in this network are PPARγ (FC 4.268, FDR 2.90x10-3), SPP1 or osteopontin (FC 28.159, FDR 3.30x10-3), CAV1 (FC -6.282, FDR 3.10x10-5), NOS2 and VEGFA. Other highly dysregulated molecules in this network are DKK1 (FC 34.030, FDR 7.57x10-5) and angiotensin I converting enzyme (ACE, FC -26.315, FDR 8.19x10-6). Our upstream analysis identified Kruppel-like factor 2 (KLF2) (z-score -2.396) as an important inhibited transcription factor. Downstream effectors of KLF2

include mediators of inflammation (CCL2, CXCL8, PTGS2), vascular development (ANGPT2) and lung development (PPARγ, ACE) (Supplementary Table S5.4).

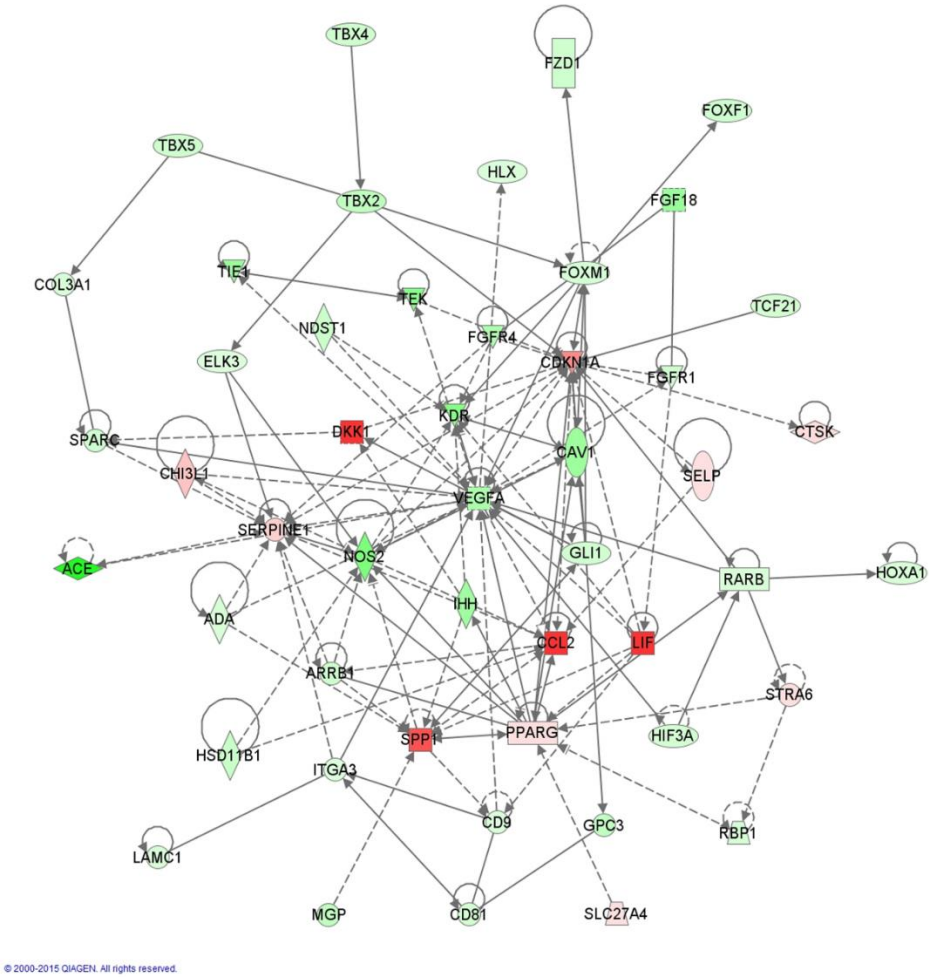

Figure 5.4. Network of dysregulated molecules involved in IPA-function 'Formation of lung'. *Central, broadly connected molecules are SPP1 (FC 28.159), CAV1 (FC -6.282), PPARγ (FC 4.268), NOS2 (FC -8.495) and VEGFA (FC 5.539). Relevant mediators of lung development mentioned in the following references are added (242-245)*

Vascular development

VEGFA (FC -5.539, FDR 1.00x10-4) is down regulated in our model. TEK tyrosine kinase (TEK or TIE2, FC -7.844, FDR 1.90x10-5) and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1, FC -6.540, FDR 1.82x10-5), the molecular receptors of the angiopoetins, are down regulated while angiopoetin 2 (ANGPT2, FC 12.089, FDR 7.00x10-4, z-score 2.612), a

receptor antagonist, is upregulated and also recognized as a significantly activated upstream regulator.

ROS metabolism

Several enzymes involved in scavenging and detoxifying the reactive oxygen species are upregulated, like superoxide dismutase 2 (SOD2, FC 2.349, FDR 8.90x10-3), thioredoxin reductase 1 (TXNRD1, FC 7.262, FDR 1.20x10-3) and sulfiredoxin 1 (SRXN1, FC 42.437, FDR 2.00x10-5). Furthermore several cytochrome P450 (CYP) enzymes are down regulated, especially CYP1A1 (FC -17.727, FDR 2.00x10-4). A major upstream regulator influencing the above mentioned enzymes is nuclear factor erythroid 2-like 2 (NFE2L2, z-score 4.796), downstream effectors include inflammatory mediators (IL1β, PTSG2, …) as well as VEGFA (Supplementary Table S5.5).

Discussion

Under the assumption that a rabbit model has several advantages over rodent models for studying BPD with regard to lung development and the potential of experimental therapeutic interventions, we used this preterm rabbit model (130) to comprehensively describe possible pathways involved in the pathophysiology of BPD using modern techniques. Herein, we used whole transcriptome analysis as this offers a comprehensive amount of expression information, which allows to document the intricate and complex processes that occur in the damaged developing lung. Lung injury score demonstrated results that parallel lung damage seen in clinical BPD-samples (219). This confirms the relevance of insights in the molecular mechanisms of BPD obtained in our model. We focused on four relevant biological networks in this context: inflammation, lung development, vascular development and ROS metabolism.

Several inflammatory mediators were significantly dysregulated in our model, confirming an important role for inflammation in the pathogenesis of hyperoxia-induced lung injury. The pattern of dysregulation of these molecules is consistent with a pro-inflammatory state. This is in line with data from other animal models (246) and clinical studies (247). The increased TNFα-activity induces many of the transcriptional changes seen in our model, some of these downstream mediators have been described in hyperoxia exposed term rabbit lungs (248). It remains unclear if this TNFα-activity is increased as a protective pathway or merely secondary to the inflammation in the preterm lung. Experimental therapy both with TNFα and anti-TNFα

show an improvement in rats exposed to hyperoxia (249, 250). To date this has not been corroborated in larger animal models.

Many preclinical studies have reported on molecules involved in lung development (242, 243, 251, 252) that can account for the developmental arrest in BPD. Several of these lung development molecules are also dysregulated in our model.(Fig 4) PPARγ, which was also an upregulated molecule in our study, is important for normal lung maturation (244). Furthermore, beneficial effects have been achieved with stimulation of PPARγ, e.g. by rosiglitazone in the rat model of hyperoxia-induced lung injury (253-255). A second upregulated molecule is DKK1, which is an inhibitor of WNT-signaling. Its known involvement in branching morphogenesis and alveolization could account for a role in the developmental arrest of BPD (245, 251). Other dysregulated factors of pulmonary development include SPP1 (downregulated during secondary septation (243)), CAV1 (involved in alveolar septation (256) and acute lung injury (257)) and ACE (involved in secondary septation (258)).

We also suggest a role for KLF2, which we identified as an important upstream regulator. It is highly expressed in adult mouse lung tissue and was demonstrated to be important for late stage lung development (saccular and alveolar phase) (259). Furthermore, KLF2 is involved in endothelial homeostasis and angiogenesis (260) and has negative regulatory effects on immune cell activation (261).

Normal lung development depends, certainly in the later stages, on vascular development and the development of mesenchymal structures: blood vessels and respiratory epithelium are in constant interaction. Several molecular factors responsible for angiogenesis in the lung (242) are changed in our hyperoxia model. VEGFA seems a central molecule in most of our generated networks. The administration of VEGFA to attenuate lung damage has been tried in different models with varying results (262, 263). Furthermore knockdown of VEGF abolishes the protective effects of mesenchymal stem cells in a rat model of hyperoxic lung injury thereby demonstrating the critical role of VEGF (264).

The upregulation of several enzymes involved in scavenging and detoxifying of ROS can be considered as protective against the potential adverse effects of hyperoxia. NFE2L2 plays a critical role in the induction of this response (265). Another important player we identified, is CYP1A1. Induction of CYP1A1 via the aryl hydrocarbon receptor (AHR) pathway, using β-

naphthoflavone or omeprazole attenuates hyperoxic lung injury, which suggests a protective effect of CYP1A1 (266, 267).

We acknowledge certain limitations of our study. First, in this model we use only hyperoxia to create lung injury. This is an imperfect approximation for human 'new' BPD (36, 232). In human BPD, several other factors (barotrauma, infections, malnutrition, fluid imbalance, antenatal corticosteroids, surfactant, …) are involved, yet are not included in this experimental model. Secondly, there are some methodological limitations. mRNA sequencing was performed on whole, mixed, lung tissue which offers an overview of expression changes in all pulmonary cell types. This results in loss of 'spatial resolution' as specific up regulation in individual cell types constituting the airways, vasculature and parenchyma cannot be quantified in this manner. Furthermore, our samples were only examined at a single time point (7 days of hyperoxic exposure), a loss of temporal resolution has to be considered as well. Although in our previous study no functional differences were found between term and preterm animals held in normoxia (130), no transcriptome analysis has been performed on term animals. Some of the expressional differences might be explained by prematurity alone without a clear hyperoxic cause. A final limitation is the use of selective filtering of the obtained results. First, filtering for small RPKM's and fold changes may result in the loss of meaningful subtle dysregulated master switch molecules. The used software, IPA (Upstream Regulator Analysis and Network Generation Algorithm) is also dependent on prior knowledge and many expression-regulating relationships remain still to be discovered or described. This may bias our analysis, in favor of molecules which have been frequently investigated.

Despite these theoretical limitations, other results of our experiment show obvious similarities with previous studies analyzing specific molecules which may be involved in the pathophysiology of BPD. Of those, two studies (243, 268) examined the changes in expression profile during the last stages of lung development in rats without any hyperoxic lung damage. Important genes which were upregulated during the saccular stage were those involved in development. Both studies found the Wnt signaling pathway to be affected by different genes like Fzd1 (frizzled-1) or Ptn (pleiotrophin). Our data demonstrated DKK1, an inhibitor of Wnt signaling to be upregulated after exposure to hyperoxia. In the study of Bhattacharya (269) whole lung tissue was used from mice exposed to 100% hyperoxia for 10 days. They found Ahr to be a key regulator in the pathophysiology of hyperoxic lung damage. This receptor is known to activate CYP1A1, one of the main dysregulated

molecules in our database. Furthermore, Cdkn1a (cyclin-dependent kinase inhibitor 1a), which is a cell cycle regulator, is upregulated both in mice as in our rabbit lung tissue. The exact mechanism how this gene is involved in the pathophysiology of hyperoxic lung injury remains to be determined.

To conclude, our study is the first to perform whole transcriptome analysis on hyperoxia-induced, preterm rabbit lung tissue identifying several central molecular mediators. The major pathophysiological features of BPD we identified are inflammation, lung developmental arrest, dysregulated vascular development and increased ROS metabolism. We will use the molecular master switches revealed in this analysis for the future definition of novel preventive strategies to avoid or reduce the occurrence of BPD.

Appendix

Supplementary Table S5.1. *All transcription changes*

Supplementary Table S5.2. *All upstream regulators*

Supplementary Table S5.3. *Downstream effectors of TNFα*

Supplementary Table S5.4. *Downstream effectors of KLF2*

Supplementary Table S5.5. *Downstream effectors of NFE2L2*

All supplementary material can be found online on the website of PLOS One: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0136569

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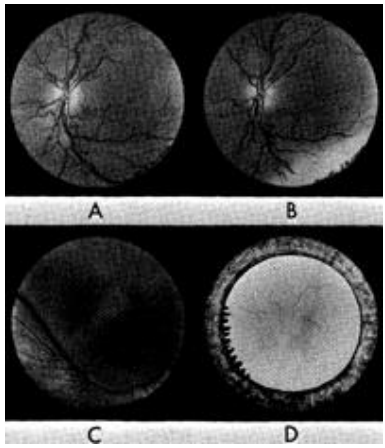

Description of a new eye disease called retrolental fibrodysplasia (RLF) in surivors of preterm birth by Owens and Owens (1948):
Free administration of oxygen resulted in a reduced mortality of hyaline membrane disease, but at the cost of RLF.

Chapter 4

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

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Revised version submitted to Pediatric Research

Abstract

Background – 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_2) or hyperoxia (95% O_2) and within each condition to treatment with 5mg/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 BPD 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.

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 (3, 270). 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 (59), the rabbit model combines hyperoxia with structural ànd functional prematurity (1). 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 (173). 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 (130, 146). 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 (178). 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, angiopoetin 2 (ANGPT2) and secreted phosphoprotein (SPP1) (Supplemental Figure S6.1). 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 (271, 272). 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 (273, 274). Furthermore, in an adult hyperoxia based mice model for ALI, simvastatin resulted in decreased lung inflammation and endothelial permeability (275).

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 KU Leuven. 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 (35mg/kg; Nimatek®; Eurovet Animal Health BV, Bladel, The Netherlands) and xylazin (6mg/kg; XYL-M®; VMD, Arendonk, Belgium), placed in supine position and euthanized with a mixture of 200mg embutramide, 50mg mebezonium and 5mg tetracain hydrochloride (intravenous bolus of 1ml 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% O2, 32°C and 50% humidity.

Randomization and treatment

After one hour, surviving pups were weighed and marked for inclusion in the experiment. Pups were randomly assigned to either normoxia (N; 21% O_2) or hyperoxia (H; >95% O_2) conditions, and within each condition to a group receiving 5mg/kg of simvastatin per day (N+SS5 and H+SS5) or to a control group (N and H) (Figure 6.1A). In a second round of experiments, a higher dose of 20mg/kg of simvastatin per day was tested. In this experiment pups were randomized in again 4 groups (N, H, H+SS20, N+SS20) (Supplemental Figure S6.6A). Randomization was performed in such a way that pups from the same litter were evenly distributed over the 4 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 [146]. 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 5min/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 200ml/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 described [146]. Pups were anesthetized with intramuscular ketamine (35mg/kg) and xylazin (6mg/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 8ml/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 25cm H_2O for 24 hours in a purposely designed pump. Samples were subsequently embedded in paraffin, and 5µm 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

(500µmx500µm) using Stepanizer software (223). Calculations were performed as described earlier (148). To assess lung tissue inflammation, the acute lung injury score was used in 20 randomly selected fields (100µmx100µm) 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 (110).

Vascular morphometry was performed on sections stained with Miller's elastic staining. In at least 20 peripheral arteries with an external diameter between 30-100 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 x 100%), as previously described (148).

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 (500µmx500µm) relative to the whole field.

Finally, for every lung 2 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_2O_2 in methanol) and protein blockage. Smooth muscle cells and myofibroblasts were stained with a mouse anti-human α smooth muscle actin (αSMA) antibody at 0.71µg/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 10 airways per lung was measured, and normalized to airway perimeter (276). Furthermore the αSMA-positive area in the parenchymal tissue was quantified on 20 randomly selected lung fields (250µmx250µm) 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 10.25µg/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 (277). In order to quantify this on 2D images, at least 20

high power fields (100µmx100µm) 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, IL1β, 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 Supplementary Table S6.2. 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 ΔΔCt-values, while 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 (146). We defined tissue damping as the primary outcome measure. To pick-up a 50% correction of the effect of hyperoxia on tissue damping, with a power of 80% and an α of 0.0167 (Bonferroni correction for 3 comparisons) in a two-tailed t-test, 7 surviving pups in each group were needed.

Simvastatin 5mg/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 (Supplementary Table S6.3). 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 5mg/kg did not alter survival in H, however it did reduce survival in N (p=0.04) (Figure 6.1B). Weight gain was not significantly different among the groups (Supplementary Table S6.3).

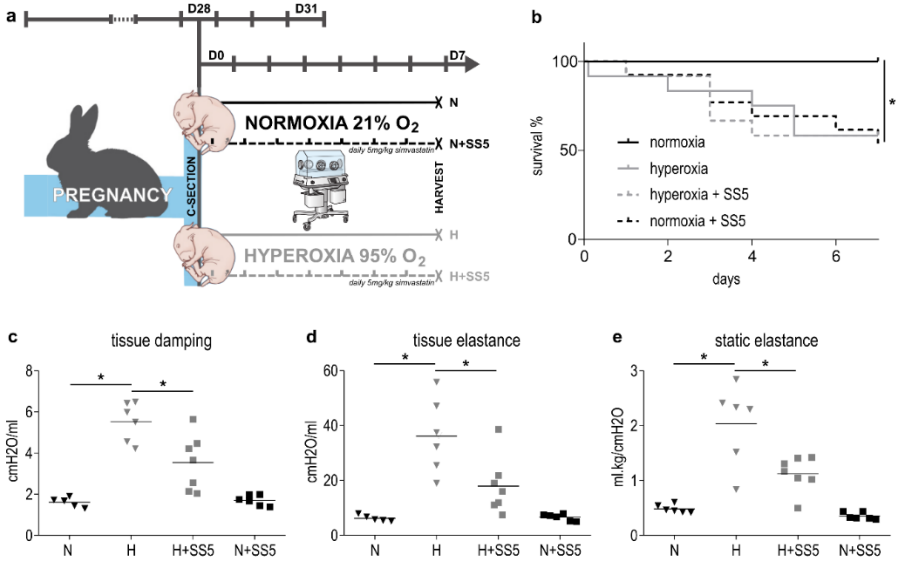


Figure 6.1. *(A) At 28 days of gestation pups are delivered by cesarean section, and randomized to 4 groups (n=7-13): two groups are raised in normoxia (21% O_2) and two are raised in hyperoxia (95% O_2) with in both conditions a control group and a group treated with 5mg/kg simvastatin per day. All pups were harvested at day 7. (B) Survival is significantly decreased by 5mg/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*

Simvastatin 5mg/kg improves lung function in hyperoxia

Tissue damping and tissue elastance were increased in H (versus N; both p<0.0001). Simvastatin (5mg/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) (Figure 6.1C-D).

Comparably, static elastance was increased in H (versus N; p<0.0001), and this effect was partially prevented by 5mg/kg simvastatin (H+SS5 versus H; p=0.001). Simvastatin had no significant effect in normoxia on static elastance (Figure 6.1E). Furthermore, inspiratory capacity was decreased in H (versus N; p<0.0001). In N+SS5 (versus N) a non-significant trend towards increased inspiratory capacity in 5mg/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 S6.4).

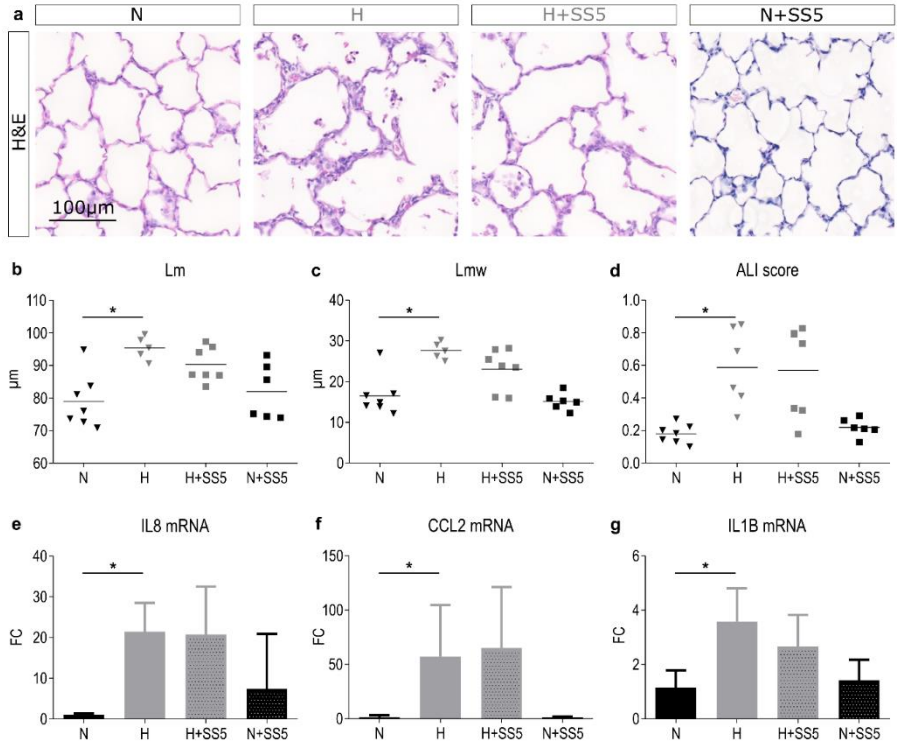


Figure 6.2. *(A) Representative pictures of HE-stained lung slides depict altered alveolar structure and inflammation in hyperoxia, which is not prevented by simvastatin. (B) Lm (mean linear intercept) and (C) Lmw (mean transsectional wall length) reflect alveolar structure and are not significantly affected by 5mg/kg simvastatin. (D) ALI score is a composite score of septal thickening, neutrophil invasion and exsudates, protein debris and reflects lung injury or inflammation, and is not affected by 5mg/kg simvastatin. (E-G) Expression of IL8, CCL2, IL1β as measured by qPCR is increased in hyperoxia, but not affected by 5mg/kg simvastatin (n=5-7). *p<0.05*

Observations on dynamic compliance and elastance in the different subgroups are in parallel with their static counterparts, however 5mg/kg simvastatin only affected these dynamic lung function measures in hyperoxia,

not in normoxia. Airway resistance was neither affected by hyperoxia, nor 5mg/kg simvastatin (Supplemental Table S6.4).

Simvastatin 5mg/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 transsectional wall length (Lmw, H versus N; p=0.0004). Simvastatin (5mg/kg) did not affect alveolar morphometry significantly (H+SS5 versus H; p= 0.53 and p=0.17 for Lm and Lmw respectively; Figure 6.2A-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 5mg/kg treated pups (Figure 6.2A and 6.2D). Similarly, high mRNA-expression of the pro-inflammatory 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 5mg/kg simvastatin, nor is their expression affected in normoxia (Figure 6.2E-G).

Simvastatin 5mg/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 αSMA 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 5mg/kg simvastatin (H+SS5 versus H; p=0.13). Simvastatin (5mg/kg) did not alter collagen or αSMA content in normoxia (Figure 6.3A, B, D, E). Finally, we observed a thicker (αSMA-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; Figure 3C, F).

Simvastatin 5mg/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 5mg/kg simvastatin resulted in a complete prevention of the effect of hyperoxia (H+SS5 versus H; p=0.004). There was no effect of 5mg/kg simvastatin in normoxia (Figure 6.4A, 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 (5mg/kg), however, did not alter pulmonary capillary conformation, in contrast to arterial medial thickness (Figure 6.4B, D).

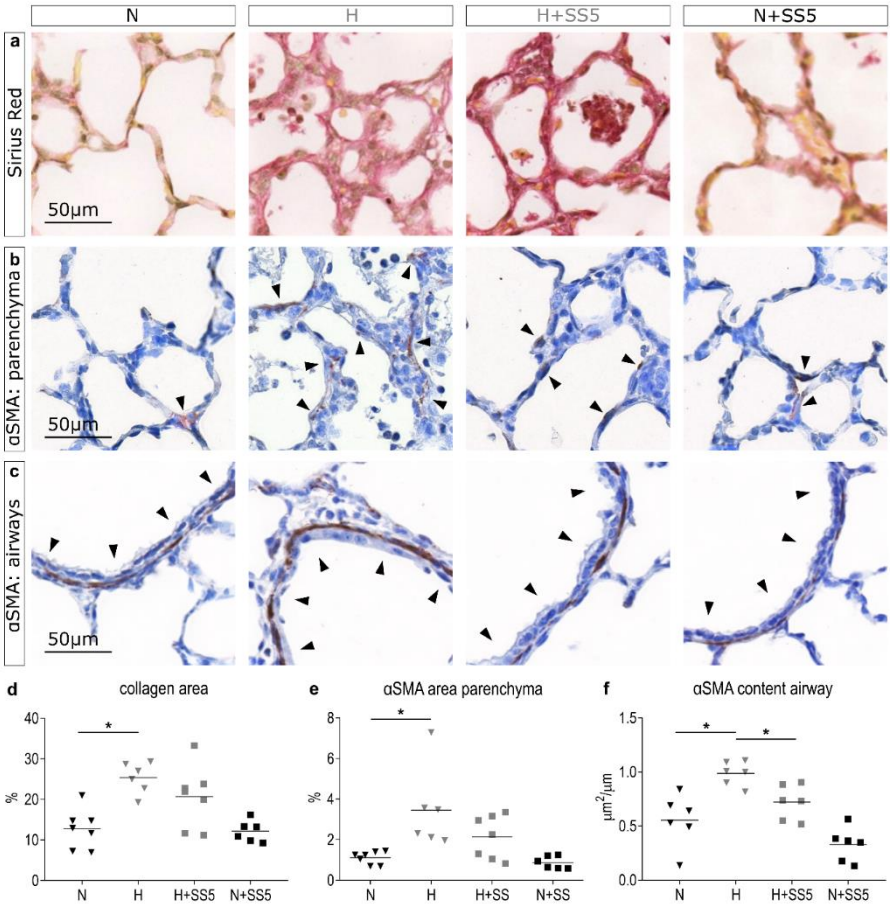


Figure 6.3. *(A) Representative pictures of Sirius Red-stained lung slides showing collagen deposition in red, quantified in pane d. (B) Representative pictures of αSMA-stained lung slides showing brown αSMA-positive areas in septal tissue (myofibroblasts or pericytes, indicated by arrows), quantified 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 5mg/kg simvastatin. (E) Quantification of αSMA-positive area in parenchymal tissue indicates more positivity in hyperoxia, not significantly affected by 5mg/kg simvastatin. (F) Quantification of αSMA-positivity in airway walls indicates an increased smooth muscle cell layer in hyperoxia, significantly prevented by 5mg/kg simvastatin. *p<0.05*

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 (5mg/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 (Figure 6.4E-F).

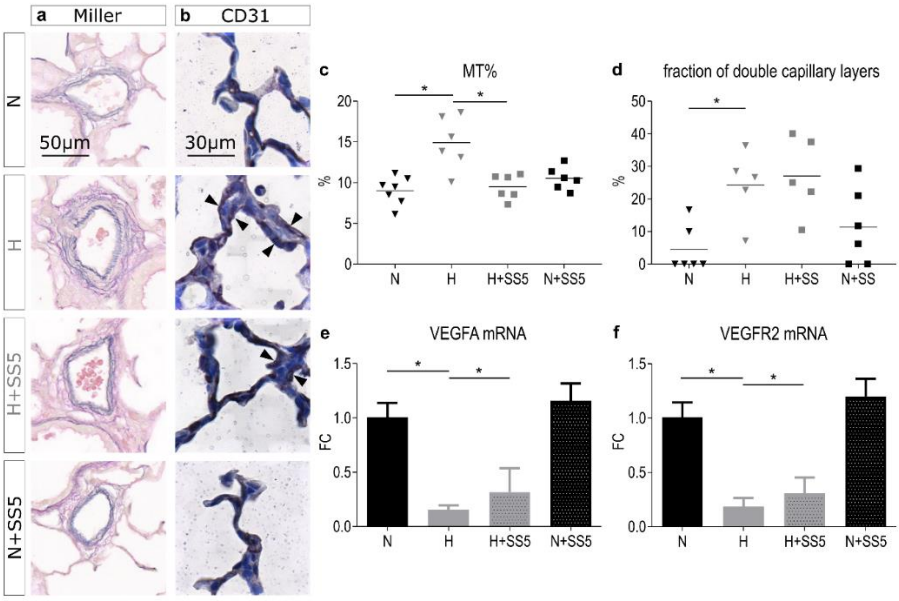


Figure 6.4. *(A) Representative pictures of lung slides stained with Miller's elastic staining showing the tunica media of lung arteries in between the lamina elastica interna and externa, quantified in pane c. (B) Representative pictures of CD31-stained lung slides showing brown capillaries in interalveolar 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 5mg/kg simvastatin. (D) Hyperoxia exposure leads to an increased amount of double capillary layers suggesting delayed microvascular maturation, which is not prevented by 5mg/kg simvastatin. (E-F) Expression of VEGFA, VEGFR2 as measured by qPCR is decreased in hyperoxia, and partially restored by 5mg/kg simvastatin (n=5-7). *p<0.05*

Simvastatin 20mg/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 S6.5). Survival was significantly and importantly decreased by 20mg/kg of simvastatin in both hyperoxia and normoxia (p=0.006 and p=0.0003 respectively, Supplemental Figure S6.6B). 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 20mg/kg (Supplemental Figure S6.6C-H). In contrast to the findings in 5mg/kg, 20mg/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 Figure S6.6I-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 previous reports on the beneficial effects of simvastatin on developmental lung diseases such as neonatal pulmonary hypertension and congenital diaphragmatic hernia (273, 274) 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 (278, 279)). Unlike these studies we could not demonstrate a reduction of lung inflammation and epithelial injury which can explain the functional effect at 5mg/kg. At a higher dose of 20mg/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 α-SMA). This is in line with findings in adult lung fibrosis models, demonstrating anti-fibrotic effects of simvastatin (280, 281) and clinical studies on idiopathic pulmonary fibrosis, suggesting a role for adjuvant statin therapy (282).

Additionally, there was a significantly thinner smooth muscle cell layer in conductive airways of treated animals. This suggests an effect on airway remodelling, as also demonstrated in experiments with simvastatin in asthma models (283). Obstructive airway disease remains an important consequence of BPD and this is thus particularly interesting (270). 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 (146). 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 (284, 285). In the other direction, pulmonary vascular disease is also crucial for alveolar developmental arrest, which is described as the hallmark of BPD (105). 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 (273) and persistent pulmonary hypertension of the newborn (274). It should be noted however that large clinical trials with simvastatin in adult pulmonary hypertension, showed overall negative results(286-288). This could be explained by a lower dose, different characteristics of adult pulmonary hypertension, or the discrepancy between well controlled animal experiments and the heterogeneity of a patient population. 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 Figure S6.1), 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 (Figure 6.2E-G, Figure 6.4E, Supplemental Figure S6.6J-K, Supplemental Figure S6.7 (178)). 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 in to account (289).

The pleiotropic effects of statins have generally been attributed to alteration of post-translational protein modification (290). 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 (post-translational protein prenylation) (291). 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 (292, 293). In vascular smooth muscle cells for instance it attenuates proliferation, induces apoptosis, and also alters contractility (294-296). Lung fibroblast to myofibroblast

differentiation is limited by statins (decreased αSMA-expression, decreased production of collagen) through the same pathway (297, 298). These RhoA-mediated effects might explain the observed phenotype of improved lung function, trends towards a decreased collagen and αSMA 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 20mg/kg, but also in the 5mg/kg experiments. This contrasts findings in other animal models such as mice and rats (273, 274). 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, which is definitely a limitation of our study. The most well-known side effect of simvastatin is rhabdomyolysis, which has also been described in adult rabbits, although at higher doses (299). Furthermore, rabbits are known to be susceptible to centrilobular hepatic necrosis due to exposure to simvastatin (300). 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 5-20mg/kg are much higher than what is currently used for cholesterol lowering (10-80mg 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.

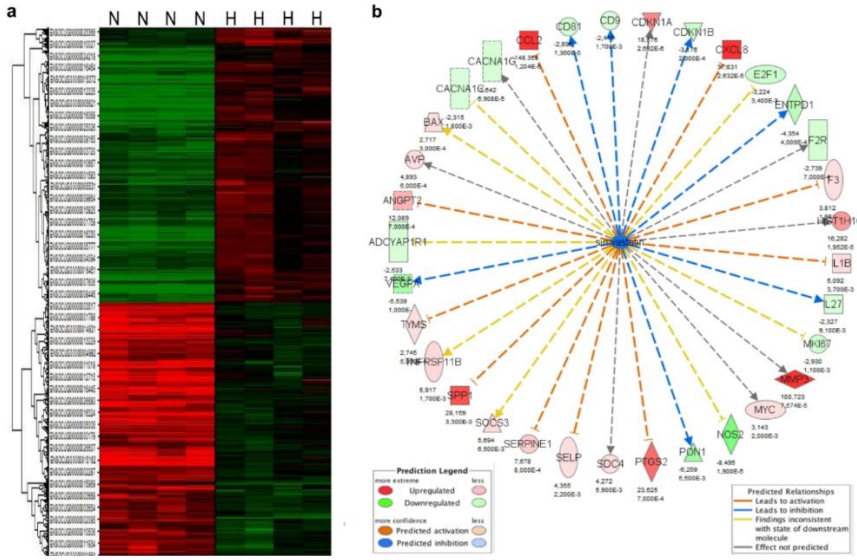
The major limitation of our study is the lack of in depth molecular data (such as Western blot) on the exact working mechanism of simvastatin. This relates to the limited availability of reagents, inherent to the rabbit model. A second limitation is the use of enteral simvastatin, for which there is 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 (301). 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).

Appendix

Supplementary Figure S6.1. *(A) Heat map of 2217 gene transcripts (mRNA) which are differentially expressed in lungs of preterm rabbits exposed to normoxia versus hyperoxia. (B) Upstream regulator analysis diagram for simvastatin and its 33 dysregulated, predicted downstream effectors. Color of the downstream molecules refers to observed dysregulation in H versus N. Color of the arrow refers to the direction of the relationship based on literature findings and determines the prediction for simvastatin.*



Supplementary Table S6.2. *Primer sequences used for qPCR in this study.*

Supplementary Table S6.2. Primer sequences.	
IL8 FW	CCACACCTTTCCATCCCAAAT
IL8 RV	CTTCTGCACCCACTTTTCCTTG
CCL2 FW	GAAGAATCAACAGCACCAAGTG
CCL2 RV	GTGTCCGGGTGGAAGATAAAT
IL1β FW	GTGGTGTTCTCCATGAGTTT
IL1β RV	CAGGGTAGGTTTATCGTCTTTC
VEGFA FW	CTTGCTGCTCTACCTCCACC
VEGFA RV	CTTTGGTCTGCATTCACATTTG
VEGFR2 FW	CCCCTGATTACACTACGCCC
VEGFR2 RV	TGTAGTCTTTGCCACCCTGC
ANGPT2 FW	CCCGTGAACTTTGTCACTTA
ANGPT2 RV	CTCTCAACCTTGAACTCATCTT
SPP1 FW	GCAAGATGCCAGCCAATA
SPP1 RV	CACGAAACAACAAACTACACTAC
YWHAZ FW	TGATTAGTGATGATGAACCG
YWHAZ RV	CACACAGAGGGCTACAATG
HPRT FW	GGTCTGGCCCTTAACTTCTCTGTGTTCTA
HPRT RV	GCGTGCTGTCTTTGTATGATTCTTCACTT
ACTB FW	CAAGCGTGGCATCCTGAC
ACTB RV	CTCGTTGTAGAAGGTGTGGTG

Supplementary Table S6.3. Baseline weight characteristics of pups included in this experiment.

Supplementary Table S6.3. Baseline characteristics					
	Normoxia **N**	**Hyperoxia** **H**	**Hyperoxia + simvastatin** **H+SS5**	**Normoxia + simvastatin** **N+SS5**	
	mean ± sd	mean ± sd	mean ± sd	mean ± sd	*ANOVA p*
Day 0					
Number of pups (n)	7	12	12	13	*N/A*
Birth weight (g)	39.8±5.5	36.6±6.1	38.7±7.2	37.1±5.3	*0.64*
Day 7					
Survival (n, %)	7/7 (100%)	7/12 (58%)	7/12 (58%)	7/13 (54%)	*N/A*
Weight on day 7 (g)	58.3±7.0	49.9±8.2	55.9±5.9	53.3±6.8	*0.17*
Weight gain (%)	47±15	33±12	38±13	35±8	*0.08*

Supplementary Table S6.4. *Lung function read-outs obtained with Flexivent.*

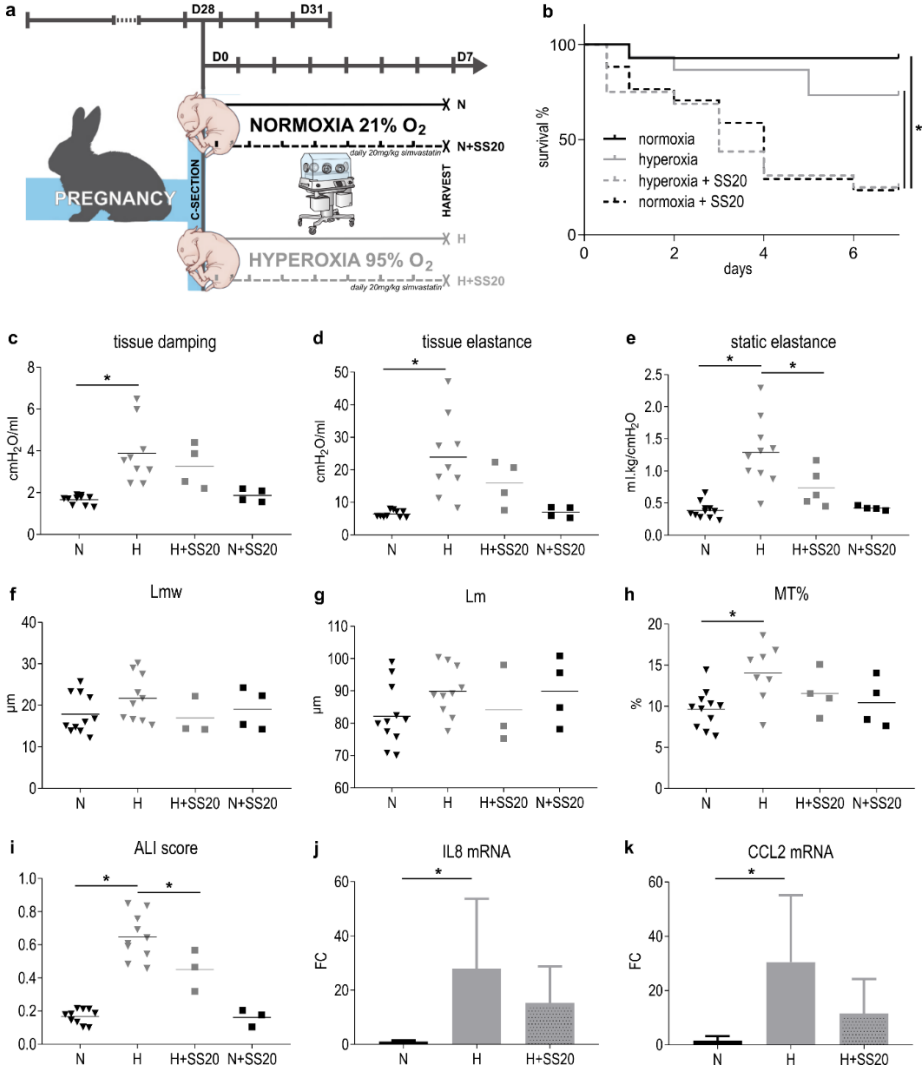
Supplementary Table S6.4. Lung function read-outs							
	Normoxia	**Hyperoxia**		**Hyperoxia + simvastatin**		**Normoxia + simvastatin**	
	N	**H**		**H+SS5**		**N+SS5**	
	mean ± sd	mean ± sd	*H vs N p**	mean ± sd	*H+SS5 vs H p**	mean ± sd	*N+SS5 vs N p**
Static measures							
Inspiratory capacity (ml/kg)	60.7±3.4	28.9±7.1	*<0.001*	35.3±8.2	*0.20*	69.6±3.4	*0.06*
Static compliance (ml/cmH_2O.kg)	2.1±0.3	0.6±0.3	*<0.001*	1.0±0.5	*0.21*	2.9±0.5	*0.07*
Static elastance (cmH_2O.kg/ml)	0.48±0.07	2.04±0.73	*<0.001*	1.12±0.32	*0.001*	0.35±0.07	*0.92*
Forced oscillation							
Tissue damping (cmH_2O/ml)	1.6±0.2	5.5±1.0	*<0.001*	3.5±1.3	*0.002*	1.7±0.3	*0.99*
Tissue elastance (cmH_2O/ml)	6.2±1.2	36.2±13.6	*<0.001*	17.9±10.5	*0.004*	6.6±1.2	*0.99*
Airway resistance (cmH_2O.s/ml)	0.25±0.21	0.15±0.10	*0.46*	0.17±0.07	*0.99*	0.14±0.03	*0.35*
Single frequency oscillation							
Dynamic compliance (ml/cmH_2O.kg)	2.8±0.3	0.5±0.3	*<0.001*	1.1±0.5	*0.06*	3.0±0,4	*0.91*
Dynamic elastance (cmH_2O.kg/ml)	0.35±0.04	2.21±0.69	*<0.001*	1.12±0.53	*0.007*	0.34±0.06	*0.99*

**p-values adjusted for multiple comparisons (Bonferroni-Sidak)*

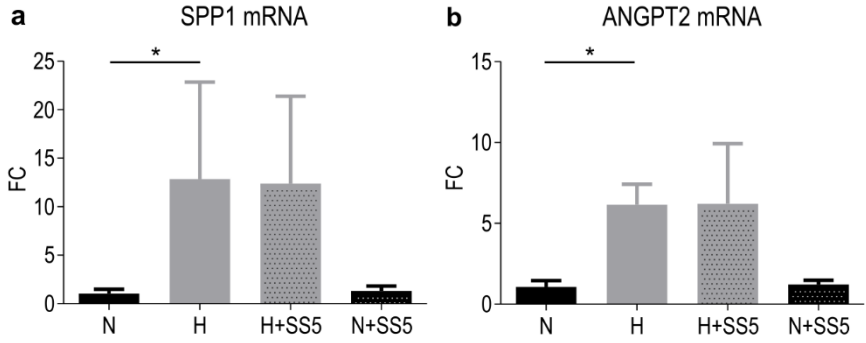
Supplementary Table S6.5. *Baseline weight characteristics of pups included in this experiment.*

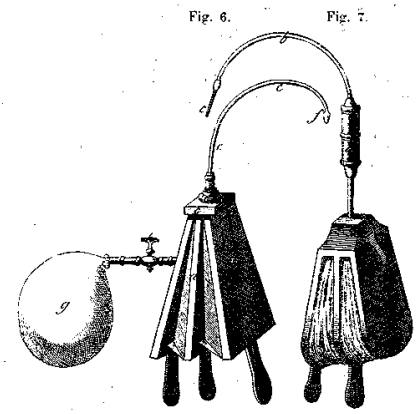
Supplemental Table S6.5. Baseline characteristics of the 20mg/kg experiment					
	Normoxia **N**	**Hyperoxia** **H**	**Hyperoxia + simvastatin** **H+SS20**	**Normoxia + simvastatin** **N+SS20**	
	mean ± sd	mean ± sd	mean ± sd	mean ± sd	*ANOVA p*
Day 0					
Number of pups (n)	13	15	17	17	*N/A*
Birth weight (g)	37.9±5.9	37.3±4.6	38.2±4.4	36.8±4.1	*0.84*
Day 7					
Survival (n, %)	12/13 (92%)	11/15 (73%)	5/17 (29%)	4/17 (24%)	*N/A*
Weight on day 7 (g)	52.2±7.2	49.7±6.8	52.0±3.4	54.2±1.7	*0.60*
Weight gain (%)	36±8	35±12	37±14	31±7	*0.82*

Supplementary Figure S6.6. *(A) At 28 days of gestation pups are delivered by cesarean section, and randomized to 4 groups (n=7-13): two groups are raised in normoxia (21% O_2) and two are raised in hyperoxia (95% O_2) with in both conditions a control group and a group treated with 20mg/kg simvastatin per day. All pups were harvested at day 7. (B) Survival is significantly decreased by 20mg/kg simvastatin in normoxia, but not in hyperoxia. (C-E). Lung function read-outs tissue damping, tissue elastance and static elastance at day 7 suggest an effect of 20mg/kg simvastatin in hyperoxia. (F) Lm (mean linear intercept) and (G) Lmw (mean transsectional wall length) reflect alveolar structure and are not significantly affected by 20mg/kg simvastatin. (C) Thickness of the tunica media (MT%) of lung arteries increases in hyperoxia and seems to decrease with 20mg/kg simvastatin. (I) ALI score is a composite score of septal thickening, neutrophil invasion and exsudates, protein debris and reflects lung injury or inflammation. It is increased in hyperoxia and is significantly reduced by 20mg/kg simvastatin. (J-K) Expression of IL8, CCL2 as measured by qPCR is increased in hyperoxia, but not significantly affected by 20mg/kg simvastatin (n=4-7). *p<0.05, all statistical analyses in this figure should be treated with caution given the low number of surviving animals (underpowered experiment).*



Supplementary Figure S6.7. *(A-B) qPCR does not confirm the prediction that simvastatin normalizes the upregulated expression of SPP1 (n=3-6) and ANGPT2 (n=5-7) in hyperoxia. *p<0.05*





Der Scheintod der Neugeborener - Knapp (1898):
Drawing of a device to insufflate air in to the trachea of a newborn.

Chapter 5

Local pulmonary surfactant delivery in the preterm rabbit: feasibility and efficacy of daily intratracheal injections

Salaets T., Gie A., Jimenez J., Aertgeerts M., Gheysens O., Vande Velde G., Koole M., Murgia X., Casiraghi C., Ricci F., Salomone F., Villetti G., Allegaert K., Deprest J., Toelen J.

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Abstract

Recent clinical trials in newborns have successfully used surfactant as a drug carrier for an active compound, in order to minimize systemic exposure. To investigate the translational potential of surfactant-compound mixtures and other local therapeutics, a relevant animal model is required where intratracheal (IT) administration for maximal local deposition is technically possible and well tolerated. Preterm rabbit pups (born at 28 days of gestation) were exposed to either hyperoxia or normoxia and randomized to receive daily IT surfactant, daily IT saline or no injections for 7 days. At day 7, the overall lung function and morphology were assessed. Efficacy in terms of distribution was assessed by microPET-CT on both day 0 and day 7. Lung function as well as parenchymal and vascular structure were altered by hyperoxia, thereby reproducing a phenotype reminiscent of BPD. Neither IT surfactant nor saline affected the survival or the hyperoxia-induced BPD phenotype of the pups. Using PET-CT, we demonstrate that 82.5% of the injected radio-active tracer goes and remains into the lungs, with a decrease of only 4% after 150 minutes. Surfactant and saline can safely and effectively be administered in spontaneously breathing preterm rabbits. The described model and method enable researchers to evaluate IT pharmacological interventions for the treatment of BPD.

Introduction

Despite significant advances in perinatal care, chronic respiratory morbidity remains an important sequel of preterm birth. Antenatal steroids and surfactant replacement have increased the survival of preterm neonates, but since their introduction rates of BPD have been rising. Defined as oxygen dependency at 36 weeks' postmenstrual age or at discharge, BPD still occurs in about 45% of survivors of extremely preterm birth (<28 weeks of gestational age) (3). Even beyond BPD, preterm birth results in significant lung function abnormalities and increased hospitalization rates for respiratory tract infections during infancy and childhood (270, 303). It has even been suggested that preterm lungs are prone to develop COPD in adulthood (304). The need for novel therapies thus remains high.

Many pharmacological interventions have been investigated to treat or prevent BPD, however, just a few products have shown therapeutic potential (12). For instance, studies on systemic corticosteroids in different regimens have shown potent effects in reducing oxygen dependency at a

postmenstrual age of 36 weeks. However, significant adverse events have been reported, including gastro-intestinal perforations, growth failure, hypertrophic cardiomyopathy and, most importantly, a worsened neurocognitive outcome (305, 306).

Overall, newborns are more prone to develop adverse events, which could be reduced by avoiding systemic drug exposure (307). Therefore local pulmonary delivery of novel drug candidates could be safer and reduce off-target organ exposure (308). In this regard, a recent and successful approach has been reported by Yeh *et al.* consisting of the combined intra-tracheal (IT) administration of a surfactant-budesonide mixture (16). Surfactant is a life-saving treatment for preterm neonates with established respiratory distress but it may also serve as a carrier for pulmonary drug delivery, as it possesses unique biophysical properties to efficiently spread along the air-liquid interface (309). The use of surfactant as a drug carrier could result in increased local drug deposition in the lungs and decreased systemic side effects (310, 311). Along the same lines, also other carriers of active compounds (e. g. exosomes, polymeric carriers) are being considered for the use in neonatal lungs, with the same purpose of reducing systemic exposure (310, 312).

Animal models play an indispensable role during the preclinical development of pharmacological treatments, including for BPD. Unfortunately, commonly used animal models have important limitations: mice and rats models are not preterm and do not allow easy tracheal access due to their small size, while lambs are very expensive and the use of baboons is ethically questioned. The preterm rabbit model could be an advantageous alternative as it includes the major driver of BPD: prematurity. Preterm rabbit pups born at day 28 of gestation depict altered lung function at birth, reminiscent of neonatal respiratory distress syndrome (205), are born in the early saccular stage of lung development (155) and have immature defense mechanisms against oxidative stress (173). Furthermore the animals combine prematurity with relatively low housing and care investments and have a size that allows for technical manipulations (130, 313). When exposed to hyperoxia, they develop morphological and functional manifestations comparable to human BPD. Previously, we evaluated the efficacy of several systemically administered compounds in this preterm rabbit model (181, 183, 314).

In this study we established a method for direct access to the airways, through transcutaneous, IT injections on spontaneously-breathing preterm rabbits. We evaluated the feasibility and safety of daily IT instillations of saline or

surfactant, and their effect on the phenotype of the preterm rabbit BPD model. Furthermore, we determined the pulmonary distribution of surfactant and saline following IT administration.

Methods

Cesarean Section

Time-mated pregnant rabbits (New Zealand White and Dendermonde cross-breed) were provided through the animal facility of KU Leuven. All experiments were approved by the Ethics Committee for Animal Experimentation (project numbers P090/2016 and P080/2017) and conducted according to current guidelines on animal welfare. Cesarean section was performed at 28 days of pregnancy (early saccular stage of lung development, term = 31 days). Does were sedated with 35 mg/kg of intramuscular ketamine (Nimatek®; Eurovet Animal Health BV, Bladel, The Netherlands) and 6 mg/kg of xylazin (XYL-M®; VMD, Arendonk, Belgium). After adequate sedation, does were placed in the 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 International BV, Boxmeer, The Netherlands). Immediately afterwards the abdomen was opened and all pups were extracted through hysterotomy.

Neonatal rabbit care

At delivery, the pups were dried, stimulated and placed in an incubator at 32° C and 50% of humidity. Oxygen concentration was continuously monitored with a Palm O2 D% Analyzer® (Analytical Industries, Pomona, US). Neonatal rabbit care has been described before (130, 146). Briefly, pups were fed twice daily via an orogastric tube with increasing quantities of a milk replacer (FoxValley 30/50, Arizona, USA, containing 10g/100ml fat, 6g/100ml protein and <1g/100ml carbohydrates), supplemented with vitamins and probiotics (6g/100ml Biolapis, Protexin, Somerset, UK) and immunoglobulins on the first 2 days of life (4g/100ml Col-o-Cat, Sanobest, 's Hertogenbosch, The Netherlands). Furthermore, intramuscular vitamin K on D2 (0.002 mg/kg Konakion pediatric, Roche, Belgium) and antibiotics from D2-D7 (benzylpenicillin (20,000 I.U./kg Penicilline, Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg, Amukin, Bristol-Myers Squibb, New York, US)) were administered. The pups remained in the incubator for 7 days except for the feeding and interventions.

Randomization

After an initial 1-hour adaptation period, surviving pups were weighed, numbered, and randomly assigned to one of six different groups, ensuring an equal distribution within nests: 1) normoxia control group (21% oxygen; N; n = 11), 2) hyperoxia control group (≥95% oxygen; H, n = 11), 3) hyperoxia group treated with daily intratracheal (IT) injections of 100mg/kg of surfactant (Curosurf®, Chiesi Farmaceutici, Parma, Italy; Hsurf; n = 13), 4) hyperoxia group treated with daily IT injections of an equal volume of saline (Hsal; n = 13), 5) normoxia group treated with daily IT injections of 100mg/kg of surfactant (Nsurf; n = 10) and 6) normoxia group treated with daily IT injections of an equal volume of saline (Nsal; n = 11) as visualized in Figure 7.1A. Sample size calculation was performed using GPower 3.1. To pick-up a 1/3 correction of the effect of hyperoxia on tissue damping (historical data hyperoxia versus normoxia (146)), with a power of 80% and an α of 0.01 (Bonferroni correction for 5 comparisons) in a two-tailed t-test, 8 pups in each group were needed. In order to obtain this number at day 7, 10-13 pups were randomized to each experimental group (Table 7.1).

Intratracheal injections

Rabbit pups were anesthetized with isoflurane 2.5% (ISO-VET®, EuroVet, Heusden-Zolder, Belgium) in 2L oxygen/min and placed in supine position. We initially evaluated different techniques to obtain tracheal access (including intubation with curved catheters, intubation with a small scope or nasal intubation), however these attempts failed in neonates because of the small size and long curvature of the rabbit snout. Eventually we chose the transcutaneous access because of its feasibility. The laryngeal and tracheal cartilage was stabilized with an Allis-forceps and catheterization of the trachea was performed transcutaneously with a 19mm long 26G catheter (Neoflon®, BD, New Jersey, US). Correct insertion of the catheter was checked by water valve in the transparent catheter. Subsequently either 1.25mL/kg of surfactant or saline was slowly injected with a 30G blunt Hamilton needle inserted through the catheter (technique visualized in Figure 7.1C). In post mortem test pups this technique resulted in delivery above the carina. A gentle position check and instillation was used, as initial attempts using a slightly more aggressive strategy resulted in overinflation of lungs and mortality. In these optimization experiments, position in the trachea was checked by air aspiration and after administration of the surfactant a fast air bolus of 0,5ml was applied. This resulted in procedural mortality in the surfactant group, but not in the saline group. Necropsy of the animals dying after surfactant administration revealed plump and (over)expanded lungs.

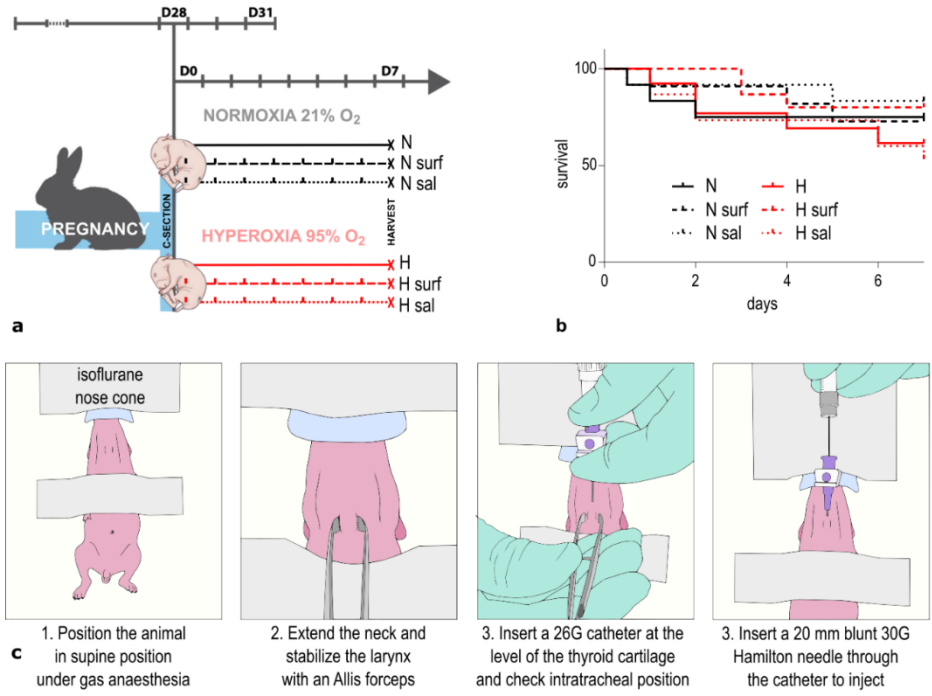


Figure 7.1. Study design and survival. *(A) At 28 days of gestation pups are delivered by cesarean section, and randomized to 6 groups (n = 10-13). Three groups are raised in normoxia (21% oxygen): a control group (N, n = 11), a surfactant group (N surf, n = 10) and a saline group (N sal, n = 11) (both daily IT administration). In parallel 3 groups are raised in hyperoxia (>95% oxygen): control (H, n = 11), surfactant (H surf, n = 13) and saline (H sal, n = 13). All pups are harvested for functional and histological analysis at day 7. (B) Survival analysis does not reveal any differences between the study groups. (C) Illustration of the intratracheal injection strategy*

Lung function testing

At day 7, invasive lung function testing was performed using the Flexivent system (FlexiVent® 5.2; SCIREQ, Montreal, Canada), as previously described (130). After anesthesia with 35 mg/kg of ketamine and 6mg/kg of xylazin, the trachea of the pups was surgically exposed. An 18G metal needle was inserted in the trachea and connected to the ventilator with the following settings: 120 breaths/min at a tidal volume of 8mL/kg. Thereafter, four respiratory manoeuvers were performed: a recruitment manoeuver (deep inflation), a pressure-volume loop (PVRV), a single frequency forced oscillation manoeuver (Snapshot90v5.1), and a broadband forced oscillation manoeuver (Prime8). The mean of 3 consistent repetitions is reported for each parameter measured.

Morphometric assessment

After lung function testing, deeply anesthetized animals were euthanized by exsanguination. A thoracotomy was performed and the lungs were removed "en bloc". Left lungs were fixed with 4% paraformaldehyde under a constant hydrostatic pressure of 25cmH_2O for 24h in the airway. Afterwards, left lung volume was measured through water immersion (Scherle's principle) (104). Lungs were embedded in paraffin. A central sagittal cut per lung was stained with hematoxylin and eosin (HE) and Miller´s elastic stain.

Lung sections were scanned with a slide scanner (AxioScan® Slide Scanner, Zeiss, Oberkochen, Germany). Afterwards, a purpose-designed Fiji-plug-in randomly selected fields of 500x500 µm. Lung morphometric measurements were semi-automatically performed with a self-designed Fiji-plugin, based on overlapping a 64-point grid on 20 fields per lung. For each lung Lm (mean linear intercept), Lmw (transsectional wall length) and S (total alveolar surface) corrected for body weight were calculated (104). The semi-automatic method was validated to manual counting for 8 lungs using the STEPanizer-tool (223). For vascular morphometry, at least 15 arteries with an external diameter of 30-100 µm were measured on Miller-stained lung sections to obtain the internal and external diameter of the *tunica media* (muscular layer), to calculate MT% (medial thickness) as a ratio of the thickness of the tunica media to the vessel diameter (315).

Micro-PET-CT

Biodistribution of injected surfactant and saline was assessed with micro-PET-CT in a separate litter of 6 pups. Surfactant (100mg/kg, 1.25 mL/kg) or equal volumes of saline were mixed with 2-deoxy-2-(18F)fluoro-D-glucose (^{18}F-FDG) (25µCi, max 20% of the volume). Surfactant+^{18}F-FDG (n=3) or saline+^{18}F-FDG (n=3) was delivered IT at day 0 and day 7. PET-CT-imaging was performed under isoflurane anesthesia (1.5-2% isoflurane in 100% oxygen) at both 15 and 150min following IT delivery (Figure 7.4A). Each animal was fixed on an exchangeable Styrofoam bed in the prone position. First micro-PET was performed using a Concorde Focus 220 micro-PET (Siemens/Concorde Microsystems, Knoxville, TN, USA), in a static protocol. Images were reconstructed with Fourier rebinning and 2D OSEM iterative reconstruction (Ordered Subsets Expectation Maximization, 16 subsets - 10 iterations) at zoom 2, resulting in a voxel size of 0,5x0,5x0,8mm (316). Consequently, the Styrofoam bed was placed in a dedicated low-dose small-animal micro-CT scanner (SkyScan 1278, Bruker micro-CT, Kontich, Belgium) while the animal remained in the same position. Respiratory gated micro-CT images were

acquired using the following parameters: 50 kVp X-ray source voltage, 1 mm Al filter, 918 µA source current, 55 ms exposure time, 9 projection images per 0.9° rotation step over a total angle of 180° in list mode and retrospectively gated. This resulted in four reconstructed 3D datasets with 50 µm isotropic reconstructed voxel size corresponding to four different phases of the breathing cycle (4D). Data reported here is at end of expiration. Software provided by the manufacturer (TSort, NRecon, DataViewer, TCONV, DicomCT and CTan) was used to respectively gate, reconstruct, visualize, convert, process and analyze µCT data (201). Fusion of the micro-PET and –CT images was done in PMOD 3.7, based on the position of 4 reference points on the animal bed. Consequently lungs (right and left), upper airway and stomach were manually delineated, and activity in these volumes of interest was expressed as percentage of total activity. For evaluation of internal distribution in the lungs we defined the smallest possible volume containing 90% of the pulmonary activity, based on histograms, and expressed it as a fraction of the total delineated lung volume. Furthermore the coefficient of variation (COV) for the activity of the voxels was calculated by dividing the standard deviation by the average voxel activity.

Statistical analysis

All statistical analysis was performed in Prism (Graphpad Software, La Jolla, CA, USA). A Grubbs' test with an α of 0.05 was used to identify outliers. For all lung function, morphometric and vascular read-outs, one-way ANOVA was used. With a Bonferroni-Sidak test for 5 comparisons, the uninjected hyperoxia-group was compared to the uninjected normoxia-group, and both injected groups were compared to the uninjected control group in the same condition (hyperoxia or normoxia). Survival was compared between groups with a log-rank (Mantel-Cox) test. As the relative organ distribution of the PET-CT data is not normally distributed, non-parametric testing was performed (Wilcoxon signed rank test, Kruskal-Wallis with Dunn multiple comparisons test). For the distribution of the activity within the lung, parametric tests were used (paired t-test and ANOVA with a Bonferroni-Sidak multiple comparisons test).

Results

IT injections do not increase mortality in preterm rabbit pups

A total of 98 pups were delivered from 9 different does. From these, 29 (30%) died during a 1-hour adaptation period after respiratory distress symptoms or apnea. The remaining pups were randomized. Of them 52 (75%) survived

to harvest at 7 days. Baseline characteristics of the pups (birthweight, body weight) did not differ significantly between groups (Table 7.1). We did not observe any immediate mortality after IT injections with surfactant or saline. Overall survival was not significantly affected by surfactant or saline administration, or by hyperoxia exposure (Figure 7.1B).

Table 7.1. Baseline characteristics

	N mean ± sd	**H** mean ± sd	**H surf** mean ± sd	**H sal** mean ± sd	**N surf** mean ± sd	**N sal** mean ± sd	*ANOVA p*
Day 0							
Number of pups (n)	11	11	13	13	10	10	*N/A*
Birth weight (g)	38.7 ± 7.6	36.8 ± 7.4	36.9 ± 6.9	35.3 ± 6.6	34.5 ± 7.1	34.5 ± 7.1	*0.64*
Day 7							
Survival (n, %)	9 (82%)	8 (73%)	11 (85%)	8 (62%)	7 (70%)	9 (82%)	*N/A*
Birth weight (g)	38.0 ± 6.1	37.9 ± 3.9	37.2 ± 5.4	38.5 ± 5.3	36.5 ± 6.6	31.6 ± 4.5	*0.09*
Weight on day 7 (g)	51.8 ± 9.7	48.7 ± 5.1	48.3 ± 9	49.6 ± 6.6	49.0 ± 8.6	42.3 ± 7.6	*0.24*
Weight gain (%)	35 ± 7	29 ± 8	29 ± 12	29 ± 9	35 ± 9	34 ± 12	*0.53*

Table 7.1. Survival and weight characteristics of included pups. *Weights of the pups included in the experiment (n=69), and of the pups harvested at day 7 (n=52). Mean ± SD are shown. Data analyzed with ANOVA.*

IT injections of surfactant or saline do not affect the development of the BPD phenotype

Seven days of hyperoxia affected inspiratory capacity, static elastance, dynamic compliance and elastance, as well as tissue mechanics at forced oscillation (tissue damping and tissue elastance). No effect on airway resistance was observed. IT injections of surfactant or saline, did not alter any of these parameters, neither in hyperoxia nor in normoxia groups (Table 7.2).

Hyperoxia exposure resulted in lower left lung volume at day 7 (Figure 7.2B). Morphometric assessment revealed a significantly increased mean transsectional wall length (Lmw) and a trend towards higher mean linear intercept (Lm), suggesting thicker septations and larger airspaces in the hyperoxia groups (Figure 7.2A, 7.2C-D). In total, this resulted in a decreased parenchymal surface area (S) corrected for body weight (Figure 7.2E). The morphometric characteristics were not significantly influenced by IT injections of surfactant or saline (Figure 7.2).

Table 7.2: Lung functional, structural and vascular read-outs.						
	N	**H**	**H surf**	**H sal**	**N surf**	**N sal**
	mean ± sd	mean ± sd	mean ± sd	mean ± sd	mean ± sd	mean ± sd
Lung function						
Vend/wt – Inspiratory capacity (ml/kg)	63.9 ±14.3	40.4 ±6.0*	43.6 ±14.8	48.6 ±12.9	66.5 ±11.8	72.2 ±26.3
Cst – Static compliance (mL/cmH2O.kg)	2.1 ±0.4	1.1 ±0.3	1.5 ±1.1	1.3 ±0.6	2.8 ±1.4	3.4 ±2.7
Est - Static elastance (cmH2O.kg/mL)	0.47 ±0.14	1.06 ±0.42*	0.94 ±0.75	0.73 ±0.24	0.42 ±0.17	0.39 ±0.22
Parenchymal structure						
R - Resistance (cmH2O.s/mL)	0.31 ±0.07	0.47 ±0.11	0.46 ±0.12	0.56 ±0.33	0.25 ±0.08	0.31 ±0.12
C - Dynamic compliance (mL/cmH2O.kg)	3.4 ±0.8	1.5 ±0.6*	1.9 ±0.9	1.8 ±1.0	3.6 ±1.0	4.0 ±1.4
E - Dynamic elastance (cmH2O.kg/mL)	0.31 ±0.06	0.63 ±0.21*	0.57 ±0.36	0.48 +0.15	0.29 ±0.08	0.27 +0.08
Vascular structure						
Rn - Airway resistance (cmH2O.s/mL)	0.12 ±0.05	0.15 ±0.06	0.16 ±0.08	0.16 ±0.06	0.08 ±0.04	0.11 ±0.08
G - Tissue damping (cmH2O/mL)	1.6 ±0.2	2.5 ±0.6*	2.2 ±0.5	2.1 ±0.3	1.6 ±0.5	1.6 ±0.2
H - Tissue elastance (cmH2O/mL)	6.0 ±1.1	11.5 ±3.8*	9.8 ±3.9	8.4 ±2.0	6.0 ±1.8	6.0 ±0.8

Table 7.2. Lung function read-outs. *Hyperoxia controls are compared to normoxia controls, both saline and surfactant injected groups are compared to the controls in the same condition (hyperoxia or normoxia) (n=7-11): *p<0,05 adjusted for multiple comparisons according to Bonferroni-Sidak.*

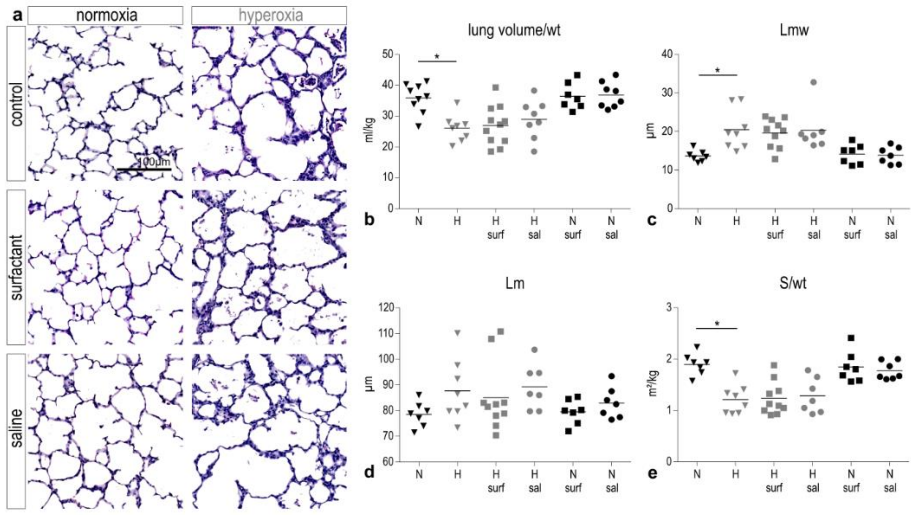


Figure 7.2. Lung volume and alveolar morphometry. *(A) Representative pictures of HE-stained lung slides. (B) Left lung volume as measured by water immersion is decreased in animals exposed to hyperoxia, and unaffected by daily surfactant or saline IT administration (n=7-11). (C) Lmw (mean transactional wall length) is increased in animals exposed to hyperoxia, suggesting thicker alveolar walls, but is unaffected by daily surfactant or saline IT administration (n=7-10). (D) Lm (mean linear intercept) tends to increase in hyperoxia, suggesting larger airspaces, but is unaffected by daily surfactant or saline IT administration (n=7-10). (E) Parenchymal surface area is decreased in hyperoxia, but unaffected by daily surfactant or saline IT administration (n=7-10).*p<0,05 (significance level adjusted for multiple comparisons according to Bonferroni-Sidak)*

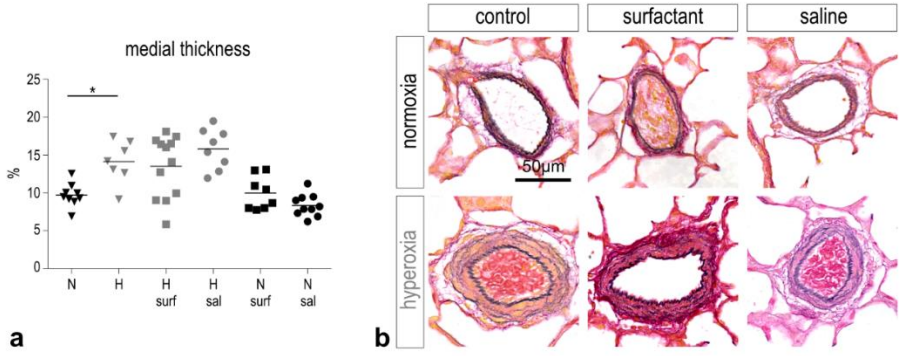


Figure 7.3. Vascular morphometry. *(A) MT% (medial thickness, or thickness of the tunica media corrected for vessel diameter) is increased by hyperoxia, but remains unaffected by daily surfactant or saline IT administration (n=7-11). (B) Representative pictures of lung arteries on Miller-stained lung sections. *p<0,05 (significance level adjusted for multiple comparisons according to Bonferroni-Sidak)*

A marked increase in pulmonary artery medial thickness (MT%) was noted in the pups exposed to hyperoxia, reminiscent of pulmonary vascular disease in BPD. MT% was not significantly affected by daily IT injections of surfactant or saline (Figure 7.3).

IT injections result in high pulmonary deposition

PET-CT was used to evaluate the bio-distribution of IT-delivered surfactant or saline (mixed with an ^{18}F-FDG radio-isotope) in spontaneously breathing rabbit pups. The median fraction of radio-activity in the lungs was 78.3%, while 10.2% and 6.1% was detected in the stomach and upper airways respectively (Figure 7.4C-D). No systemic absorption (activity in kidneys or bladder) was noted within the 150min time frame. A significant, however small, decrease in the pulmonary fraction of activity occurred between the first scan, performed 10 min after injection, and a second scan at 150 min (median of 82.5% to 78.3%, p=0.02, Figure 7.4D). At both time points the pulmonary fraction did not differ significantly between injections with surfactant or saline at day 0 or 7.

About two thirds of the pulmonary fraction (64.8±15.7%) was located in the right lung, which is in line with the larger volume of the right lung (58.3%±5.3%, Figure 7.4B). There was no significant difference in activity between both lungs, when corrected for total lung volume (p=0.11). Right-to-left lung distribution did not change between the first scan at 10 min and the second scan at 150 min (p=0.37).

On average, we observed that 90% of the activity was present in 44.6±5.9% of the lung volume. This corresponds to a coefficient of variation of 1.38±0.22. Visual inspection of the images suggests a rather central than peripheral deposition in all animals (Figure 7.4C). The internal lung distribution was not significantly changed between the first and the second scans (43.9±6.3% and 45.2±5.7% respectively, p=0.38). Also for internal distribution no significant differences were noted between injections with surfactant or saline at day 0 or 7 (Figure 7.4E). However, when data from the surfactant and saline groups were pooled, a significantly more homogenous distribution of the activity was noted at day 0 compared to day 7 (47.8±5,0% and 39.2±4.1% respectively, p=0.01).

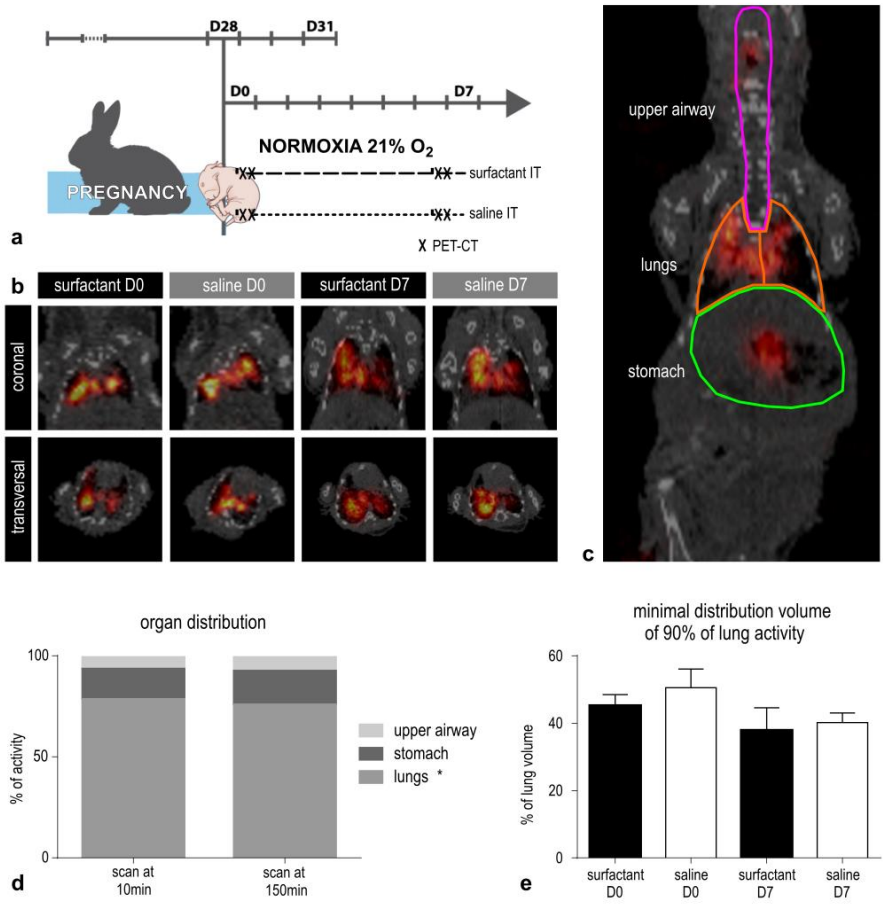


Figure 7.4. PET-CT distribution data. *(A) A separate litter was delivered at 28 days of gestation (by cesarean section), housed in normoxia, and randomized to either IT injections with surfactant + ^{18}F-FDG or saline + ^{18}F-FDG (n=3). Injections were performed at both day 0 and day 7, followed by a PET-CT scan after 10 and 150 min. (B) Representative image of a full body coronal section. Manually the different volumes of interest containing ^{18}F-FDG-activity (upper airway, right and left lung, stomach) were delineated on all scans. (C) Representative lung images of pups in both groups at day 0 and day 7, taken at 10 min after injection, suggesting an unequal and predominantly central distribution in all groups. (D) Organ distribution of the injected activity in all groups (n=12), 10 and 150 min after injection. We observe a significant but small relative decrease in lung activity (82.5% to 78.3%): *p=0,03 (nonparametric testing, adjusted for multiple comparisons according to Dunn). (E) Internal distribution within the lung, graph indicates the minimal relative lung volume containing 90% of the total activity at 10 min after injection. In perfectly homogenous distributions this would be 90% of the volume. No differences between individual groups (n=3), but when pooled higher at day 0 compared to day 7 (n=6, p=0.01).*

Discussion

From a clinical and research perspective the interest in local pulmonary delivery of molecules to promote the long term respiratory outcome in preterm neonates is increasing. The current study validates the preterm rabbit model for research on this emerging topic. This small animal model combines prematurity and oxygen toxicity, and results in a structural and functional phenotype mimicking human BPD (313). Until now a preclinical evaluation of surfactant-compound mixtures would only have been possible in larger animal models like adult rabbits, preterm lambs or baboons, at a higher financial and ethical cost (71, 72, 317). The combination of prematurity in a small animal model with tracheal access as described in this paper, opens opportunities to investigate novel intratracheal therapeutics. The preterm rabbit obviously also has some limitations such as the limited availability of commercial reagents and antibodies for the explorative analysis of rabbit tissue. Additionally there is no reliable visible assessment of male or female phenotype, making it difficult to evaluate the role of gender on preterm lung disease and therapy. Also, this model cannot replace experiments in the more human-like ventilated or non-invasively supported large animal models. A final disadvantage of the model is the need for a transcutaneous puncture, as intubation through the normal route is technically very challenging.

This study provides arguments for the use of surfactant as a safe and efficient carrier for drugs targeting the neonatal lung. As the large majority of preterm infants at risk of BPD are anyway receiving surfactant for RDS at birth, the addition of a therapeutic molecule in the surfactant emulsion to improve long term respiratory outcome is a promising strategy. This study was set-up to evaluate the feasibility, safety and distribution of daily IT administration of surfactant, as a potential drug carrier, in the preterm rabbit model.

A **first** essential prerequisite for a drug carrier is the absence of a noxious effect. The direct effect of surfactant on chronic lung disease has been a matter of debate for many years. The introduction of surfactant supplementation in the care of extreme preterm babies has been associated with higher rates of BPD (3, 318), a finding that is generally explained by the survival of infants with more immature lungs. Furthermore in prophylactically treated infants increased incidences of BPD or death have been observed, compared with infants treated in a rescue strategy (319). Additionally researchers have raised concerns on the potential acute side effects of surfactant administration, such as ventilation disturbances in formerly normally functioning lung regions (320). On the other hand long term

beneficial effects of repetitive instillations of surfactant have been described in ventilated infants (TOLSURF and CURDYS trials: (321, 322)). The rationale is that exogenous surfactant is necessary to overcome endogenous surfactant inactivation by oxidation and protein leak due to respiratory support.

Our study suggests in a relevant (non-ventilated) preterm animal that repetitive surfactant administration has no direct effect on the evolution towards chronic lung disease. Despite older data suggesting surfactant inhibition in neonatal rabbits exposed to hyperoxia (167), daily exogenous surfactant did not result in in vivo improvement in lung function in this model. Surfactant administration neither altered the alveolar nor vascular architecture.

We did not investigate the acute effects of surfactant administration on a possible RDS phenotype, but earlier work has shown beneficial effects on the overall lung mechanics of preterm rabbits (179). The increased mortality in our optimization experiments however illustrates that surfactant should be used with caution. The absence of any procedural mortality in the final, pressure-less, method, demonstrates the importance of the administration strategy.

A **second** prerequisite for a good drug carrier is an adequate distribution to and in the target organ, in this case the lung. Using the technique described above, the vast majority of the injected activity reached the lungs (82.5%) and stayed there at least 150 minutes after injection (78.3%). The slight, but significant decrease possibly reflects a small esophageal fraction that is impossible to distinguish from the pulmonary fraction, and that descends to the stomach at the second time point. This study suggests that tracheal administration of drugs or surfactant, even in the absence of a closed ventilation system and positive pressure, leads to a very high pulmonary deposition, with minimal loss. This is a clear advantage over inhalation strategies in neonates, where low lung deposition, and high loss (buccal mucosa, stomach, face and device; up to 2%) has been observed (323). A recent study with non-human primates indicated that after nebulization only 11% of the administered surfactant reached the lungs (324). IT administration thus seems to result in less systemic exposure, limiting the potential for side effects.

We conclude that the internal distribution within the lung is acceptable, even if it is not perfect. A perfectly homogenous distribution would result in 90% of the activity present in 90% of the lung volume, while in our study this

amount is present in about 45% of the lung volume. Previous studies did also show a patchy or rather central distribution of surfactant after intratracheal delivery which is comparable to our data (325-327). As these studies used less precise quantification methods it is however impossible to exactly benchmark our findings. To our knowledge, distribution of exogenous surfactant has never been assessed in human neonates.

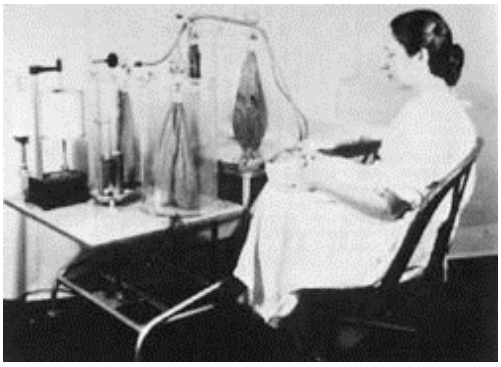
From a drug delivery perspective a more homogenous distribution might be desirable, however established pulmonary drug delivery strategies such as inhalation, have been proven to be efficient, despite comparable imperfect distribution (15, 323). Furthermore, the need for anesthesia during the PET-CTs might have negatively influenced the distribution of the tracer through hypoventilation. If allowed a longer recovery period, distribution towards the periphery of the lung might improve, however this could not be tested because of the short half-life of ^{18}F-FDG. Additionally, in our study repetitive injections (on day 0 and day 7) in the same animal resulted in different distribution patterns, increasing the total amount of lung tissue exposed. Finally, it should be noted as a limitation that this proof of concept study uses ^{18}F-FDG, as the behavior of this tracer molecule does not necessarily reflect the biophysical and biological behavior of a given investigational drug (e.g. interaction with surfactant, viscosity in solution and eventual uptake by the epithelium).

The rationale to use surfactant as a carrier is the idea that it would act synergistically by improving the internal distribution of a drug within the lung. In this study we did not see any difference in intrapulmonary distribution of ^{18}F-FDG between the groups in which surfactant or saline was used as a carrier. We have to acknowledge that our study was not powered to perform this comparison (n=3). We did see a trend towards improved distribution on day 0 versus day 7 in both the saline and surfactant treated animals. This is in line with previous observations in a sheep model that surfactant distribution is better in fluid filled lungs (328).

Our data are in line with observations from Fajardo et al. in surfactant depleted, mechanically ventilated adult rabbits. They reported a comparable central distribution of radio-actively labeled budesonide, not affected by surfactant (325). On the other hand, Huang et al. report on improved distribution of a fluorescent dye after IT injection in a surfactant mixture in spontaneously breathing adult mice, most likely by altering the biophysical characteristics of the injected liquid (329). This discrepancy nicely illustrates that the effect of surfactant on the distribution of a drug will depend, like

mentioned above, on the biophysical and biological properties of the specific drug.

In conclusion, we developed a model that allows the preclinical evaluation of intratracheal therapeutics in a BPD context. Here, we used this model to mimic IT surfactant administration, a common clinical procedure, from bedside to bench. In our experiments, we provided support for the safety and efficacy of surfactant as a drug carrier as its use 1) did not result in noxious effects on the lung, and 2) resulted in high pulmonary deposition with an acceptable internal distribution. Future perspectives include the use of this model and methods to translate innovative localized treatment strategies for BPD from the bench towards bedside.



First successful ventilation of a preterm baby by Stahlman (1961):
Stahlman created a laboratory next to the nursery, and translated her observations in the clinics to the lab and vice versa, leading to the birth of the NICU.

Chapter 6

A single dose of budesonide added to surfactant prevents hyperoxia induced lung injury in preterm rabbits

**Gie A., *Salaets T., Casiraghi C., Salomone F., Allegaert K., Deprest J., Toelen J.*
*(*shared first)*
Manuscript in preparation

Abstract

The role of postnatal steroids in the prevention of BPD is controversial because of non-pulmonary side effects, such as neurodevelopmental delay. Co-administration of budesonide with surfactant is an attractive strategy to maximize local delivery and minimize distant side effects. A repetitive dosing schedule of 0.25mg/kg budesonide in surfactant has been shown to prevent BPD. In this work we explore whether a single administration improves lung function, cytokine expression and lung structure in preterm rabbits exposed to hyperoxia.

Rabbit pups were delivered prematurely (day 28 of gestation) and exposed to hyperoxia (95% O_2). In a first round of experiments, pups were randomized to either IT budesonide added to surfactant or surfactant alone on day 0, with assessment of lung function and cytokine expression 24h later. In a second round of experiments, pups were randomized to either a hyperoxia control group or to treatment with a single (on day 0) or daily (on days 0-6) dose of 0.25mg/kg budesonide plus 100mg/kg surfactant with assessment of lung function, cytokine expression and lung structure on day 7.

The addition of budesonide to surfactant led to decreased expression of CCL2 at 24h, without effect on IL8 and lung function. A single co-administration of surfactant with budesonide on day 0 improved lung function, lung structure and CCL2 and IL8 expression at day 7, in comparison to untreated pups. A daily dose of surfactant with budesonide did not have the same beneficial effects.

In conclusion, a single dose of budesonide plus surfactant had persistent effects on lungs of preterm rabbit pups exposed to hyperoxia. This indicates further dose reduction could be envisaged in clinical trials.

Introduction

Advances in perinatal and neonatal care have led to improved survival of more extremely preterm babies, however at the cost of the respiratory outcome of survivors. Despite trends to less invasive ventilation and lower oxygen saturation targets, the incidence of BPD, defined by the need for oxygen at 28 days of life, increased over the last decade (3). BPD has important consequences with more frequent and more severe respiratory tract infections throughout childhood, pulmonary hypertension in severe cases, and obstructive lung function abnormalities in later life (137, 330, 331).

The role of postnatal steroids in the (primary and secondary) prevention of BPD has been a subject for debate for many years. Corticosteroids are potent anti-inflammatory mediators, and it is hypothesized they prevent lung tissue inflammation and damage. In the nineties up to 40% of babies born extremely preterm (<28 weeks of postmenstrual age) received postnatal steroids (3). Systemic dexamethasone, when administered both early and late, does have the potential to facilitate weaning from mechanical ventilation and diminish the rate of BPD or death, but in the late nineties it became apparent that this was at the cost of an increased risk for cerebral palsy and intestinal perforation (305, 306). This knowledge has importantly reduced the use of postnatal steroids in most neonatal intensive care units. Recently, alternative strategies for corticosteroid delivery have been developed to maximize local effect and minimize systemic exposure (16, 332).

The most promising results were obtained with IT administration of a mixture of budesonide and surfactant. This is an attractive strategy, as surfactant is anyway administered to preterm infants with respiratory distress syndrome, a population at risk for BPD. In a clinical trial with 265 mechanically ventilated preterm newborns with severe respiratory distress syndrome, an 8 hourly repeated administration of 0.25mg/kg budesonide in 100mg/kg surfactant (maximum of 6 doses) resulted in a 24% increase in BPD-free survival in the intervention group, without a deterioration of neurodevelopmental outcome (16, 333). More research however is needed to confirm these observations (PLUSS-trial is currently ongoing ACTRN12617000322336), to understand the working mechanism and to optimize the dosing regimens and formulation of such mixtures. A further dose decrease could for instance limit the potential for side effects.

Research on this type of interventions in a BPD context could, until recently, only be performed in larger preterm animal models, such as preterm lambs and preterm baboons. In our group, a hyperoxia-driven preterm rabbit model has been developed that depicts functional and structural characteristics of BPD and tolerates (daily) IT surfactant administration (302, 313). As exposure to hyperoxia resulted in an important increase in inflammatory mediators in preterm rabbit lungs, we anticipate that steroid treatment mitigates lung injury in this model (178).

We therefore evaluated in this preterm rabbit model, whether a single dose of budesonide added to surfactant prevents hyperoxia-induced lung injury.

Methods

Animal protocol

Time-mated pregnant rabbits (New Zealand White and Dendermonde hybrid) were provided by the animalium of KU Leuven. The experiments were approved by the Ethics Committee for Animal Experimentation (P080/2017) and performed according to local guidelines on animal welfare. Does were housed in individual cages, with free access to water and chow.

Cesarean section

The model was created as described before (146). Cesarean section was performed on day 28 (full term being 31 days). Does were sedated with intramuscular ketamin 35 mg/kg (Nimatek®; Eurovet Animal Health BV, Bladel, The Netherlands) and xylazin 6 mg/kg (XYL-M®; VMD, Arendonk, Belgium). After adequate sedation, does were subsequently euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (IV bolus of 1 ml T61®; Intervet Belgium, Mechelen, Belgium). Immediately following euthanasia the abdomen was opened through a low midline abdominal incision, the uterus exposed, and all pups extracted through hysterotomy.

Neonatal rabbit care

Following delivery, pups were dried, stimulated and placed in an incubator with a temperature, humidity, O_2 and CO_2-controller (OKOLAB©, Pozzuoli, Italy) at 32°C, 50% relative humidity and 95% of oxygen for the whole duration of the experiment. Oxygen concentration was continuously monitored with a Palm O2 D% Analyzer® (Analytical Industries, Pomona, US). After one to two hours, the surviving pups were randomized. Pups were fed twice daily via a 3.5 Fr orogastric tube placed prior to and removed immediately after feeding. The feeding consists of a milk replacer (Day One®, Protein 30%, Fat 50%; FoxValley, Illinois, US) mixed with water according to the manufacturer's directions. Probiotics, electrolytes and vitamins were added during the first 7 days (Bio-Lapis®; Probiotics International Ltd, Somerser, UK) and additional immunoglobulins during the first 2 days (Col-o-Cat®, SanoBest, Hertogenbosch, Netherlands). The volume of feeds is stepwise increased from a total of 80ml/kg body weight/day on day 1 of life to 200ml/kg/day on days 3-7. On day 2, vitamin K1 is administered intramuscularly (0.25 mg/kg, Konakion pediatrique®; Roche, Basel, Switzerland), and from that point until harvest, pups are given a daily intramuscular injection of benzylpenicillin

(20,000 IU/kg Penicilline®; Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg day, Amukin®; Bristol-Myers-Squibb).

IT injections

IT injections were performed under isoflurane anesthesia (2.5% in 2l/min oxygen; ISO-VET®, EuroVet, Heusden-Zolder, Belgium) as described before (302). Briefly, pups were placed supine, with the neck extended. While stabilizing the thyroid cartilage, the trachea was transcutaneously cannulated with a 26G catheter (Neoflon®, BD, New Jersey, US), through which a dose of surfactant (Curosurf®, Chiesi, Parma, Italy) or surfactant plus budesonide (Pulmicort®, AstraZeneca, Groot-Bijgaarden, Belgium), as required per individual experiment protocol, was injected with a Hamilton syringe.

Interventions

In a first experiment (Figure 8.1A) we wanted to evaluate what the effect was of adding budesonide to surfactant at short term. Pups were randomized to receive either an IT injection of 100mg/kg surfactant (Surf, 1µl/g) or 100mg/kg surfactant mixed with 0.25mg/kg of budesonide (Surf+Bud, 1,75µl/g) within 2h after birth and lung function and cytokine expression were determined 24h later.

In a second experiment (Figure 8.2A) we wanted to evaluate the effect of either a daily or single dose of a surfactant-budesonide mixture on the phenotype of hyperoxia-induced lung injury in the preterm rabbit model at day 7. Pups were randomized to 3 groups: a hyperoxia control group (H), a treated group with in total 6 daily IT injections of 100mg/kg surfactant plus 0.25mg/kg budesonide (total volume 1,75µl/g) on days 0 to 6 (H+dailySB) and a treated group with a single IT injection of 100mg/kg surfactant plus 0.25mg/kg budesonide (1,75µl/g) within 2h after birth (H+singleSB). Lung function, cytokine expression and both alveolar and vascular morphometry was determined at day 7.

Lung function testing

FlexiVent 5.2 (SCIREQ, Montreal, Canada) is used to perform forced oscillation and pressure-volume pulmonary function tests. After anesthesia with ketamine 35mg/kg and xylazin 6mg/kg the trachea of the pup is surgically exposed and an 18G metal cannula is inserted in the trachea and connected to the FlexiVent ventilator (120 breaths/min ventilation; tidal volume of 8ml/kg). A pressure volume perturbation is performed to measure inspiratory capacity and static compliance. Both measures are corrected for body weight. Prime-8 is a forced oscillation perturbation and is used to assess tissue

damping (frequency independent parenchymal energy dissipation) and tissue elastance (energy conservation of lung tissue). Three measures for each perturbation are obtained per pup and the mean value is used in the analysis.

Cytokine mRNA expression

After lung function testing, deeply anesthetized pups were euthanized by exsanguination. Right lungs were snap frozen for qPCR-assessment of cytokine mRNA expression. First, mRNA was extracted from the tissue using the Tri-Pure® Isolation reagent (Roche Diagnostics, Germany). RNA concentration was measured using the Nanodrop 1000® spectrophotometer (Thermo Scientific) and RNA integrity was assessed by checking the integrity of the 18S and 28S band by agarose gel electrophoresis. cDNA was synthesized with Taq Man® Reverse Transcription Reagents (ThermoFisher Scientific). The Platinum SYBR® Green qPCR Supermix-UDG with ROX (ThermoFisher Scientific) was used to detect expression of IL8 and CCL2. YWHAZ and HPRT were used as stable housekeeping genes to normalize mRNA levels. An overview of used primers, obtained from Integrated DNA Technologies (Haasrode, Belgium), is given in Supplementary Table S8.1. Samples were run in triplicate on a StepOne Plus® instrument (ThermoFisher Scientific). Relative quantitation was determined using the comparative Ct method. Statistics are calculated in based on ΔΔCt-values, while FC is used for visualization.

Alveolar and vascular morphometry

Left lungs were harvested for histology, and therefore pressure fixed on 25cmH_2O of paraformaldehyde. Fixed left lungs were embedded in paraffine, and for every lung a central sagittal 5µm slide was made for hematoxylin-eosin and Miller's elastic staining. Stained sections were scanned with a slide scanner (Axio Scan® Slide Scanner, Zen Zeiss, Oberkochen, Germany). Alveolar morphometry measurements are semi-automatically performed with a self-designed Fiji-plugin on 20 randomly selected fields per lung. This results in a measure for septal thickness (Lmw) and alveolar airspace size (Lma) (104). For vascular morphometry a minimum of 15 arteries with an external diameter of between 30- 100 µm were measured on Miller-stained slides to calculate medial thickness [MT% = (external diameter - internal diameter)/(2 x external diameter x 100%)], as previously described (148).

Statistical analysis

All statistical analysis was performed 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. For the

first, short term, experiment, unpaired t-testing was used to compare both groups. For the second experiment, one-way ANOVA was used to compare read-outs between groups. Post hoc Dunnett's multiple comparisons were used to compare both treated groups (H+dailySB and H+singleSB) to the control group (H). Normality was assumed after visual inspection of the data. In the analysis of Lmw, 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.

Results

Addition of budesonide to surfactant alters cytokine expression but not lung function within 24h

In the first, one-day experiment 16 pups from 3 mothers were used. Birth weights and survival did not differ significantly between the groups (Supplementary Table S8.2). The addition of budesonide to surfactant did not significantly affect inspiratory capacity, static compliance, tissue damping or tissue elastance, compared to surfactant alone when measured 24h after administration (Figure 8.1B-E). It however did significantly decrease mRNA-expression of CCL2 (p=0.02), but not of IL8 (Figure 8.1F-G).

Co-administration of surfactant and budesonide on day 0 improves lung function and cytokine levels on day 7

In total 70 pups from 10 mothers were included in the second experiment, of which 35 (50%) survived until day 7. Birth weights and survival were not significantly different between the groups (Supplementary Table S8.3, Figure 2B). A single dose of surfactant plus budesonide on day 0 improved inspiratory capacity (p=0.003) and static compliance (p<0.0001) at day 7. Repetitive daily IT injections of surfactant and budesonide however, did improve static compliance (p=0.0003) but not inspiratory capacity (Figure 8.2C-D) compared to hyperoxia controls (H). There was no significant effect of either a single or daily dose of surfactant plus budesonide on tissue damping and elastance measured by forced oscillation (Figure 8.2E-F). A single dose of surfactant plus budesonide did also significantly decrease mRNA-expression of both IL8 and CCL2 (respectively p=0.003 and p=0.02), while a daily dose did not (Figure 8.2G-H).

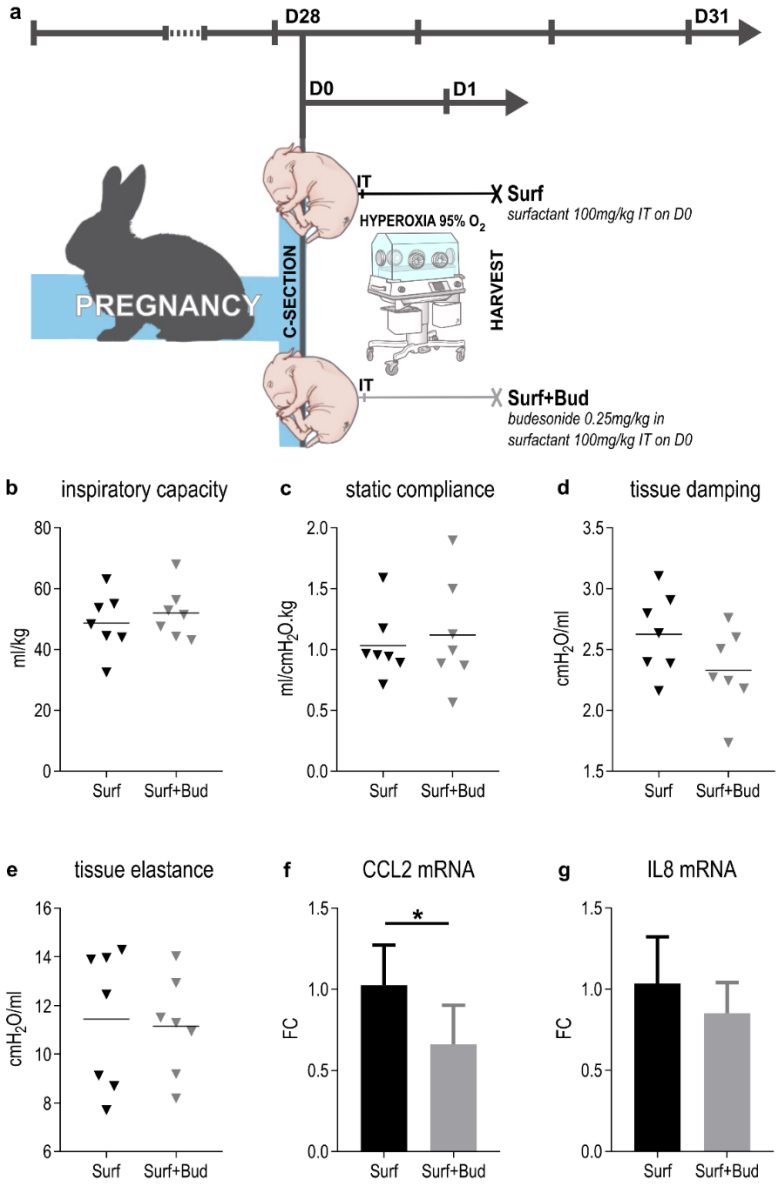


Figure 8.1. *(A) At 28 days of gestation pups are delivered by cesarean section, and receive either an IT injection of 0.25mg/kg budesonide in 100mg/kg surfactant or surfactant alone (n=8). All pups are kept in hyperoxia, until harvest 24h after injection (n=7). (B-E) Lung function read-outs inspiratory capacity, static compliance, tissue damping and tissue elastance are not affected by the addition of budesonide to surfactant. (F-G) mRNA-expression of CCL2, but not IL8, is significantly decreased by the addition of budesonide to surfactant. *p<0.05*

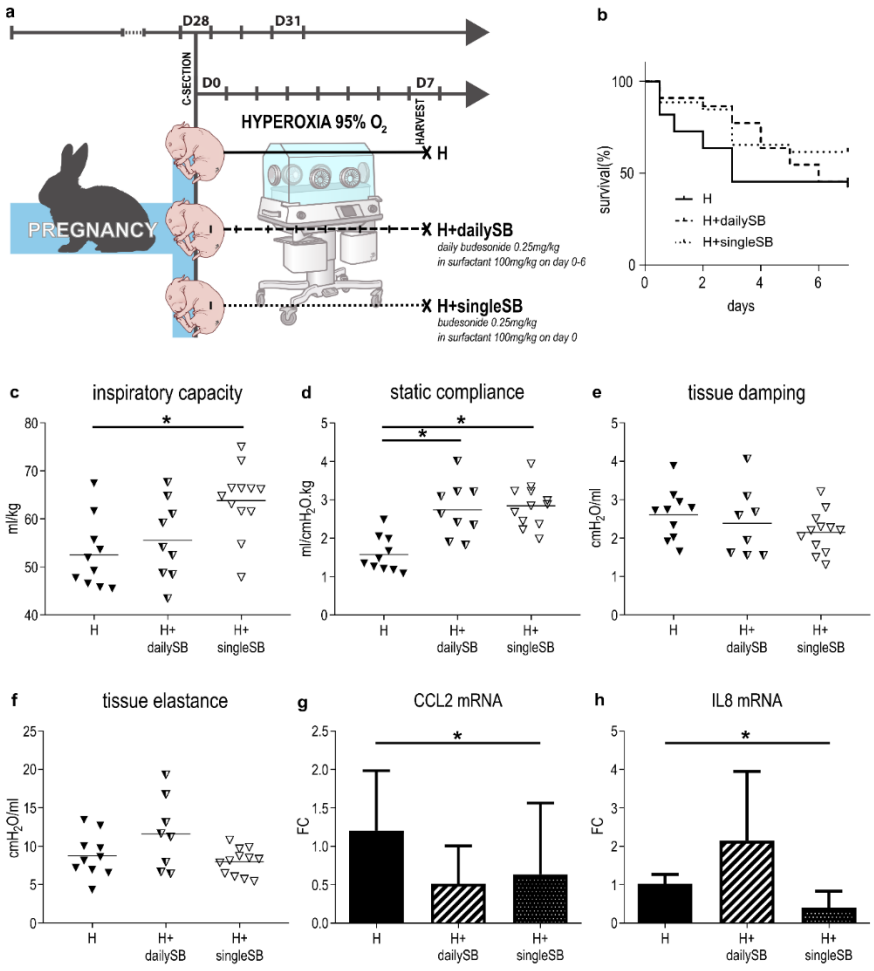


Figure 8.2. *(A) At 28 days of gestation pups are delivered by cesarean section, and are randomized to one of three groups (n=22-26): a control group, a group treated with daily IT injections of 0.25mg/kg budesonide in surfactant or a group treated with a single IT injection of 0.25mg/kg budesonide in surfactant on D0. All pups are kept in hyperoxia, until harvest at day 7 (n=9-12). (B) Survival is not significantly different between the treatment groups and controls (41-62%). (C) Inspiratory capacity is significantly increased in pups treated with a single, but not daily, dose of surfactant plus budesonide. (D) Static compliance is significantly increased by a single or a daily dose of surfactant plus budesonide. (E-F) Forced oscillation read-outs tissue damping and tissue elastance are not affected by a daily or single dose of surfactant plus budesonide. (G-H) mRNA-expression of CCL2 and IL8 is significantly decreased by a single, but not daily, dose of surfactant plus budesonide on day 0. *p<0.05*

Co-administration of surfactant and budesonide on day 0 decreases alveolar wall thickness

A single dose of surfactant plus budesonide did also alter lung tissue structure at day 7: it reduced alveolar wall thickness (Lmw; p=0.03; Figure 3A-B) without

altering alveolar size (Lma; data not shown). The daily dosing regimen however did not affect Lmw nor Lma. Finally, neither regimen did affect arterial wall thickness at day 7 (MT%; Figure 8.3A, 8.3C).

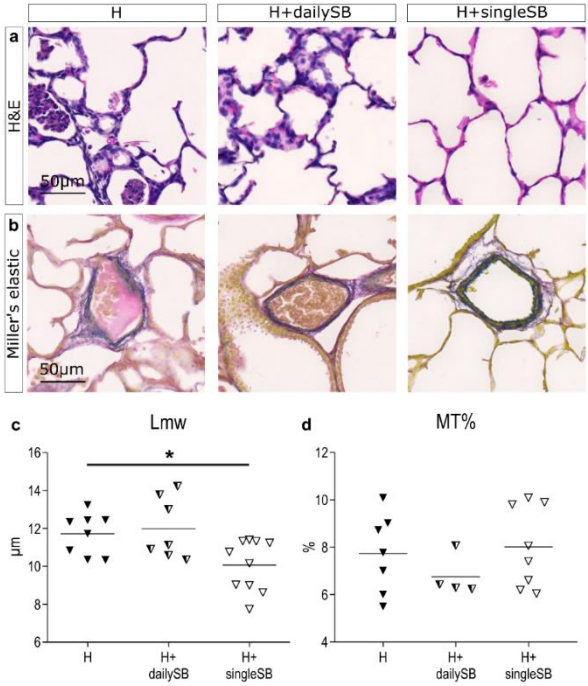


Figure 8.3. *(A) Representative pictures of HE-stained lung parenchyma. (B) Representative pictures of lung arteries stained with Miller's elastic staining. (C) Lmw (mean transsectional wall length) reflects septal wall thickness and is significantly decreased by a single, but not a daily, dose of surfactant plus budesonide. (D) Arterial medial wall thickness (MT%) is not affected by a daily or single dose of surfactant plus budesonide.*

Discussion

In this work, we tested the hypothesis whether a single dose of surfactant plus budesonide would be sufficient to attenuate the functional and structural manifestations of hyperoxia in a preterm rabbit model. We were able to show that the addition of budesonide to surfactant leads to a decreased pro-inflammatory cytokine expression at 24h after injection, and that a single dose of this mixture on day 0 indeed improves lung function, lung structure and cytokine expression at day 7 in preterm rabbit pups exposed to hyperoxia, in comparison to non-treated pups. Unexpectedly however, we did not observe the same beneficial effects in the group to which a daily dose of surfactant with budesonide was administered.

The observation that a single dose of surfactant plus 0.25mg/kg budesonide is able to attenuate preterm lung injury is in line with previous work, which shows that the addition of 0.25mg/kg budesonide to surfactant leads to improved lung physiology, decreased markers of lung injury, and decreased

systemic responses in liver and brain in ventilated preterm lambs at 2h, 6h and 24h (334). In parallel to our data, at 24h after injection in preterm lamb lungs a decrease in CCL2 is observed, but not in IL8. Our study is the first to look at longer term effects of a single dose of surfactant plus budesonide, on a BPD-phenotype. We are able to show a beneficialeffect of a single dose on lung function, structure and inflammation persisting at least until day 7. Because of the short half-life of budesonide in the lungs (311, 334), this effect is probably driven by a lasting pharmacodynamic effect (possibly by inhibiting an inflammatory cascade). This suggests future preclinical experiments and clinical trials could consider less frequent dosing (and thus lower exposure) than the repetitive 8 hourly regimen (maximum of 6 doses) of 0.25mg/kg budesonide in surfactant which has been clinically studied by Yeh et al (16).

The main working mechanism of budesonide is likely its anti-inflammatory effect, as demonstrated in ex vivo fetal lungs (335), fetal and neonatal preterm lambs (334, 336) and tracheal fluid of human preterm babies receiving budesonide (16). Our data adds that a single dose of surfactant plus budesonide is able to decrease the expression of IL8 and CCL2 in response to hyperoxia even until 7 days later. An attenuation of the inflammatory reaction to preterm birth and hyperoxia, leading to less tissue injury, could indirectly explain the improved lung compliance and structure (337). Increased compliance and thinner alveolar septa however could also be explained by a decreased water content of the neonatal lung tissue or a change in extracellular matrix. Corticosteroids can induce expression of salt transporters such as the epithelial sodium channel (ENaC), and thereby promote fluid reabsorption (338). In preterm sheep, exposure to budesonide resulted for instance in lower wet to dry lung weight ratios (336). It has also been demonstrated that steroids affect extracellular matrix composition by delaying elastin and αSMA-expression (339).

The rationale behind the combined use of surfactant and budesonide is that surfactant is regularly administered to the population at risk of BPD, which could benefit from a therapy aiming to improve long term respiratory outcome, such as budesonide. In both in vivo and in vitro experiments it has been established that budesonide does not alter the potential of surfactant to reduce surface tension and rescue respiratory distress syndrome (317). Mixing budesonide in surfactant should furthermore enhance its distribution to the distal lung (329) and reduce systemic off-target exposure via the circulation to other organs like the brain (311). This strategy likely avoids neurocognitive and other side effects of postnatal steroids as systemic exposure is reduced (333).

In our study we did not investigate distant side effects of IT steroids, however the absence of a beneficial effect of daily dosing of surfactant plus budesonide could indicate a possible toxic effect on the preterm rabbit lung of high cumulative doses of budesonide. In the daily dose group, there was an increase in compliance, however without increase in inspiratory capacity. This could indicate less lung growth resulting in smaller lungs. Furthermore daily IT injections with surfactant plus budesonide led to a (non-significant) trend towards increased expression of IL8 On CCL2 however, there is a downward trend which is not significant, due to the large variability on the ΔΔct-values. It seems unlikely that this effect is due to repetitive trauma of IT injections, as we previously could not show any adverse effect on lung function and structure of repetitive surfactant injections (302). It is finally unclear whether this effect is specific for (preterm) rabbits. It is for instance known that rabbits are very sensitive to steroid-induced immunosuppression (340).

The major strength of this study is the focus on medium term functional and structural changes in the lung after treatment with budesonide plus surfactant in a well-established model for BPD. By instilling the surfactant-budesonide mixture intratracheally in spontaneously breathing subjects, it also mimicks to some extent the current low-invasive methods to administer surfactant, without PEEP. This study also has some limitations. First, the addition of a surfactant-treated hyperoxia and normoxia control group to the 7 day experiment could help distinguish the effect of hyperoxia and repetitive injections, however in earlier work we showed that daily surfactant injections did not alter lung function or structure (302). In the 24h experiment on the other hand, the addition of an untreated control group could be of added value to evaluate the effect of surfactant alone at short term in this model. Second, we did not directly investigate lung fluid content nor extracellular matrix composition, which could help explaining the effect of budesonide. Third, there is no factor mechanical ventilation involved in our experiments. The effect of surfactant plus budesonide could be different in patients and models including volutrauma through mechanical ventilation. Finally, overall mortality in this experiment was rather high (in all groups). This reflects the prematurity of the animal model, but also leads to a selection bias of the more healthy animals in the outcome measures at day 7.

In conclusion, we demonstrate that a single dose of budesonide plus surfactant has a beneficial effect on lung function, lung structure and cytokine expression in preterm rabbit pups exposed to hyperoxia persisting until day 7. Our results also suggest caution is required for high cumulative or frequent dosing of budesonide in preterm lungs. Future experiments and clinical trials

should aim to identify the lowest possible dose that improves lung function, without resulting in (distant) side effects.

Appendix

Supplementary Table S8.1: *Primers used for cytokine qPCR*

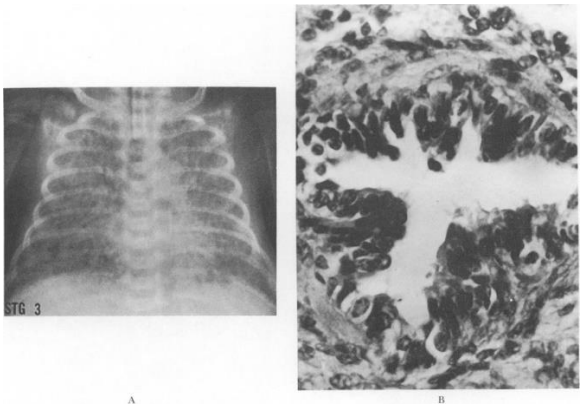
Supplementary Table S8.1. Primer sequences.	
IL8 FW	CCACACCTTTCCATCCCAAAT
IL8 RV	CTTCTGCACCCACTTTTCCTTG
CCL2 FW	GAAGAATCAACAGCACCAAGTG
CCL2 RV	GTGTCCGGGTGGAAGATAAAT
YWHAZ FW	TGATTAGTGATGATGAACCG
YWHAZ RV	CACACAGAGGGCTACAATG
HPRT FW	GGTCTGGCCCTTAACTTCTCTGTGTTCTA
HPRT RV	GCGTGCTGTCTTTGTATGATTCTTCACTT

Supplementary Table S8.2. *Weight and survival data for the 24h experiment*

Supplementary Table S8.2. Weight and survival 24h experiment			
	Surf mean ± sd	**Surf+Bud** mean ± sd	*t-test p*
Day 0			
Number of pups (n)	8	8	*N/A*
Birth weight (g)	43,2 (±3,9)	44,6 (±4,9)	*0.55*
Day 1			
Survival (n, %)	7/8 (87%)	7/8 (87%)	*N/A*

Supplementary Table S8.3. *Weight and survival data for the 7 day experiment*

Supplementary Table S8.3. Weight and survival 7 day experiment				
	H mean ± sd	**H+dailySB** mean ± sd	**H+singleSB** mean ± sd	*ANOVA p*
Day 0				
Number of pups (n)	22	22	26	*N/A*
Birth weight (g)	34,2 (±6,7)	37,2 (±5,9)	36,3 (±6,0)	*0.28*
Day 7				
Survival (n, %)	10/22 (45%)	9/22 (41%)	16/26 (62%)	*N/A*
Weight on day 7 (g)	49,5 (±6,5)	54,6 (±8,4)	53,1 (±8,4)	*0.36*
Weight gain (%)	31 (±9)%	36 (±13)%	38 (±14)%	*0.58*



Northway (NEJM, 1967):
Northway identified BPD as an adverse effect of mechanical ventilation in preterm babies

Chapter 7

Development of a neonatal adverse event severity scale through a Delphi consensus approach

Salaets T., Turner M., Short M., Ward R., Hokuto I., Ariagno R., Klein A., Beauman S., Wade K., Thomson M., Roberts E., Harrison J., Quinn T., Baer G., Davis J., Allegaert K., for the International Neonatal Consortium
Published in Archives of Disease in Childhood(341)

Abstract

Background - Assessment of the seriousness, expectedness, and causality are necessary for any adverse event (AE) in a clinical trial. In addition, assessing AE severity helps determine the importance of the adverse event in the clinical setting. Standardization of AE severity criteria could make safety information more reliable and comparable across trials. Although standardized AE severity scales have been developed in other research fields, they are not suitable for use in neonates. The development of an AE severity scale to facilitate the conduct and interpretation of neonatal clinical trials is therefore urgently needed.

Methods - A stepwise consensus process was undertaken within the International Neonatal Consortium (INC) with input from all relevant stakeholders. The consensus process included several rounds of surveys (based on a Delphi approach), face-to-face meetings, and a pilot validation.

Results - Neonatal AE severity was classified by five grades (mild, moderate, severe, life threatening, or death). AE severity in neonates was defined by the effect of the AE on age appropriate behavior, basal physiologic functions, and care changes in response to the AE. Pilot validation of the generic criteria revealed $\kappa = 0.23$ and guided further refinement. This generic scale was applied to 35 typical and common neonatal AEs resulting in the INC neonatal AE severity scale (NAESS) v1.0 which is now publicly available.

Discussion - The INC NAESS is an ongoing effort that will be continuously updated. Future perspectives include further validation and the development of a training module for users.

Introduction

An AE is defined as “any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related” (342-344). In vulnerable populations such as critically ill neonates, background rates of mortality and morbidity are high (3). This leads to a higher incidence of reported AEs, not necessarily attributed to the investigational medicinal product (IMP) or device. However, when recognized and reported in a standardized manner, AEs can be important safety signals.

Regulatory guidelines require investigators to assess whether an AE is serious and whether or not there is a reasonable possibility that it is related to IMP administration (causality) (Figure 9.1) (343). A strict regulatory definition

exists for “seriousness” (343). Although causality involves clinical judgment, algorithms have been developed to make this assessment in a neonatal population more objective and homogeneous (345, 346). Data Safety Monitoring Boards and sponsors review the investigator report of an AE and adjudicate whether or not the AE is expected, based on known side effects (Reference Safety Information), and background complication rates (343). Finally, assessing the severity of an AE enhances the reporting process by capturing medical intensity.

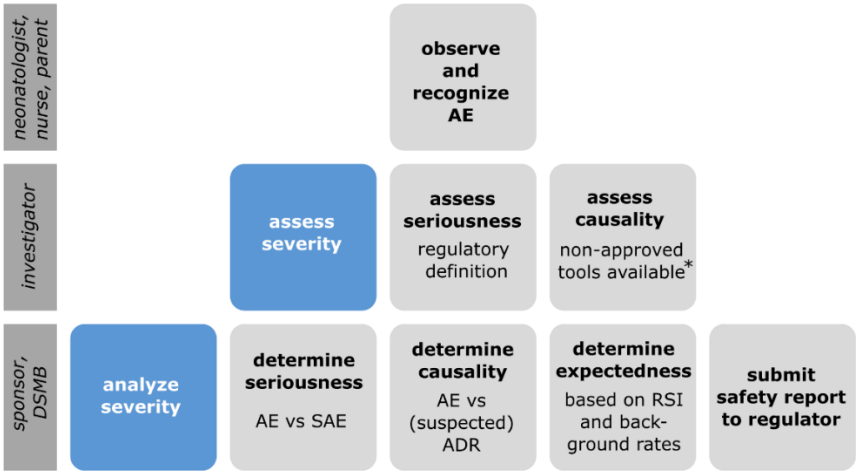


Figure 9.1. *This figure summarizes aspects of AEs that should be considered to account for safety reporting in clinical trials. It visualizes responsibilities of the different actors and the currently available criteria and guidance. The figure is not intended to illustrate sequential activities. Abbreviations not occurring in text: serious adverse event (SAE), suspected unexpected serious adverse drug reaction (SUSAR). *Causality assessment relies on regulatory guidance, however algorithms (e.g. Du et al.) have been developed for a neonatal population.*

Communication of safety data between study investigators, sponsors, and regulators remains suboptimal because of diverse ways of collection, reporting, and assessment. A common clinical research language, using standard terms and definitions, could facilitate responsible data sharing (347, 348). Within neonatology, efforts have been directed towards developing standard terminology and definitions for adverse events (349) that integrate in to larger dictionaries such as the Thesaurus of the National Cancer Institute (NCI) or the Medical Dictionary for Regulatory Activities (MedDRA) (350, 351).

Standardizing criteria to report AE severity could make safety information more comparable across centers and trials and is a reasonable next step (352). In other research fields, toxicity tables and AE severity scales are

commonly used (353-355). The most widely used example is the Common Terminology Criteria for Adverse Events (CTCAE) which was developed for oncology research (356). As the generic severity criteria of these scales are not applicable to neonates (Table 9.1), we identified the need for a NAESS with criteria forneonatal AEs. Currently only the Division of AIDS (DAIDS) Table for Grading the Severity of Adverse Events, contains some neonatal AE's, however it is very incomplete and recommends the use of local reference values for preterm neonates (353).

Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Mild	Moderate	Severe	Life threatening	Death
Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated	Moderate; minimal, local or non-invasive intervention indicated; limiting age-appropriate instrumental activities of daily living	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activities of daily living	Life-threatening consequences; urgent intervention indicated	Death related to AE

Table 1. *Table shows the generic severity criteria of CTCAE, which are commonly used for adult and pediatric patients, but are not directly applicable to neonates.*

Methods

A stepwise consensus process, based on a Delphi approach (357), was undertaken between December 2016 and September 2018. First, generic severity criteria for neonatal AEs were developed. Next, these severity criteria were applied to frequently occurring neonatal AEs. Finally, the terminology was integrated into larger terminology dictionaries (Figure 9.2). Full reports for every step can be found in the appendices.

Stakeholder input

The NAESS was developed within the INC. INC was established in 2015 in order to address regulatory and scientific challenges in the development of innovative drugs for neonates (358, 359). Throughout the neonatal AE scale development, input was requested from multiple key stakeholders involved in neonatal drug development. Academic and non-academic clinicians and

researchers, industry representatives, regulators, nursing and funding organizations and parent representatives from Canada, Europe, Japan and USA participated in the process (Figure 9.2). Respondent and participant groups were expanded at every round to incorporate feedback from a maximal number of stakeholders.

Development of generic severity criteria

Step 1: A first anonymous online survey was circulated through the network of INC and assessed in general terms regarding which aspects of AEs could be used as severity markers and provide input on the framework of NAESS.

Step 2: A second anonymous online survey presented the results and feedback of the first survey together with a proposal for generic severity criteria based on these results (Delphi approach (357)).

Step 3: In a face-to-face meeting, all components of the scale were discussed in the context of the feedback received in the surveys in order to achieve consensus on the generic severity criteria.

Step 4: Pilot validation was undertaken to test the validity and inter-observer agreement of the consensus generic severity criteria. Nineteen written case reports of AEs were provided by the University of Liverpool (360). All reports contained information on parameters, clinical and technical examinations, drug exposure, and any resulting care changes. Twelve observers with different backgrounds from Canada, Europe, Japan and USA graded the severity of the 19 AEs using the proposed full generic severity scale and all individual markers (see Appendix 9.1). Of these 12 observers, 8 had previously contributed to the scale as respondent or participant in any of the previous steps. The results were analyzed by calculating a free-marginal multi-rater kappa as a measure of inter-observer reliability (361). The generic criteria were subsequently improved based on the results of this exercise.

Development of event specific severity criteria

Step 5: During a subsequent *face-to-face meeting*, thematic subgroups (neurological, cardiovascular, respiratory, gastrointestinal and infectious/general neonatology) drafted severity criteria for a list of specific neonatal AEs provided in step 1.

Step 6: The resulting specific severity criteria were evaluated in *a final anonymous online survey*. For all AEs with more than 20% disagreement, modifications were made in order to align all the key stakeholders. All modified criteria were approved in a final teleconference.

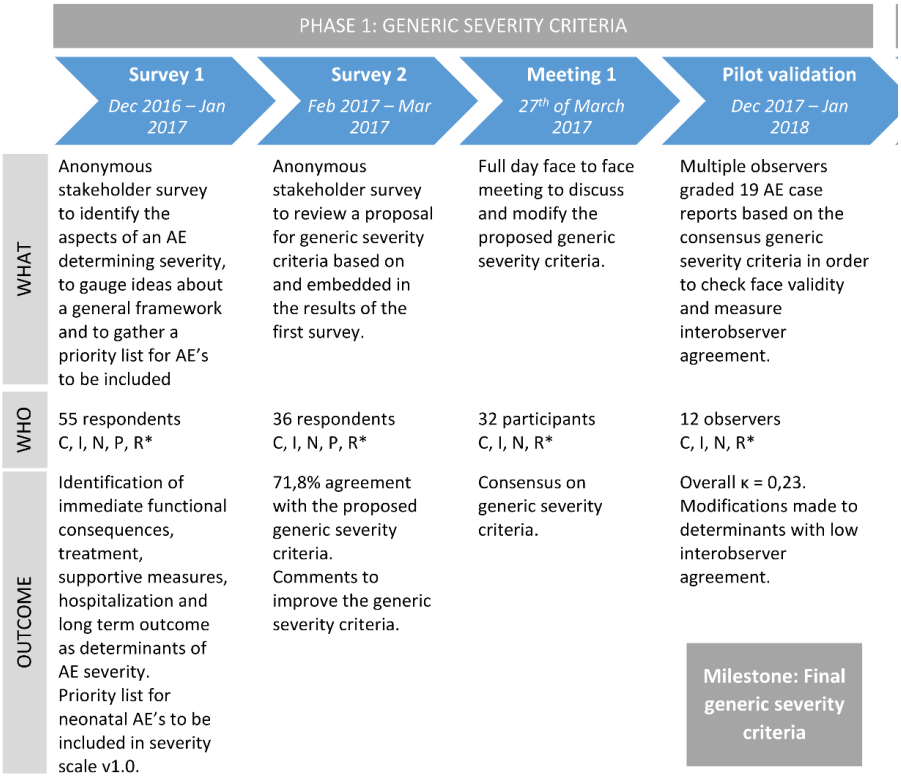


Figure 9.2 (a). This figure gives an overview of the development process of the NAESS. Stakeholder involvement is indicated by C (clinicians), I (industry), N (nursing representatives), P (parent representatives) and R (regulatory authority employees)

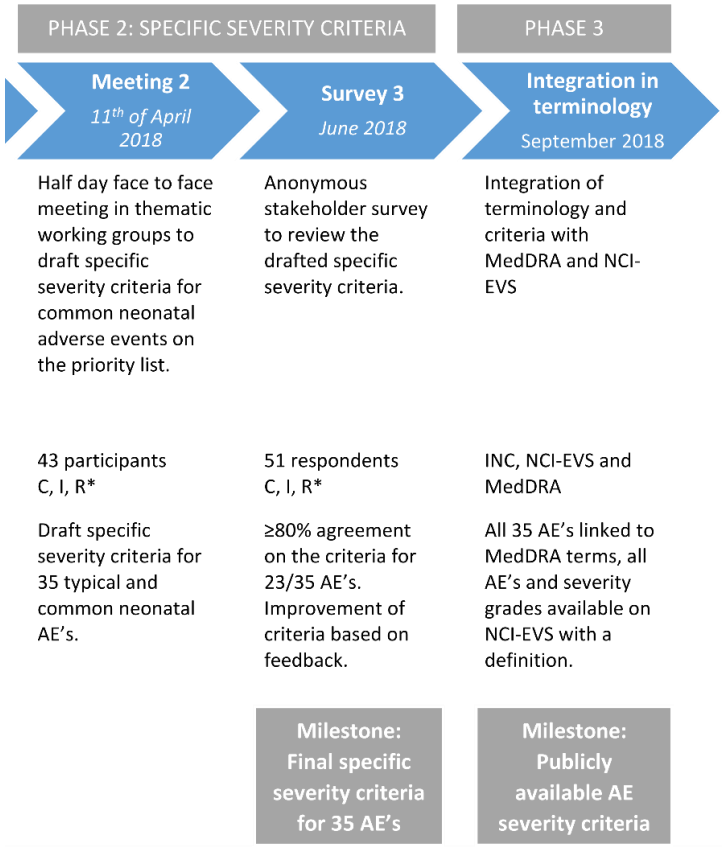


Figure 9.2 (b). This figure gives an overview of the development process of the NAESS. Stakeholder involvement is indicated by C (clinicians), I (industry), N (nursing representatives), P (parent representatives) and R (regulatory authority employees)

Linking to existing terminology

Step 7: For each AE, a definition was used from the National Institute of Child Health and Human Development (NICHD) Pediatric AE Terminology if appropriate (349). Every AE was linked to the corresponding MedDRA Lowest Level Terms (LLTs). MedDRA Maintenance and Support Services Organization (MSSO) was contacted with a proposal to adjust or add terms if no suitable LLT was available. All criteria were added to the NCI Thesaurus.

Results

In total, 109 members participated in the process leading to consensus on INC NAESS v1.0. Participant numbers and background for all steps is summarized in Figure 9.2 and Appendix 9.2. All participants who were involved in at least one step are listed in the Acknowledgement section.

Development of generic severity criteria

Step 1: We received 55 responses to the first survey. Immediate functional consequences (accepted by 81% of respondents), changes in treatment (82%), prolongation of hospitalization (75%), supportive measures (85%), and long-term outcome (73%) were accepted as indicators of AE severity. Many comments referred to the feasibility of using long-term outcome to classify AE severity (see Appendix 9.3).

Step 2: Thirty-six respondents completed the second survey. Of the respondents, 72% agreed with the proposal for generic severity criteria based on immediate functional consequences, treatment (including supportive measures), and prolongation of the initial hospitalization. The remaining 28% of respondents suggested adjustments (see Appendix 9.4).

Step 3: Thirty-nine experts participated in the first face-to-face meeting trying to define generic severity criteria for neonates. Consistent with other AE severity scales, severity was subdivided into 5 categories: mild, moderate, severe, life-threatening, and death. Immediate functional consequences (on age appropriate behavior and basal physiological functions), together with resulting care changes were established as the parameters of the generic AE severity scale. Additionally, the stakeholder group agreed that this AE severity scale would pertain to neonates <44 weeks postmenstrual age (196, 362).

Step 4: The proposed consensus scale resulted in a multi-rater free-marginal κ of 0.23 (ranging from -0.03 to 0.59 for individual AEs). Severity evaluations based on 'care changes' alone resulted in suboptimal agreement.

Furthermore, marked intra- and inter-observer variability was noted in how the 12 observers weighted the different factors involved in the determination for the final severity grade (see Appendix 9.1).

Examples of minor and major care changes were added for clarification. It was also specified that if individual determinants of severity resulted in a discrepant severity grade, the highest grade would be reported. These changes resulted in the final generic severity criteria as shown in Table 9.2.

Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Mild	Moderate	Severe	Life threatening	Death
Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; no change in baseline age-appropriate behavior*; no change in baseline care or monitoring indicated	Moderate; resulting in minor changes of baseline age-appropriate behavior*; requiring minor changes in baseline care or monitoring***	Severe; resulting in major changes of baseline age-appropriate behavior* or non-life-threatening changes in basal physiological processes**; requiring major change in baseline care or monitoring****	Life-threatening; Resulting in life-threatening changes in basal physiological processes**; requiring urgent major change in baseline care	Death related to AE
*Age-appropriate behavior refers to oral feeding behavior, voluntary movements and activity, crying pattern, social interactions and perception of pain. **Basal physiological processes refer to oxygenation, ventilation, tissue perfusion, metabolic stability and organ functioning. ***Minor care changes constitute: brief, local, non-invasive or symptomatic treatments ****Major care changes constitute: surgery, addition of long-term treatment, upscaling care level				
If the different factors of this scale result in conflicting severity grades, **the highest grade** should be reported.				

Table 9.2. *Table shows the generic severity criteria of INC NAESS, developed for use in neonates.*

Development of event specific severity criteria

<u>*Step 5:*</u> Forty-two professionals, in 5 thematic subgroups, drafted the specific severity criteria for the 35 common neonatal AEs (Table 9.3) during a second face-to-face meeting. Specific disease markers were used as severity criteria if it was determined that they reflected the factors incorporated in the generic severity scale. AEs based on laboratory values were not included in this first version because reliable reference values for normality were not available (for postmenstrual and postnatal age).

Step 6: Among the 51 respondents of the final survey, there was ≥80% agreement for 23 out of 35 of the draft criteria (indicated with an asterisk*). For the remaining 12 AEs, criteria were then adjusted (see Appendix 9.5) and approved in a final teleconference. The resulting specific severity criteria for all selected 35 AEs can be found in the INC AE severity scale v1.0 (Appendix 9.6).

AEs in INC neonatal AE severity scale	
Neurological	**Respiratory**
Neonatal Convulsion	Infantile Apnea
Neonatal Epileptic Seizure	Neonatal Respiratory Insufficiency
Neonatal Intraventricular Hemorrhage*	Neonatal Respiratory Distress Syndrome
Retinopathy of Prematurity*	Neonatal Pulmonary Hemorrhage*
Hypoxic Ischemic Encephalopathy	Persistent Pulmonary Hypertension of the Newborn*
Periventricular Leukomalacia*	Neonatal Pneumothorax*
Infant Irritability	Bronchopulmonary Dysplasia
Infant Sedation*	**Gastrointestinal**
Cardiovascular	Necrotizing Enterocolitis
Neonatal Hypotension	Neonatal Diarrhea*
Neonatal Hypertension*	Infantile Vomiting*
Neonatal Sinus Tachycardia*	Feeding Intolerance
Neonatal Sinus Bradycardia	Neonatal Gastrointestinal Bleeding*
Neonatal Tachyarrhythmia*	Neonatal Spontaneous Intestinal Perforation*
Neonatal Bradyarrhythmia	Neonatal Constipation*
Neonatal Edema*	**General**
Neonatal Coagulation Disorder*	Neonatal Rash*
Infectious	Neonatal Administration Site Complication*
Neonatal Culture Positive Sepsis*	Neonatal Fever*
Neonatal Culture Negative Sepsis*	

Table 3. *Table shows the AEs included in the current version of INC NAESS. For these 35 AEs, specific severity criteria were defined.*

Linking to existing terminology

For twenty-three (66%) of the AEs in v1.0 definitions were taken directly from the NICHD Pediatric AE Terminology. Nineteen (54%) of the AEs could be linked to an existing Lowest Level Term in MedDRA Version 21.0. New LLTs were added in MedDRA v22.0 to match the remaining AEs. For all AEs and severity grades, specific codes for the NCI Thesaurus were generated. The terminology was made publicly available on the NCI Thesaurus (https://evs.nci.nih.gov/ftp1/INC/Adverse_Events_Terminology/)

Discussion

This paper describes the development of the INC NAESS. It provides criteria that guide investigators and clinicians in assessing severity of AEs. It was developed to increase the quality of safety information.

Generic severity criteria

The common framework of the AE severity scale is contained in generic criteria shown in Table 9.2. It defines suitable severity markers for neonates, in contrast to criteria used in other populations (e.g. CTCAE; Table 9.1). First, impact on age appropriate activities is included, describing the signs exhibited by the neonate (feeding behavior, voluntary movements, activity, crying, social interactions, and signs of pain). The impact on basal physiological processes (changes in oxygenation, ventilation, circulation, metabolic stability, and organ function) is a second severity marker. A third severity marker is the change in care in response to the AE, as it reflects the severity of the underlying event and is indicative of additional stress. All of this information is readily available after the AE occurs and permits immediate severity grading.

It is important to note that for all determinants, only changes from the baseline condition due to the AE should be considered. Also, even though these criteria provide guidance, some form of clinical judgement (and thus subjectivity) remains inherent in the severity assessment.

The final decision was not to include long term outcome as a marker of AE severity, as it might be difficult to establish a direct causal link. Furthermore, the overall goal of this scale is to create reliable and immediate safety signals prompting awareness, which is not compatible with assessing the severity of an event when the final outcome is only known years later. Despite this decision, examining the long term outcome associated with a neonatal drug exposure remains a crucial effort that should be encouraged (196).

Validation

The goal of a standardized severity scale is to reduce subjectivity in severity assessments and thus reduce interobserver variability. Our pilot validation exercise on the consensus generic severity criteria revealed only fair agreement ($\kappa = 0.23$) among observers of different backgrounds. This seems less rigorous than what is published for other severity scales (e.g. CTCAE (363), SAVES-2 (364)). It should be noted that our results were obtained by applying a generic scale, which is purposely broad and thus provides less

direct guidance. Furthermore, our pilot validation was performed as a part of an optimization process, resulting in improved final generic severity criteria. Future plans include measurement of inter-observer agreement with the final scale on prospectively collected data in order to show the benefit of using standardized criteria for the severity of neonatal AEs. For this validation we will ask 2 observers, in 5 centers around the world to grade the severity of 30 adverse events occurring in there unit, before and after being trained to use NAESS v1.0. In parallel a retrospective validation using AE case reports from a clinical trial is being planned. In the meantime there also has been interest from clinical trials, to use this instrument in their protocol.

INC NAESS v1.0

The current version (v1.0) contains specific severity criteria for 35 routinely encountered neonatal AEs and more will be added. For instance, laboratory based AEs will be included when age appropriate reference values become available. The severity of AEs currently not included in INC NAESS v1.0 can be estimated by applying the generic criteria.

This INC NAESS v1.0 is now publicly available in the NCI Thesaurus. This platform allows end users to embed the criteria relevant to a particular project within the concept information. Its presence in the Thesaurus improves dissemination to a relevant audience. Finally, NCI Thesaurus users can request the addition of new AEs or modifications of existing criteria which permits the terminology to be revised as needed. This ensures the sustainability of the INC NAESS.

The INC NAESS is a continuous work in progress. We are preparing to expand the number of AEs included and encourage readers to suggest new AEs for addition or comment on existing criteria if deemed not appropriate through the NCI Thesaurus website.

Anticipated benefits

Severity assessments provide a nuanced clinical appreciation of an AE. Standardizing this information leads to more reliable and comparable information which can facilitate regulatory safety evaluations of drugs. Furthermore, it can improve scientific communication on AEs in publications. The availability of a standardized severity scale can also facilitate the conduct of clinical trials in neonates. For example dose reductions can be recommended in response to severe AEs. In the neonatal population where the background rates of clinical AEs are high, protocols could stratify reporting obligations for different severity grades and background rates of

AEs. This could enhance consistent AE reporting and reduce the administrative burden associated with neonatal research.

Finally, the scale could also be used in routine clinical care and for post-marketing pharmacovigilance. Neonates are vulnerable to adverse drug reactions (ADR) in clinical care, but these events are underreported (365). NAESS was developed to classify AEs, which would complement neonatal algorithms used to identify ADRs (345, 346). A standardized evaluation of severity would add information that is useful for pharmacovigilance, quality improvement projects, observational studies or registries and can help establish risk/benefit ratios of common therapies (366).

Conclusion

In conclusion, a neonatal AE severity scale containing both generic and specific criteria for 35 common neonatal AEs was developed in collaboration with key stakeholders involved in neonatal drug development. The use of this tool can improve the quality of drug and device safety evaluations and can facilitate the conduct of neonatal clinical trials. Future perspectives include validation research to assess inter-observer agreement and the addition of more AEs.

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Furthermore we would like to acknowledge Anna Davis and Rebecca Spencer from the University College London for their help in initiating this process.

Appendix

Appendix 9.1: Full report of pilot validation exercise.

Appendix 9.2: Background data of participants to all steps of consensus process

Appendix 9.3: Full report of survey 1 (on generic criteria)

Appendix 9.4: Full report of survey 2 (on generic criteria)

Appendix 9.5: Full report of survey 3 (on specific criteria)

Appendix 9.6: INC NAESS v1.0

The appendices are available on https://adc.bmj.com/content/early/2019/09/18/archdischild-2019-317399.long.

Appendix 6, or the current version of INC NAESS, is also available online: https://evs.nci.nih.gov/ftp1/INC/Adverse_Events_Terminology/



Baby cage, London (1930's):
In the 1930's window cages were very popular in urban areas. Parents thought that giving babies fresh air would purify their blood and help their lungs grow.

Chapter 8

Parental perspectives long term after neonatal clinical trial participation

Salaets T., Lavrysen E., Smits A., Vanhaesebrouck S., Rayyan M., Ortibus E., Toelen J., Claes L., Allegaert K.
Manuscript in preparation

Abstract

Importance – Although including babies and infants in trials are ethically challenging, clinical trials remain essential to improve neonatal care. There is a lack of empirical data on the parental perspectives following participation of their neonate in a clinical trial, especially at long term.

Objective – To assess experiences and emotions of parents, long term after trial participation in an interventional drug trial.

Design – Survey.

Setting – Single academic neonatal intensive care unit in Leuven, Belgium.

Participants – Parents of former neonatal trial participants.

Exposure – Participation of their neonate in an interventional drug trial, between 3 and 13 years ago.

Main outcomes – Parental contentment with trial participation, perceived influence of the trial on care and health, emotional consequences of participation and awareness of typical clinical trial characteristics.

Results – The survey was completed by 123 parents (31% of potential respondents, representing 52% of involved families). Twenty percent of parents did not remember participation in the clinical trial. Those who remembered participation reported high contentment with trial participation, but rather low contentment with follow-up after participation. Most parents did not perceive any influence of the trial on the care for and health of their child. The perception of a positive effect was more common than the perception of a negative effect. Almost all parents reported satisfaction and pride, while a minority of parents reported anxiety, stress or guilt related to trial participation. A relevant minority was unaware of typical trial characteristics such as equipoise, the possibility of side effects, the presence of a control group, blinding or randomization.

Conclusions and Relevance – Overall, parents reported positive experiences and little emotional distress long term after participation. Future efforts to improve the practice of neonatal clinical trials should focus on ensuring effective communication about the concept and characteristics of a clinical trial during consent discussions and on the follow-up after the trial.

Introduction

Every year, an estimated 15 million babies are born preterm (367). Neonatal mortality and morbidity can be reduced with evidence-based interventions, which need to be tailored to this specific population (196). Unfortunately, many drugs are still being used off-label in (pre)term neonates, without evidence (368). Clinical trials can identify better care practices, uncover useless or harmful therapies, and reveal new knowledge gaps (369). The need for high quality clinical trials in neonates thus remains high.

Balancing the interests of a trial for future patients against the interests of an individual study participant remains a difficult ethical exercise (370). In clinical trials study participants are exposed to a treatment of which safety and efficacy is not yet established. To deliver reliable and unbiased information on the effect of an experimental therapy, participants included in a blinded randomized clinical trial are randomly assigned to either a study or control (placebo) group. Parents and clinicians are unaware of the treatment allocation (blinding). This concept of a clinical trial does not necessarily serve the individual interests of study participants.

Clinical research in neonates has additional challenges. Enrollment in trials in the neonatal intensive care unit (NICU) often occurs at a moment when parents are overwhelmed by stress and emotions. The validity of informed consent in this population has therefore been questioned (371). After trial participation, parents reported that they did not always understand the purpose, potential benefits and risks of the trial (372). Often the process of randomization is not well understood, sometimes even resulting in astonished and angry reactions during in-depth interviews afterwards (373).

There is a lack of empirical data on how parents evaluate participation in a neonatal drug trial and on how the trial affects their emotional well-being (374). Currently available evidence, shows that parents overall think of trial participation as a positive experience (375-378). However, these studies evaluated parents of neonates exposed to smaller non-pharmacological experimental interventions, and focused on the parental experience during the trial. We are unaware of any study evaluating the effect of participation in a neonatal clinical trial at long term.

In the current study, we assessed the perspectives and emotions of parents long term after trial participation, in an interventional drug trial conducted in the NICU.

Methods

Parental perspectives were evaluated through an online questionnaire for parents who participated in 1of 5 interventional clinical trials. This survey was approved by the Ethical Committee of the University Hospitals Leuven.

Setting

All 5 included trials were interventional clinical trials in which neonates were exposed to a novel treatment (novel product or novel dosing regimen), however these trials had different objectives and methods. The SMOF-trial was a double blinded, randomized controlled clinical trial comparing a new lipid emulsion to a conventional soy emulsion for parenteral nutrition in preterm infants (379). NIRTURE was a multicenter open label, randomized controlled trial evaluating the effect of early insulin therapy on mortality and sepsis in very low birth weight infants (380). The DORIPENEM-study was a multicenter phase I clinical trial evaluating the pharmacokinetics, safety and tolerance of a single dose of intravenous infusion of doripenem in pre(term) neonates (381). The LAIF-trial was a multicenter double blinded, placebo-controlled, randomized trial to test the efficacy of adding recombinant bile salt-stimulated lipase to the feeding of preterm infants to promote growth (382). NEOPROP was a dose-finding study to evaluate safety and efficacy of different doses of propofol for sedation during semi-elective intubation (383). The main characteristics of the 5 included trials can be retrieved from their initial publications, and are summarized in Table 10.1.

Questionnaire

The items of this questionnaire were developed after reviewing literature and interviewing different stakeholders: neonatologists, psychologists, neonatal research nurses, representatives of the Flemish preemie parent alliance (VVOC) and parents of former study participants. Face validity of the resulting items was evaluated by testing the questionnaire in the same stakeholder group. To make the questionnaire as personalized as possible, adapted versions were made (mentioning the right trial name, referring to both children when both members of a twin participated, referring to the death of a child for bereaved parents). A secured web survey platform of KU Leuven was used, in which parents could log in with an individual access code.

The online survey consisted of 4 parts formulated in Dutch, an English translation of the items can be found in Supplementary Tables S10.1 and S10.2. A ***first part*** aimed to obtain general parental information like age and sex. Education of respondents was classified as lower secondary, higher

	SMOF	NIRTURE
Full name (if acronym)	//	Neonatal Insuline Replacement Therapy in Europe
Epoch	2004-2006	2005-2007
Patient population	Infants <34 weeks GA*, 500-2000g, with an estimated requirement for parenteral nutrition of >7days	Very low birth weight infants (<1500g)
Intervention	SMOFlipid 20% in parenteral nutrition	Continuous IV infusion of insulin (0.05 u/kg/h)
Comparison (control group)	Conventional soy bean emulsion	Normal neonatal care
Blinding	√	open label
Randomization	√	√
Postnatal age at start of study	Mean = 7 days	Median = 13.4 hours (before 24h of age)
Consent procedure	Postnatal written informed consent	Postnatal written informed consent + continuing consent
Duration of intervention	7-14 days	First 7 days
Additional procedures related to the study	5 additional blood samples	Subcutaneous glycaemia sensor, frequent finger pricks, 4 additional blood samples
Follow-up	No a priori defined follow-up	No a priori defined follow-up

Table 10.1 (a). *Summary of the characteristics of the 5 interventional drug trials in neonates (379-383). *GA: gestational age; **INSURE: intubation-surfactant administration-extubation; †NIRS: Near Infra-Red Spectroscopy; ‡aEEG: amplitude-integrated electro-encephalography.*

	DORIPENEM	LAIF	NEOPROP
Full name (if acronym)	//	Lipase Added to Infant Feeding	Exploratory Propofol Dose Finding Study In Neonates
Epoch	2009-2011	2011-2014	2012-2014
Patient population	Preterm (and term) neonates <12 weeks postnatal age, at risk of infection	NICU patients, born before 32 weeks GA*	NICU patients needing sedation for semi-elective intubation (mostly INSURE** procedures)
Intervention	Single dose of IV doripenem, through separate line	rhBSSL (recombinant bile salt-stimulated lipase) added to normal feeding	Single dose of IV propofol bolus (dose finding approach)
Comparison (control group)	N/A	Normal feeding	N/A
Blinding	open label	√	open label
Randomization	N/A	√	N/A (randomized in strata)
Postnatal age at start of study	Mean = 3-52 days (in different groups)	Mean = 3.2 weeks (100ml/kg of feeding)	Mean = 33.9 hours
Consent procedure	Postnatal written informed consent	Postnatal written informed consent	Postnatal written informed consent
Duration of intervention	1 hour infusion	4 weeks	12 hours
Additional procedures related to the study	4 additional blood samples within 7h	1 additional blood sample (follow-up consultation)	NIRS† and aEEG‡ monitoring during procedure 2 blood samples for PK
Follow-up	7 days after infusion	3 months after treatment and 12 months corrected age	No a priori defined follow-up

Table 10.1 (a). *Summary of the characteristics of the 5 interventional drug trials in neonates (379-383). *GA: gestational age; **INSURE: intubation-surfactant administration-extubation; †NIRS: Near Infra-Red Spectroscopy; ‡aEEG: amplitude-integrated electro-encephalography.*

secondary, higher non-university and university education. Furthermore, age-adjusted quality of life of the child (or both children if twins) who participated in the trial was assessed by the validated parent-reported PEDsQL-scale, ranging from 0 to 100 (384). In the *second part*, parents were asked whether they remembered participation in the trial. Only respondents who answered "yes", were directed to part 3 and 4. *Part 3* assessed contentment of parents with different aspects of the trial, whether the trial influenced care for and health of their child and emotional consequences through a set of 52 items. Every item was a statement on which respondents were asked to express their level of agreement on a 6 point Likert scale ranging from 1 (strong disagreement) to 6 (strong agreement). Finally, *part 4* inquired awareness on 5 typical clinical trial characteristics, only when applicable to the specific trial. A brief, lay term, explanation of equipoise, the possibility of adverse events, placebo or control groups, blinding and randomization was given. Parents indicated if they were aware of these characteristics (1 item), and whether knowing this causes any distress (3 items). Only parents of children who participated in SMOF, NIRTURE and LAIF (randomized clinical trials) were directed to part 4.

All items of part 3 and 4 were organized in 17 scales, measuring comparable experiences or perceptions. Negative items were inverted to load in the same direction on the scales. Cronbach's α coefficients were calculated to assess the scale reliability (385). For part 3, α's of all scales ranged from 0.757 to 0.957, while for part 4 all α's ranged from 0.639 to 0.908, indicating overall good reliability (Supplementary Tables S10.1 and S10.2). Cronbach's α coefficients did not change considerably when only the first respondent was taken into account (Supplementary Table S10.3).

At the end of the questionnaire contact details of the study team were provided for parents having questions or doubts. The survey was accessible from October 2017 until March 2018, being 3 to 13 years after the original trials. Parents were asked to complete the survey individually.

Bayley scales of infant development

All preterms with a gestational age (GA) below 32 weeks, born in the University Hospitals Leuven are invited for standardized neurodevelopmental follow-up, with assessment of Bayley Scales of Infant Development (BSID). We retrospectively collected age-corrected mental and motor scale scores of all infants for whom parents responded to our questionnaire (and gave permission to access the follow-up data). We aimed to use scales around the corrected age of 24 months, however any earlier assessment was considered

if no assessment at the age of 24 months was available. For most infants BSID-II was used, while starting from 2016 BSID-III was obtained. The BSID-III motor scale scores were excluded, BSID-III cognitive and language scale scores were recalculated to BSID-II mental scale scores (386).

Recruitment

Parents of infants who participated in the above mentioned trials were eligible. Families were excluded if they were not familiar with Dutch language or in case of loss of parental authority. Each eligible family received an invitation for an online survey by postal mail, along with 2 unique access codes (1 for each parent). Non-responding families were invited a second time by postal mail and by phone in a third attempt. For bereaved parents a separate protocol was followed, which included only 1 adapted invitation. If hospital records indicated death of the child, parents were only contacted once. Eligibility and response rates (RR1, considering only complete responses) were calculated according to the standard definitions of the American Association of Public Opinion Research (387).

Data analysis

All analyses were performed using SPSS (version 25, IBM). Response distributions are described as medians and quartiles (non-normal distribution, Shapiro-Wilk), while normally distributed baseline variables are described by means and standard deviations (sd). For parents of twins who both participated in the trial, we only used the lowest value of the PedsQL, BSID-II mental and motor scale scores of both twins as a baseline variable ("worst case scenario"). Scale scores were calculated by averaging all involved items of the particular scales. For visual representation scale values were binned to integers (1 for 1-1.5; 2 for 1.5-2.5; …; 6 for 5.5-6) (Table 10.3 and 10.4). Remembrance of trial participation was compared between sexes and trials with a X^2-test. Scales were compared across sexes and trials using Mann-Whitney U and Kruskal-Wallis-tests respectively. Spearman's ρ was calculated to study correlations.

Results

Respondents

In total, the 5 trials selected for this study contained 267 participants, from 229 families. Of those, 197 non-bereaved and 14 bereaved families were considered eligible for participation. In total, 123 non-bereaved and 2 bereaved parents completed the survey. For 20 non-bereaved families, both mother and father completed the questionnaire. We focus here on the non-

bereaved group, of which we reached 31% of possible respondents, representing 52% of the eligible families (Figure 10.1).

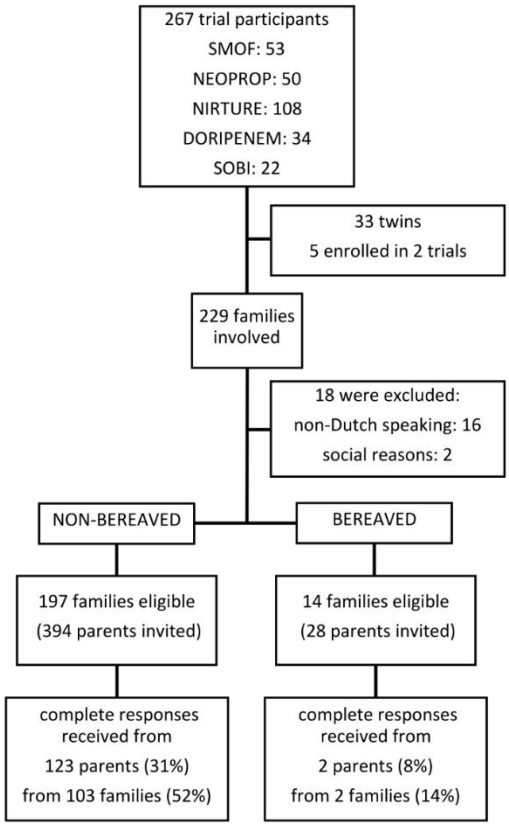


Figure 10.1. *Flow chart of the study, indicating eligibility and response rate (RR1, only considering complete responses).*

Respondents were predominantly mothers (71%), and higher education outside university was the most common educational attainment. Twenty percent of respondents had twins that both participated in the trial. The median time since participation in the trial was 11.5 (range 3.5-13.1) years. Average BSID-II mental and motor scale scores, a median total PedsQL score and other baseline characteristics per respondent are displayed in Table 10.2. The median corrected age at assessment of BSID was 20 months (range 7 – 31 months). No BSID mental and motor scale scores were obtained for 22 and 19 families (singletons or twin pairs) respectively, due to absence of indication for follow-up, loss of follow-up or insufficient cooperation. Baseline characteristics per family are comparable and given in Supplementary Table S10.4.

Table 10.2. Baseline characteristics per respondent	
Sex, n (%)	
Father	*36 (29)*
Mother	*87 (71)*
Parent of twins, n (%)	50 (41)
Parent of twins that were both in trial, n (%)	24 (20)
Partner also responded to survey, n (%)	20 (16)
Age at completion of questionnaire, mean in years (±sd)	41.8 (±5.6)
Age at participation in study, median in years (Q1-Q3)	31.7 (28.9-34.9)
Time since participation in study, median in years (Q1-Q3)	11.5 (5.1-12.2)
Trial, n (%)	
SMOF	*30 (24)*
NIRTURE	*46 (37)*
LAIF	*7 (6)*
DORIPENEM	*9 (7)*
NEOPROP	*31 (25)*
Education, n (%)	
Lower secondary education	*11 (9)*
Higher secondary education	*31 (25)*
Higher education outside university	*44 (36)*
Academic education (university degree)	*37 (30)*
BSID-II, mean (±sd)*	
Mental index	*99.0 (±18.5)*
Motor index	*95.8 (±19.8)*
Total PedsQL, median (Q1-Q3)*	81.7 (73.3-91.7)

Table 10.2. *Baseline characteristics of survey respondents (n=123). *BSID-II (Bayley scale of infant development II) or PedsQL (pediatric quality of life scale) of the respondent's child that participated in the trial. If both members of a twin participated in the trial only the lowest value is reported.*

Remembrance of trial participation

Twenty-five respondents (20%) did not remember participation of their child in a clinical trial. Remembering trial participation was not significantly correlated to sex, age or educational level (Supplementary Table S10.5). Surprisingly, the longer the time period since participation in the trial, the more respondents remembered participation (ρ=0.202; p=0.025). Remembrance was also strongly correlated to the trial (p=0.016), with especially lower remembrance (61%) in NEOPROP, the most recent clinical trial (Supplementary Table S10.6). Finally, remembering trial participation was not significantly correlated to the outcome of the infant (BSID-II mental or motor scale scores; PedsQL; Supplementary Table S10.5).

Contentment on trial participation

Overall, parents reported high levels of contentment with trial participation (median score 5.00), with only 2% of the respondents indicating disagreement (Table 10.3). Respondents were also rather happy with the information they received about the trial (median score 4.33), however 26% of respondents somehow to strongly disagreed. The recruitment and consent procedure of the trial was overall positively evaluated (median score 4.58). Seven percent of respondents was not happy with trial recruitment. In contrast, the majority of parents (63%) was unsatisfied with the follow-up after the trial (median score 3.00). We did not find significant correlations between contentment and sex, education, time since trial participation, trial, BSID-II mental or motor scale scores and PedsQL (Supplementary Table S10.7). Mothers however tended to be slightly less happy with the recruitment process than fathers (p=0.098).

Perceived influence of the trial on care and health

None of the respondents thought that participation in the trial affected the routine care (or attention of the clinical staff) for their child (median score 2.00; Table 10.3). Only 1 respondent thought that participation influenced the health of his child (median score 2.43). If all items referring to positive and negative effects of the trial on care and health were grouped, 34% of respondents somewhat to strongly agreed that the trial was beneficial for their child (median score 3.00), while no respondents thought it was negative (median score 1.75). Parent-reported quality of life of the child was negatively correlated to the perception that participation in the trial influenced care (p=0.021), and tended to be negatively correlated to the perception that it influenced health (p=0.059; Supplementary Table S10.7). Furthermore, the perception that participation had a positive influence on care and health, was more common in participants with a lower educational attainment (p=0.042) and in parents of infants with lower BSID-II mental scale scores (0.031).

Emotional consequences of trial participation

High levels of satisfaction and pride were reported (median score 4.40), with 14% of respondents disagreeing (Table 10.3). Parents reported very low levels of anxiety and distress (median score 1.44) and guilt (median score 1.33). Only 1 person felt somewhat anxious about trial participation, while another felt somewhat guilty. Educational attainment was negatively correlated to satisfaction and pride, and positively correlated to guilt (Supplementary Table S10.7). No significant correlations emerged between emotional consequences and sex, time since participation, type of trial and outcome..

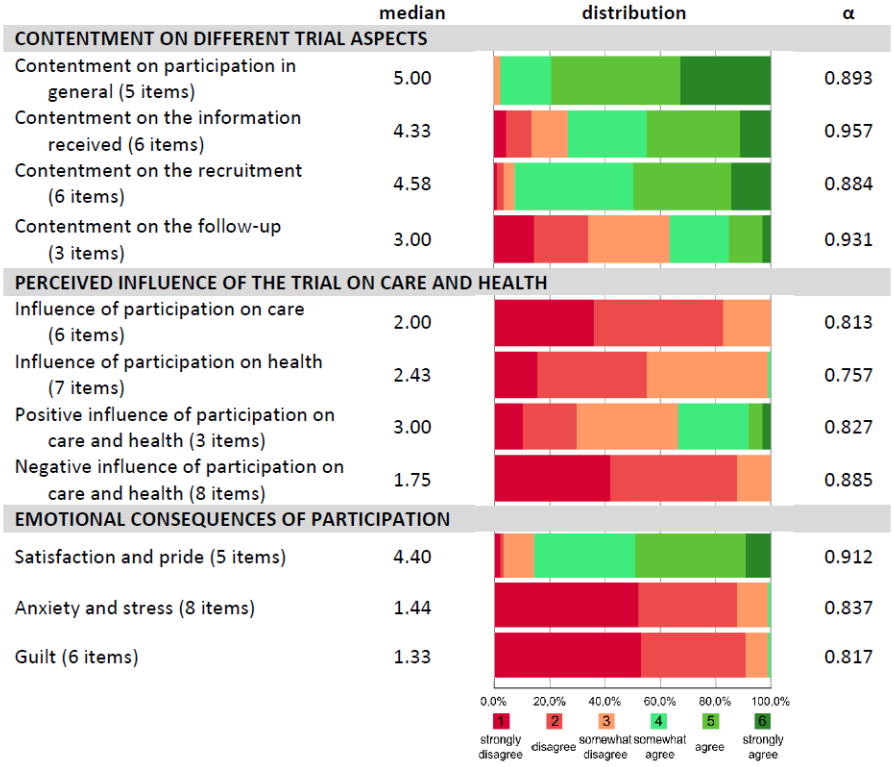


Table 10.3. *Descriptive results of the construct scales evaluating (i) contentment on trial participation, (ii) perceived influence of the trial on care and health and (ii) emotional consequences of trial participation (n=98). Median values of construct scales are expressed on 6 point Likert scales indicating agreement from 1 (strong disagreement) to 6 (strong agreement). Color bars show relative data distribution of binned scales (%). Cronbach's α indicates reliability of the construct scales.*

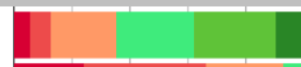
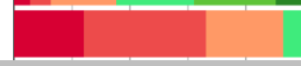
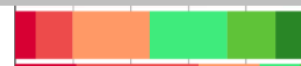
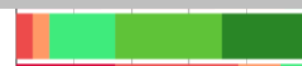
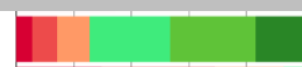
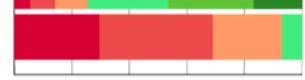
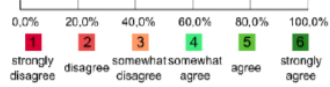
	median	distribution	α
Awareness about trial characteristics (4-5 items)	4.20		0.890-0.874
EQUIPOISE (n=71)-			
Awareness about equipoise (1 item)	4		
Distress about equipoise (3 items)	2.00		0.757
POSSIBILITY OF SIDE EFFECTS (n=71)			
Awareness about the possibility of side effects (1 item)	4		
Distress about the possibility of side effects (3 items)	2.33		0.795
PLACEBO OR CONTROL GROUP (n=71)			
Awareness about presence of control group (1 item)	5		
Distress about placebo (3 items)	2.00		0.639
BLINDING (n=35)			
Awareness about blinding (1 item)	5		
Distress about blinding (3 items)	2.00		0.908
RANDOMIZATION (n=71)			
Awareness about randomization (1 item)	4		
Distress about randomization (3 items)	2.00		0.722
			

Table 10.4. *Descriptive results of the construct scales and items evaluating awareness of and distress about typical clinical trial characteristics (n=35-71). Median values of construct scales are expressed on 6 point Likert scales indicating agreement from 1 (strong disagreement) to 6 (strong agreement). Color bars show relative data distribution of binned scales (%). Cronbach's α indicates reliability of the construct scales.*

Awareness of typical trial characteristics

Overall, parents were aware of the characteristics of the clinical trial in which their child participated (median score 4.20; Table 10.4). Twenty-seven percent of respondents however disagreed to be aware of these characteristics. Looking at individual characteristics, awareness was lowest for the possibility of adverse events, followed by equipoise, randomization, the presence of a control group and blinding (medians 4; 4; 4; 5 and 5 respectively). For all 5 trial characteristics, a large majority of respondents did not feel distressed after reading a brief explanation on the topic (medians ranging from 2 to 2.33). There was a strong negative correlation between awareness and distress for each individual characteristic (p values ≤0.001; Supplementary Table S10.8).

Sex played an important role here, with fathers being overall more aware (p-values ranging from 0.008 to 0.178) and less distressed (p-values ranging from 0.008 to 0.128) than mothers about the characteristics of the trial (Supplementary Table S10.9). Overall, awareness about typical trial characteristics tended to be related to the trial in which the respondent's child participated (p=0.0665). Awareness about the presence of a control group was significantly correlated to the trial (p=0.042), with parents of NIRTURE participants scoring lowest. Overall awareness about typical trial characteristics was strongly correlated to contentment on trial participation, on the information received, on the recruitment and on the follow-up (p-values ≤0.002; Supplementary Table S10.10). Furthermore awareness was correlated to the perception that trial participation influenced the health of the participating child (p=0.036), and to the perception that the trial had beneficial effects on care and health (p=0.001). Finally, awareness was correlated to satisfaction and pride (p=0.011), and negatively correlated to anxiety and stress and to guilt (p=0.015 and 0.001 respectively).

Bereaved parents

Only 2 bereaved parents (from 2 different families) responded the survey. One parent did not remember trial participation, while the other reported overall high levels of contentment with trial participation, but low levels of awareness of typical trial characteristics.

Discussion

Positive perspectives on trial participation

Overall, parents looked back at trial participation as a positive experience. They were overall contented with their participation, and reported high levels of satisfaction and pride, and low levels of anxiety, stress and guilt. These findings could provide comfort to researchers, who often doubt whether it is acceptable to burden the already distressed family of a newborn with recruitment for a clinical trial (388). Furthermore, it is reassuring that there was no correlation between contentment on trial participation or emotional consequences and the neurodevelopmental outcome of the child or its quality of life, suggesting that parents of babies with worse outcome are equally comfortable with trial participation. Other studies have previously documented comparable positive experiences among parents of neonatal clinical trial perspectives (375-377). The current study however is the first to evaluate the perspectives of parents of neonates participating in interventional pharmacological trials, with a relatively invasive experimental intervention. While previous studies assessed the experiences of parents during the trial, our study also assessed the effect of participation once long term outcome is known.

Validity of consent

At the moment of consent, parents are often overwhelmed by emotions and stress. It has often been questioned whether parents of sick newborns are able to give voluntary, competent and informed consent for trial participation (371, 388-391). In our study, parents were relatively satisfied with the information they received and the recruitment procedure. Furthermore, overall good levels of awareness on the characteristics of the clinical trial in which their child participated, indicate that most parents (regardless of education) understood to what they consented. These results are comparable to earlier studies with parents of pediatric study participants (392), but contrast qualitative work demonstrating poor understanding of the concept of a clinical trial and randomization in a sample of parents of neonatal study participants (373). In our data, parents who were less aware about any trial characteristic, were significantly more distressed about trial participation when confronted with a brief explanation on the topic. Additionally, awareness on trial characteristics was strongly correlated to positive perspectives and emotions related to participation. Being well aware of the specific concept of a clinical trial thus seems to be beneficial. Researchers asking for consent should ensure that parents capture and retain the concept

of the trial properly. Effectively explaining the concept and potential risks and benefits of a clinical trial to parents going through an emotional rollercoaster, however is a difficult exercise. Strikingly, in our sample fathers were significantly more aware and less distressed about typical trial characteristics. Mothers also tended to report lower contentment with the recruitment procedure. We hypothesize that mothers are more emotionally distressed at the moment of consent and often still bear the consequences from medical conditions leading to preterm birth that also affect cognitive functioning and possibly memory (393, 394). In this context it is also remarkable that fewer parents remembered participation in NEOPROP, a rather short trial for which consent very early after birth was required, and visible study procedures were limited in time. Recently, professional stakeholders and parent representatives made recommendations to ensure effective communication in consent procedures in this setting (395). Alternatively, other types of consent procedures avoiding recruitment during a stressful period (such as antenatal consent, continuing consent and deferred consent) could be explored (371, 396).

Therapeutic misconception

The goal of a clinical trial is to assess whether a new experimental treatment is effective, ineffective or even deleterious. Logically, this means that it is not always in the best interest of an individual neonate to participate in a clinical trial (370). Though a minority, still 35 and 46% of the respondents were unaware of the uncertainty concerning the effectivity (equipoise) of the experimental intervention and the possibility to cause adverse effects respectively. This failure to discriminate the different nature of research from clinical care is called therapeutic misconception, and often leads to disproportional optimism regarding the beneficial effects of a trial (397, 398). Sometimes this optimism contrasts the results of the trial. For example, in NIRTURE the experimental treatment showed little clinical benefit and there were even concerns of increased mortality at 1 time point (380). In our study, most parents did not think the trial affected care or health. Nevertheless 34% of respondents perceived positive effects of the clinical trial, while no respondents perceived negative effects. Parental education was positively correlated to the perception of positive effects of the trial and feelings of satisfaction and pride, but negatively correlated to feelings of guilt. This suggests that parents with a lower educational attainment are more likely to overestimate the beneficial effects of the trial and underestimate the side effects. This point should however be interpreted with caution, as self-reported awareness on clinical trial characteristics such as equipoise and the

possibility of side effects was not correlated to education. Additionally, parents reporting high awareness of clinical trial characteristics were more likely to perceive a positive effect of the clinical trial. This discrepancy could be explained by self-overestimation of awareness on the nature and characteristics of the clinical trial. Also, reporting a perceived positive effect, is not necessarily equal to inappropriate optimism. The experimental treatment might also have had a true positive effect, and regardless of being in the intervention or control group, there are data that trial participation leads to better outcomes (inclusion benefit) (399).

Follow-up

Finally, parents report rather low contentment with the follow-up after the trial. We did not ask parents about their unmet expectations on follow-up and can therefore only hypothesize on possible solutions. Future trials could provide more intensive follow-up of participants to reassure parents. Researchers could also, during the consent procedure, provide more information on the planned follow-up in the context of the trial protocol. Furthermore, research teams should inform parents of participants about the results of the trial and the impact of their contribution for science and future care (400).

Limitations

This study has some limitations. First, one has to be careful generalizing our findings. Despite relatively high response rates for this type of survey (long after trial participation), it is possible that our study is biased by selection of parents at least remembering trial participation, or having a strong opinion on it. Our results on remembrance of the trial and awareness of different trial characteristics indicate that the perspectives of parents might also be different depending on incidental aspects of the specific trial. In our study we did not observe major differences in parental evaluation between trials, which justifies the pooling of the 5 trials in this report. Parents had in general positive perspectives on these 5 diverse trials in this single center survey, but we do not know whether this accounts for all interventional drug trials in neonates. Our results might encourage clinicians to approach parents of neonates for study consent. However, we cannot generalize to parents that have not been asked for a trial, or even declined participation. Second, the possibility that some results are biased by social desirability. In our personal experience many parents of former preterm neonates, even long after admission, still feel connected to the neonatal intensive care team and admire their work. This might have led to a favorable evaluation of the trial. A qualitative study would be able to better explore how important this aspect

was for our results, and whether parents had truly in depth understanding of the concept and risks of the trial in which they participated (373). Third, despite our focus on long term perspectives, the long recall time (median 11.5 years) might have made it difficult to give a thought out opinion on some of the questions in the survey. Fourth, we did not assess how the clinical status of the infant (and the parental perception of it) during the trial affected parental evaluation of participation. Finally, with an adapted and less aggressive recruitment strategy, we were only able to recruit two bereaved parents. For the perspectives of this group we can refer to the BRACELET-study that has shown that most bereaved parents consider the trial as irrelevant to their child's death (401).

Conclusions

In conclusion, parents report positive perspectives and low levels of emotional distress at long term after trial participation of their neonate. This finding can reassure clinicians, researchers and review boards who worry about the emotional effect of trial participation on parents of neonates. Future efforts to improve the practices of neonatal clinical trials should focus on ensuring effective communication of the concept, risks and benefits of the trial to already distressed parents. Special attention should go to mothers early after birth and parents with lower educational attainment. Finally, efforts could go to better match the planned study follow-up and the needs of parents.

Appendix

Supplementary Table S10.1. *Descriptive results of the construct scales and individual items evaluating contentment on trial participation, perceived influence of the trial on care and health and emotional consequences of trial participation (n=98). Median and quartiles values are expressed as 6 point Likert scales indicating agreement from 1 (strong disagreement) to 6 (strong agreement) for all individual items, and averaged for all items in a construct. Cronbach's α indicates reliability of the construct scales.*

	median	Q1-Q3	α
CONTENTMENT ON DIFFERENT TRIAL ASPECTS			
Contentment on participation in general	**5.00**	**4.60-5.80**	**0.893**
I am contented that I let my son/daughter participate in the trial	5	5-6	
I would, if I was in the same situation, participate again in the trial.	5	5-6	
I would recommend participation in the trial to other parents in a comparable situation.	5	5-6	

Participation of my son/daughter in the trial was a positive experience for me.	5	4-6	
Participation of my son/daughter in the trial was a negative experience for me. *(inverted)*	2	1-2	
Contentment on the information received	**4.33**	**3.50-5.00**	**0.957**
I'm contented with the information I received about the trial.	5	3-5	
I was, on beforehand, well informed about the course of the trial.	4.5	4-5	
I was, on beforehand, well informed about the possible consequences of the trial.	4	3-5	
The information forms about the trial were useful.	4	3-5	
I was sufficiently informed to make a good decision about the participation of my son/daughter in the trial.	4	4-5	
I could turn to someone with my questions on the trial.	4	3-5	
Contentment on the recruitment	**4.58**	**4.17-5.17**	**0.884**
I'm contented with the conversation in which I was asked for participation of my son/daughter in the trial.	5	4-5	
I'm contented with the way how I was asked for participation of my son/daughter in the trial.	5	4-5	
I'm contented with the timing of the conversation in which I was asked for participation of my son/daughter in the trial.	4	4-5	
I have the feeling I was under pressure to let my son/daughter participate in the trial. *(inverted)*	2	1-2	
I think I was, at the moment when I was asked for participation of my son/daughter in the trial, capable of taking this decision.	5	3.75-5	
I think the person that asked for my consent on the participation of my son/daughter in the trial was well placed to do so.	5	4-5.25	
Contentment on the follow-up	**3.00**	**2.00-4.00**	**0.931**
I'm contented with the follow-up after the trial.	3.5	2-4	
I think there were enough follow-up consultations after the trial.	3	2-4	
I was well informed about the results of the trial.	3	2-4	
PERCEIVED INFLUENCE OF THE TRIAL ON CARE AND HEALTH			

Influence of participation on care	**2.00**	**1.17-2.33**	**0.813**
I have the feeling that participation in the trial influenced the normal care for my son/daughter.	2	1-2	
I think the quality of the care for my son/daughter decreased because of participation in the trial.†	1	1-2	
I think my son/daughter received better care because of his/her participation in the trial.*	2	1-3.25	
I have the feeling that infomation about the health status of my child was too much mixed with information about the trial.†	2	1-2	
I have the feeling that the interest of my child was conflicted by the interest of the trial by the doctors.†	2	1-2	
I have the feeling that doctors were more interested in the trial than in my child.†	1	1-2	
Influence of participation on health	**2.43**	**1.86-2.86**	**0.757**
I think participation in the trial has influenced the health of my son/daughter.	2	1-3	
I think my child has experienced medical benefits because of his/her participation in the trial.*	3	2-4	
I think participation in the trial was good for the health of my child.*	4	3-4	
I think participation in the trial was bad for the health of my trial.†	2	1-3	
I think the study procedure was burdensome for my child.†	2	1-3	
I think my child has experienced adverse events in the trial.†	2	1-2	
I attribute the current health problems of my child to participation in the trial.†	1	1-2	
Positive influence of participation on care and health*	**3.00**	**2.33-4.00**	**0.827**
Negative influence of participation on care and health†	**1.75**	**1.12-2.12**	**0.885**
EMOTIONAL CONSEQUENCES OF PARTICIPATION			
Satisfaction and pride	**4.40**	**3.80-5.00**	**0.912**
The participation of my son/daughter in the trial satisfies me.	4	3.75-5	
I am proud that I let my son/daughter participate in the trial.	4	3.75-5	
It satisfies me that the participation of my son/daughter helped advancing science.	5	4-6	

It satisfies me that I, by letting my son/daughter participate in the trial, have exploited all possible medical means for my son/daughter.	5	4-6	
It satisfies me that I, by letting my son/daughter participate in the trial, was able to have an important contribution myself to the health of my son/daughter.	4	3-5	
Anxiety and stress	**1.44**	**1.12-2.00**	**0.837**
Participation of my son/daughter in the trial, still causes fear.	1	1-2	
Participation of my son/daughter in the trial, still makes me uncertain.	1	1-2	
Participation of my son/daughter in the trial, still makes me worried.	1	1-2	
Participation of my son/daughter in the trial, still causes stress.	1	1-2	
Participation of my son/daughter in the trial, still causes an unsafe feeling.	1	1-2	
I brood sometimes on the possible consequences of participation in the trial.	1	1-2	
Participation of my son/daughter in the trial does not influence my peace of mind. *(inverted)*	5	3.75-6	
Trials, like the one my child participated in, are being performed to solve uncertainties in medical care. It worries me that this means that doctors are actually uncertain about many therapies.	2	1-3	
Guilt	**1.33**	**1.17-2.00**	**0.817**
Participation of my son/daughter in the trial, causes a guilty feeling.	1	1-2	
I feel guilty about my decision to let my son/daughter participate in the trial.	1	1-2	
I regret my decision to let my son/daughter participate in the trial.	1	1-2	
I have the feeling I harmed my son/daughter with my decision to let him/her participate in the trial.	1	1-2	
I have the feeling I have to make up something with my son/daughter because of my decision to let him/her participate in the trial.	1	1-2	
I have the feeling I did something good for my son/daughter with my decision to let him/her participate in the trial. *(inverted)*	4.5	4-5	

Supplementary Table S10.2.*Descriptive results of the construct scales and items evaluating awareness of and distress about typical clinical trial characteristics (n=35-71). Median and quartiles values are expressed on 6 point Likert scales for all individual items, and averaged for all items in a construct. Cronbach's α indicates reliability of the construct scales.*

	mean	sd	α
Feeling of being informed about typical trial characteristics*	**4.20**	**3.50-5.00**	**0.890-0.874**
EQUIPOISE (n=71) Clinical trials, like the one in which your child participated, are being performed to test new treatment strategies. Doctors have good reasons to investigate these treatments, but do not know for sure on beforehand if the new treatment is better than standard care. This uncertainty is the reason to perform the study. There is a possibility, that after the trial, the new treatment is less effective than standard of care. The experimental treatment that was studied in the trial in which your son/daughter participated, could thus be either better, equal or worse than the treatment that other children receive in the same situation.			
I think I was, before participation in the trial, well informed about this.*	4	3-5	
Distress about equipoise	**2.00**	**1.67-2.67**	**0.757**
I am, after reading this explanation, still happy that my son/daughter participated in the trial. *(inverted)*	5	4-5	
I am, after reading this explanation, worried about the participation of my son/daughter in the trial.	2	1-3	
I would, after reading this explanation, fill in the first part of this questionnaire differently.	2	1-3	
POSSIBILITY OF SIDE EFFECTS (n=71) In a clinical trial, like the one in which your child participated, not only the effectivity, but also the safety of a new treatment is studied. No matter how small, there is always a risk that the new treatment will cause adverse reactions. Also you son/daughter ran, by participation in the trial, a theoretical risk for adverse reactions.			
I think I was, before participation in the trial, well informed about this.	4	3-5	
Distress about the possibility of side effects	**2.33**	**2.00-3.00**	**0.795**
I am, after reading this explanation, still happy that my son/daughter participated in the trial. *(inverted)*	4	4-5	
I am, after reading this explanation, worried about the participation of my son/daughter in the trial.	2	1-3	
I would, after reading this explanation, fill in the first part of this questionnaire differently.	2	1-3	
PLACEBO/CONTROL GROUP (n=71) To be able to conclude that the new studied treatment is effective and safe, it is compared to other children in the same trial that receive a fake treatment ('placebo'). Some children			

that participate in a trial thus receive a new experimental treatment, while others receive a fake treatment (on top of the current standard treatment of course). The fact that your son/daughter participated in the trial, thus does not mean that he/she received the new experimental treatment.			
I think I was, before participation in the trial, well informed about this.	5	4-6	
Distress about placebo	**2.00**	**1.33-2.67**	**0.639**
I am, after reading this explanation, still happy that my son/daughter participated in the trial. *(inverted)*	5	4-5	
I am, after reading this explanation, worried about the participation of my son/daughter in the trial.	2	1-3	
I would, after reading this explanation, fill in the first part of this questionnaire differently.	2	1-3	
BLINDING (n=35) Sometimes it is very subjective to assess whether a drug or treatment was effective or not. To avoid that parents and doctors, based on expectations, will over- or underestimate this effect, most clinical trials are organized in such a way that both parents and doctors do not know whether a patient receives the new study treatment or a placebo treatment. This is called double blinding. This means that both you and the doctors taking care of you son/daughter did not know whether he/she received the study treatment or a fake control treatment.			
I think I was, before participation in the trial, well informed about this.	5	4-6	
Distress about blinding	**2.00**	**1.00-2.33**	**0.908**
I am, after reading this explanation, still happy that my son/daughter participated in the trial. *(inverted)*	5	4-6	
I am, after reading this explanation, worried about the participation of my son/daughter in the trial.	2	1-3	
I would, after reading this explanation, fill in the first part of this questionnaire differently.	2	1-3	
RANDOMIZATION (n=71) In a clinical trial, we try to avoid that the effect of a study drug or treatment is over- or underestimated because of unequal distribution of patients. One can imagine that when all the sickest patients for instance are assigned to the control group, the effect of the study treatment (in comparison to the placebo or control treatment) can be severely overestimated. The distribution or assignment of patients to the study or control groups is therefore performed as random as possible. In clinical trials it is a computer that decides to which group a patient is assigned, based on randomness and not based on patient characteristics (health status, age, sex). This was also the case for the trial in which your child participated.			
I think I was, before participation in the trial, well informed about this.	4	3-5	

Distress about randomisation	**2.00**	**1.33-2.67**	**0.722**
I am, after reading this explanation, still happy that my son/daughter participated in the trial. *(inverted)*	5	4-5	
I am, after reading this explanation, worried about the participation of my son/daughter in the trial.	2	1-3	
I would, after reading this explanation, fill in the first part of this questionnaire differently.	2	1-3	

Supplementary Table S10.3. *Reliability of construct scales per family expressed as Cronbach's α. Only the first responding parent per family is considered for this analysis.*

	α per respondent (n=98)	**α per family** (n=84)
CONTENTMENT ON DIFFERENT TRIAL ASPECTS		
Contentment on participation in general	0.893	0.886
Contentment on the information received	0.957	0.955
Contentment on the recruitment	0.884	0.875
Contentment on the follow-up	0.931	0.928
PERCEIVED INFLUENCE OF THE TRIAL ON CARE AND HEALTH		
Influence of participation on care	0.813	0.795
Influence of participation on health	0.757	0.746
Positive influence of participation on care and health	0.827	0.835
Negative influence of participation on care and health	0.885	0.862
EMOTIONAL CONSEQUENCES OF PARTICIPATION		
Satisfaction and pride	0.912	0.914
Anxiety and stress	0.837	0.804
Guilt	0.817	0.778
	α per respondent (n=35-71)	**α per family** (n=32-64)
AWARENESS AND DISTRESS ABOUT TYPICAL TRIAL CHARACTERISTICS		
Feeling of being informed about typical trial characteristics	0.890-0.874	0.872-0.886
Distress about equipoise	0.757	0.760
Distress about the possibility of side effects	0.795	0.787
Distress about placebo	0.639	0.607
Distress about blinding	0.908	0.889
Distress about randomisation	0.722	0.697

Supplementary Table S10.4. *Baseline characteristics of families of survey respondents (n=103). *BSID-II or PedsQL of the family's child that participated in the trial. If both members of a twin participated in the trial only the lowest value is reported. †PedsQL reported by the first responding parent.*

Baseline parameters respondents per family	
Twins, n (%)	38 (37)
Twins, both in study, n (%)	18 (17)
Time since participation in study, median in years (Q1-Q3)	11.6 (5.4-12.2)
Trial, n (%)	
SMOF	26 (25)
NIRTURE	40 (39)
LAIF	7 (7)
DORIPENEM	8 (8)
NEOPROP	22 (21)
BSID-II, mean (±sd)*	
Mental index	98.9 (±18.3)
Motor index	96.0 (±20.1)
Total PedsQL, median (Q1-Q3)*†	81.7 (71.7-91.7)

Supplementary Table S10.5. *Correlations with remembrance of trial participation (n=94-123). *X^2-test expressed as X^2-statistic (p-value). †Spearman's ρ expressed as ρ (p-value). Light grey boxes indicate significance.*

	Remembrance of participation
Sex*	0.024 (0.876)
Age at completion of questionnaire in years†	-0.026 (0.776)
Age at participation in study in years†	-0.134 (0.139)
Education†	0.055 (0.547)
Time since participation in study in years†	0.202 (0.025)
Trial*	12.240 (0.016)
BSID-II-mental scale† (n=95)	-0.028 (0.786)
BSID-II-motor scale† (n=94)	-0.102 (0.328)
Total PedsQL†	-0.096 (0.292)

Supplementary Table S10.6. *5x2 table of remembrance of trial participation versus trial (n=123). X^2-test indicates significant correlation between remembrance and trial (p=0.016).*

	SMOF	**NIRTURE**	**LAIF**	**DORIPENEM**	**NEOPROP**	**Total**
Does not remember	2 (7%)	10 (22%)	0 (0%)	1 (11%)	12 (39%)	25
Remembers	28 (93%)	36 (78%)	7 (100%)	8 (89%)	19 (61%)	98
Total	30	46	7	9	31	123

Supplementary Table S10.7. *Correlations with construct scales on contentment on diferent aspects of the trial, perceived influence on care and health and emotional consequences of trial participation (n=74-98). *Mann-Whitney U-test comparing male to female respondents expressed as p-value. †Spearman's ρ expressed as ρ (p-value). ‡Kruskal-Wallis test, expressed as p-value. Light grey boxes indicate significance.*

	Sex*	**Educa-tion†**	**Time since trial†**	**Trial‡**	**BSID-II-mental †** (n=75)	**BSID-II-motor †** (n=74)	**Peds-QOL†**
CONTENTMENT ON DIFFERENT TRIAL ASPECTS							
Contentment on participation in general	0.456	-0.108 (0.292)	0.093 (0.364)	0.104	0.116 (0.324)	-0.012 (0.919)	0.146 (0.153)
Contentment on the information received	0.556	0.009 (0.931)	-0.104 (0.306)	0.148	0.037 (0.750)	0.031 (0.795)	0.069 (0.498)
Contentment on the recruitment	0.098	-0.091 (0.371)	-0.004 (0.966)	0.474	-0.007 (0.953)	-0.006 (0.959)	0.055 (0.587)
Contentment on the follow-up	0.848	-0.109 (0.286)	-0.026 (0.799)	0.108	0.009 (0.939)	0.005 (0.963)	0.105 (0.304)
PERCEIVED INFLUENCE OF THE TRIAL ON CARE AND HEALTH							
Influence of participation on care	0.293	-0.025 (0.810)	-0.076 (0.458)	0.502	-0.092 (0.435)	0.016 (0.891)	-0,234 (0.021)
Influence of participation on health	0.617	-0.105 (0.305)	0.149 (0.142)	0.185	-0.215 (0.065)	0.044 (0.710)	-0,192 (0.059)
Positive influence of participation on care and health	0.653	-0.206 (0.042)	0.056 (0.548)	0.141	-0.249 (0.031)	-0.002 (0.988)	-0,158 (0.119)
Negative influence of participation on care and health	0.684	0.030 (0.770)	-0.025 (0.810)	0.515	-0.045 (0.702)	0.017 (0.887)	-0,165 (0,105)
EMOTIONAL CONSEQUENCES OF PARTICIPATION							
Satisfaction and pride	0.959	-0.269 (0.007)	0.044 (0.665)	0.660	0.010 (0.931)	0.044 (0.711)	0,175 (0.084)
Anxiety and stress	0.493	0.109 (0.288)	-0.087 (0.396)	0.439	-0.006 (0.959)	0.151 (0.200)	-0,120 (0.238)
Guilt	0.299	0.243 (0.016)	0.139 (0.171)	0.160	0.022 (0.852)	0.007 (0.956)	-0,036 (0.724)

Supplementary Table S10.8. *Correlations of awareness on individual clinical trial characteristics to distress about the same characteristic (n=35-71). †Spearman's ρ expressed as ρ (p-value). Light grey boxes indicate significance.*

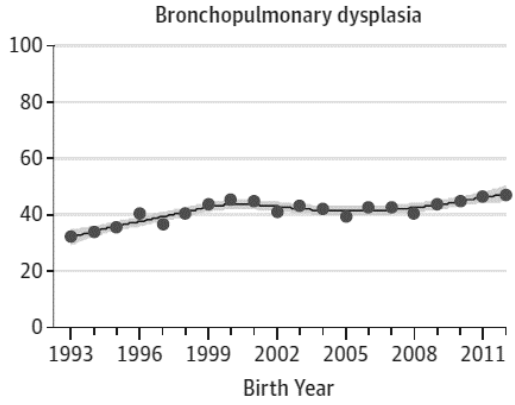
	Correlation of awareness x distress†
Equipoise	-0.579 (<0.001)
Possibility of side effects	-0.722 (<0.001)
Presence of a control group	-0.390 (0.001)
Blinding	-0.559 (<0.001)
Randomization	-0.512 (<0.001)

Supplementary Table S10.9. *Correlations with construct scales and items about awareness on typical clinical trial characteristics (n=27-71). *Mann-Whitney U-test comparing male to female respondents expressed as p-value. †Spearman's ρ expressed as ρ (p-value). ‡Kruskal-Wallis test, expressed as p-value. Light grey boxes indicate significance.*

	Sex*	**Educa-tion†**	**Time since trial†**	**Trial‡**	**BSID-II-mental †** (n=61)	**BSID-II-motor †** (n=62)	**Peds-QOL†**
Awareness about typical trial characteristics	0.018	0.111 (0.359)	-0.001 (0.994)	0.065	0.041 (0.751)	0.068 (0.598)	-0.030 (0.801)
EQUIPOISE (n=71)							
Awareness about equipoise	0.178	0.057 (0.636)	0.047 (0.697)	0.327	-0.012 (0.929)	0.013 (0.919)	0.112 (0.354)
Distress about equipoise	0.031	-0.007 (0.954)	0.042 (0.728)	0.923	0.034 (0.795)	0.108 (0.403)	-0.156 (0.193)
POSSIBILITY OF SIDE EFFECTS (n=71)							
Awareness about the possibility of side effects	0.066	0.057 (0.638)	0.002 (0.985)	0.414	-0.146 (0.263)	-0.008 (0.954)	-0.034 (0.778)
Distress about the possibility of side effects	0.016	0.001 (0.991)	-0.123 (0.309)	0.817	0.032 (0.806)	0.031 (0.811)	-0.129 (0.285)
PLACEBO OR CONTROL GROUP (n=71)							
Awareness about the presence of a control group	0.018	-0.010 (0.932)	0.077 (0.524)	0.042	0.018 (0.892)	0.224 (0.081)	-0.043 (0.721)
Distress about placebo	0.010	-0.046 (0.703)	0.028 (0.814)	0.670	0.015 (0.907)	0.071 (0.585)	-0.171 (0.155)
BLINDING (n=35)							
Awareness about blinding	0.009	0.125 (0.474)	-0.042 (0.810)	0.698	0.014 (0.946)	-0.086 (0.663)	0.053 (0.761)
Distress about blinding	0.128	-0.224 (0.195)	0.028 (0.875)	0.658	-0.095 (0.637)	0.026 (0.896)	-0.340 (0.046)
RANDOMISATION (n=71)							
Awareness about randomization	0.008	0.104 (0.389)	0.011 (0.930)	0.375	-0.019 (0.884)	0.101 (0.436)	-0.049 (0.685)
Distress about randomisation	0.008	-0.016 (0.895)	0.065 (0.591)	0.678	0.201 (0.121)	0.063 (0.626)	-0.084 (0.486)

Supplementary Table S10.10. *Correlations of awareness about typical trial characteristics to construct scales on contentment on trial participation, perceived influence on care and health and emotional consequences of participation (n=71). †Spearman's ρ expressed as ρ (p-value). Light grey boxes indicate significance.*

	Awareness about typical trial characteristics†
CONTENTMENT ON DIFFERENT TRIAL ASPECTS	
Contentment on participation in general	0.360 (0.002)
Contentment on the information received	0.746 (<0.001)
Contentment on the recruitment	0.726 (<0.001)
Contentment on the follow-up	0.596 (<0.001)
PERCEIVED INFLUENCE OF THE TRIAL ON CARE AND HEALTH	
Influence of participation on care	0.019 (0.876)
Influence of participation on health	0.249 (0.036)
Positive influence of participation on care and health	0.402 (0.001)
Negative influence of participation on care and health	-0.120 (0.317)
EMOTIONAL CONSEQUENCES OF PARTICIPATION	
Satisfaction and pride	0.300 (0.011)
Anxiety and stress	-0.289 (0.015)
Guilt	-0.373 (0.001)



From Stoll et al. JAMA Pediatrics (2015):

Despite (or due to) the improvements in neonatal care, the incidence of BPD has increased importantly.

Discussion

Some paragraphs adapted from:
Salaets T, Gie A, Tack B, Deprest J, Toelen J. Modelling Bronchopulmonary Dysplasia in Animals: Arguments for the Preterm Rabbit Model. Curr Pharm Des. 2017;23(38):5887-5901. (1)

The objective of this thesis was to advance the search for novel therapeutic strategies for BPD along different stages of the drug development pathway. We wanted to develop and improve the methods for pre- and clinical research on BPD, and use these methods to advance the search for novel therapeutic strategies.

Improving methods for explorative and preclinical research on BPD

In a first group of chapters we focused on improving the animal models for drug discovery and development and preclinical research.

Despite advances in (3D) cell culture methods (207, 402), there are currently no in vitro models mimicking the complexity of a developing lung. BPD research thus relies mainly on animal models. It is not easy to develop an ideal animal model that both mimics the morphological, functional and vascular manifestations of BPD and provides a good representation of the human pathophysiology. Neonatology is a constantly changing field and this affects the utility of the different animal models. Where aggressive iatrogenic insults (high FiO_2 and mechanical ventilation) were the cornerstones of the so called "old BPD", this may no longer hold true for the BPD that neonatologists currently encounter (36). Modern BPD remains a multifactorial disease, but the consistent common denominator in the whole disease spectrum is extreme prematurity. We therefore advocate the use of preterm animals, because of their translational value. In chapter 2 we demonstrated that preterm rabbits born at a gestational age of 28 age are both functionally and structurally immature at birth. This information, together with historical data suggesting that preterm rabbits are more susceptible to oxidative injury (172, 173), supports the use of a preterm rabbit model for BPD research.

In chapter 1 we also wanted to assess whether prematurity alone, in the absence of hyperoxia, was enough to cause a developmental arrest, as described in BPD. As a premature transition from fetal life to room air already involves exposure to a supraphysiological oxygen tension (207), we anticipated that preterm pups (born on a gestational age of 28 days) exposed to room air would exhibit lung functional and structural defects in comparison to term pups (31 days) of the same postconceptional age. To our knowledge, we are the first to report on the pulmonary effect of 'pure' prematurity in an animal model. We noted functional defects, while structural defects were more subtle (catch-up of alveolar structure, but decreased total alveolar surface due to smaller lung size). This phenotype could represent a more subtle "new BPD" phenotype, as it is seen in human survivors of extremely

preterm birth that e.g. never needed mechanical ventilation nor extremely high FiO_2. We hypothesize that identifying the factors and pathways that perturb normal lung development after preterm birth, might lead to novel treatments for BPD. The subtlety of the effect of prematurity however makes the prematurity-alone model not directly suitable to assess the effect of interventions. Future efforts could be directed to develop a more extremely preterm model to make the effect of prematurity more robust. As we observed a high perinatal mortality already in the day 28 model, a more preterm model (e.g. day 27) would however require respiratory support immediately after birth (CPAP, surfactant delivery or even mechanical ventilation). For the moment (and in the rest of this thesis) we therefore continue to need hyperoxia to deliver a stable and pronounced lung phenotype that can be used in a drug development pipeline.

Biomedical research always has to allow a certain degree of limitations in the models that it uses and as such all sub-entities of the disease will probably never be covered by one single animal model. Research models in general only incorporate stable and reproducible (like 95% oxygen, or prematurity in chapter 2) insults, while a variable plethora of insults contribute to human preterm lung injury, making every case different. Novel pathophysiological insights and derived therapeutic strategies from an animal model with a specific insult, might therefore only be applicable to the subset of BPD cases resulting from a comparable etiology (e.g. infants that were exposed to very high FiO_2). We hypothesize that this explains why very few therapeutic findings in animal models could be reproduced in humans. Precision medicine and the identification of different BPD subtypes might improve translation of findings in experimental animals to clinical care in the future (34).

We conclude that a perfect animal model for BPD does not exist. However all models have their place in BPD research, and for every research question there is one that is ideal or best suited (Figure 11.1). The small animal models of mice and rats are particularly suited for basic explorative research on pathways involved in specific aspects of the disease, because of the availability of state of the art molecular techniques. On the other hand larger animal models such as preterm primates and lambs provide a good platform for the translational study of innovative therapeutic strategies in a setting similar to the neonatal intensive care unit. Practical and ethical constraints however limit extensive use, so only the most promising therapies can be tested out in these models. In our work we focused on the preterm rabbit model, with hyperoxia exposure. This is an elegant model that sits in between the small and large animals and can be used for both explorative and

translational research, as it is the smallest model that combines structural and functional prematurity. The use of the appropriate animal model for each research question, and the evaluation of preclinical study results in the context of the limitations of its model, will benefit the development of novel therapeutic strategies for BPD (1).

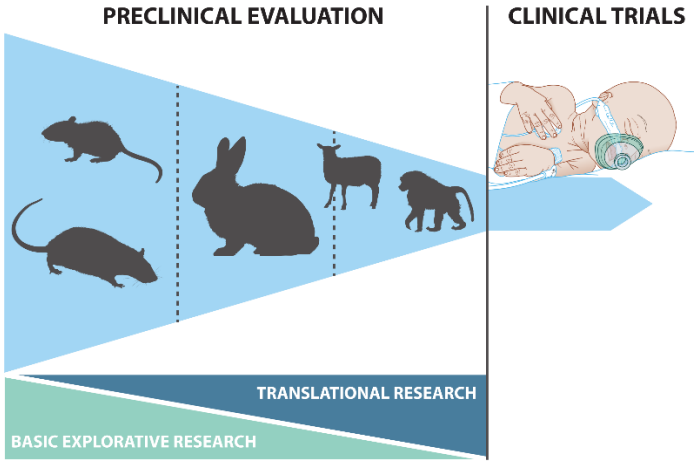


Figure 11.1. Preclinical research pipeline for BPD. *Small rodent models are ideal for basic explorative research on individual factors involved in the pathophysiology of BPD. Lamb and baboon models mimic the human disease more closely. The rabbit model elegantly sits in between.*

Overall, in all animal models, BPD researchers could do more efforts to avoid bias in their experiments. As stated in the introduction, a systematic review of animal research in BPD demonstrated that randomization, blinding, allocation concealment, prior calculation of sample size, … were only occasionally applied in the reviewed articles (19). This is a more general problem for all diseases (403), and risk of bias tools have been developed (404). For all experiments in this thesis we performed a priori sample size calculation, paid attention to careful randomization of pups within mothers and made sure that all analysis was performed blindly. A further improvement, especially now our team is expanding, would be to blind researchers for treatment allocation during the daily care for the pups. The ethical costs and potential benefits of animal research are rightly more critically appraised by society. In order to justify the use of animals in experiments, researchers should avoid bias and make sure their experiment delivers a reliable answer.

One of the primary read-outs (or main answers) in BPD research is the mean linear intercept of the airspaces (Lma), together with several other parameters of alveolar morphometry. Few researchers however use an unbiased (stereological) approach to obtain these measures (104, 222), as this is very time consuming. Furthermore, we showed unacceptable inter-observer variability after manual counting. In chapter 2 we developed and validated a computerized method to facilitate the assessment of alveolar morphometry that is compatible with a stereological approach. This semi-automatic method was markedly faster than manual counting, and provided highly reproducible results. We also showed that semi-automatic segmentation accurately aligns airspaces, however the calculated results do not completely match with the results of manual counting. As manual counting still remains the gold standard we are not able to conclude which method best matches the true morphometric characteristics of the lung. The developed semi-automatic method can furthermore easily be downloaded and works on freely available software. We think the use of this method could improve the quality of morphometric results in BPD research.

Developing novel therapies for BPD in the preterm rabbit model

In a second group of chapters we used the preterm rabbit model to develop (novel) therapeutic strategies for BPD. We followed a funnel-shaped approach, in parallel to the classic drug development process. We first focused on target identification and then continued with studies to assess efficacy of therapies aimed at some of the identified targets.

New systems-biology based approaches offer opportunities to identify novel targets for the treatment of BPD (405). In chapter 3, we performed transcriptome analysis on lung tissue of a relevant animal model. By using mRNA-seq we identified 2217 genes that were significantly up- or down-regulated in whole lungs of preterm rabbit pups exposed to hyperoxia. This is an enormous amount of information. We used several strategies in the Ingenuity Pathway Analysis software to filter out relevant therapeutic targets from this vast amount of information. First, we looked at the most up- and down-regulated molecules of the list. Several interesting molecules such as CCL2, IL8 and DKK1 were highly upregulated, while CYP1A1 was downregulated. We should however note that highly dysregulated is not equal to most relevant. Therapeutic strategies should be aimed at the molecular master switches on top of the pathway, which are not necessarily strongly dysregulated, but trigger many important downstream

dysregulations. Second, we arranged the dysregulated genes in functional networks. We identified gene networks involved in inflammation (up), lung development (down), vascular development (down) and reactive oxygen species metabolism (up) to be contributing to the lung phenotype of preterm rabbits exposed to hyperoxia. Finally, we combined our mRNA-seq results with published data on interactions between molecules in an upstream regulator analysis (289). We hypothesized that an altered activity of several master switch molecules such as NFE2L2, NF-κB and KLF-2 would be the cause of the observed dysregulations in gene expression. Molecules identified as upstream regulators could be interesting targets for drug development.

In this thesis we continued to efficacy studies with drugs aimed at 2 of the identified targets. We already mentioned KLF-2, a gene transcription factor that is predicted to be downregulated. Simvastatin is a drug that is known to induce KLF-2. If we expanded the upstream regulator analysis to exogenous molecules, simvastatin itself was furthermore predicted to reverse the dysregulation of several interesting downstream molecules (CCL2, IL8, VEGF-A, …). We thus hypothesized that simvastatin, a cholesterol lowering drug, could be repurposed for BPD-like lung disease. In chapter 4 we assessed the efficacy of simvastatin in the preterm rabbit model. A daily enteral dose of 5mg/kg attenuated the lung functional effects of hyperoxia. Most importantly it also completely prevented arterial medial thickening, which is in line with other observations on animal models for perinatal pulmonary vascular disease (273, 274). We thus successfully predicted that simvastatin has beneficial effects on the lung phenotype of preterm rabbits exposed to hyperoxia, however we did not confirm all of the predicted gene transcription changes (for instance not for IL8 and CCL2). This could be explained by the fact that upstream regulator analysis is based on molecule interactions in many in vitro or in vivo models (from different species), which can not necessarily be extrapolated to preterm rabbit lungs. This is an inherent disadvantage of this kind of big data approaches. The mechanism of the observed effect of simvastatin therefore remains largely unknown. We hypothesize that VEGF-A or maybe even Rho-kinase (as suggested in (274)) plays a role, but more research is needed. Furthermore, we also noted an unexpected and unexplained mortality in the normoxia control group treated with simvastatin, which currently limits the translational value of our findings. Furthermore, in unpublished experiments on dose seeking, a higher dose of 20mg/kg, revealed mortality up to 80% in both the hyperoxia and normoxia treated groups. Future experiments could focus on identifying the exact working mechanism to find other more targeted molecules aimed at this

pathway. Alternatively, local drug delivery could be explored, preserving the local effects of simvastatin, while omitting its systemic side effects.

A second, and more expected, target for therapeutic interventions is inflammation (CCL2, IL8 and IL1β are examples of highly upregulated genes in lungs of preterm pups exposed to hyperoxia). This idea is anything but novel. In the nineties up to 40% of babies born before 28 weeks of gestation were treated with postnatal steroids in order to prevent lung inflammation leading to BPD (3). Despite reduction in BPD, postnatal steroids however resulted in increased rates of cerebral palsy (305, 306). More recently, local delivery of steroids for the prevention of BPD have been investigated. The most successful approach was intratracheal administration of a mixture of surfactant and budesonide (16), however many questions remain on the most ideal dosing regimen or formulation.

So far, research on the long term (BPD) effects of a drug added to surfactant, was only possible in larger animal models and sheep. In chapter 5, we developed a technique for intratracheal administration in preterm rabbits. We confirmed the safety and feasibility of daily intratracheal injections with surfactant (as a vehicle for drug delivery). We also assured a good delivery to and distribution within the lungs (compared to other delivery methods such as inhalation or systemic administration). The preterm rabbit model is thus a good model to study locally administered steroids, as it combines prematurity with the ability to translate clinical procedures like intratracheal delivery. More in general, this model is therefore well suited to study neonatal applications of local pulmonary drug delivery, an area that is gaining interest (308).

Using this model and delivery method, we assessed safety and efficacy of two doses of intratracheal budesonide, mixed in surfactant in chapter 6. While Yeh et al. (16) used a repetitive dosing regimen of 0.25mg/kg budesonide in surfactant mixes, we were able to show that a single dose of 0.25mg/kg budesonide was as and even more effective than a repetitive daily dosing regimen. It improved compliance and inspiratory capacity, decreased septal wall thickening and decreased expression of inflammatory cytokines CCL2 and IL8. It remains unclear whether the lower efficacy of the repetitive dosing is a rabbit specific issue, but it draws attention to the toxic potential of high doses of steroids in a growing body. Based on the data from this experiment, we would suggest future clinical trials evaluate the safety and efficacy of a single dose of surfactant plus budesonide, early after birth.

Improving methods for clinical research on BPD

In a third and final group of chapters, we focused on improving the methods for clinical trials in newborns. Any promising innovative therapy for BPD should undergo clinical testing, in human subjects, before it gets market approval. Performing clinical trials is a difficult task in any population, in newborns however there are specific additional challenges. Important specificities are for instance the developmental changes in pharmacokinetics and –dynamics, and the enormous potential for both beneficial and deleterious long term effects that only become apparent after several years (196).

Furthermore, newborns in clinical trials encounter many adverse events, because of the severity of their underlying prematurity or disease and the high number of drugs to which they are exposed (196). Many of those adverse events go unrecognized and unreported (406). And if reported, this safety information is difficult to interpret due to the lack of standardized adverse event terminology for this population. In chapter 7, we developed, in parallel to existing terminology for other populations (354, 356), a standardized adverse event severity scale for neonatal adverse events. If an adverse event in a clinical trial (or in routine clinical care) is recognized, researchers can use the scale to assign a severity grade (mild, moderate, severe, life threatening or death) to the adverse events based on standardized definitions agreed up on by consensus. The use of this scale will make adverse event data more comparable across trials and centers. A large multidisciplinary stakeholder group was and is involved in the development of NAESS, within the context of INC. The input from clinicians, industry, regulators, nursing organizations and parent representatives in the Delphi-based consensus process was pivotal for this work. A key effort was also the integration of NAESS in existing terminology concepts for adverse events in MedDRA (351) and the vocabulary service of NCI (407), which assures its dissemination to a relative audience and sustainability. While a first version is publicly available online at present, this project remains ongoing. Future efforts to continuously update the existing criteria and increase the number of adverse events included in a second version are planned. Furthermore, we are currently planning a (prospective and retrospective) validation study, in which we hypothesize and expect to document that the use of the standardized criteria in NAESS improves inter-observer agreement of severity assessment of adverse events.

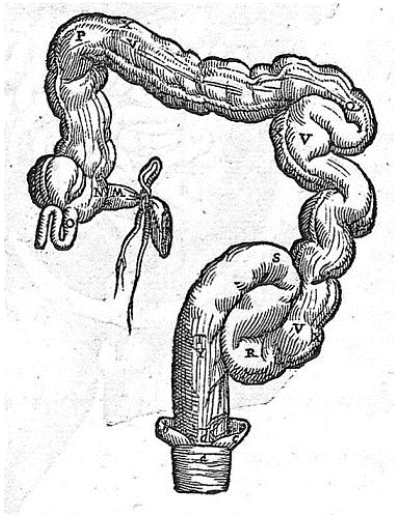
Finally, ethical issues arise when clinical trials are performed in vulnerable neonates, when parents are asked to sign informed consent in a very

emotional moment early after the birth of their (preterm) baby. There is a paucity of data on the empirical perspectives of parents on neonatal clinical trial participation (374). Smaller studies, after participation in neonatal trials with little or no invasiveness, assessed how parents evaluated trial participation and which effect it had on their emotional wellbeing (375, 376). No studies evaluated the effect of participation in an interventional drug trial. Furthermore, none of the available studies assessed the perspectives of parents at long term after participation, when outcome of the child is known. In chapter 8, we showed, with a well-designed survey among parents of former neonatal participants of interventional drug trials, that parents are overall contented with their participation. Almost no parents reported anxiety, stress or guilt when looking back at trial participation. In previous studies it was demonstrated that clinicians doubt more about asking consent for neonatal clinical research, then parents do in giving it (391). Clinicians might worry that parents are not able to give a valid informed consent, and regret participation afterwards. Our results indicate that this is not the case, and might reassure clinicians or researchers that have to ask for consent. Our study also shows that especially mothers often were not aware (anymore) about the typical characteristics of the trial. Twenty percent did also not remember participation. This indicates the difficulties in clearly transmitting information on a clinical trial at a moment when parents are overwhelmed by emotions. A recent Delphi consensus study identified the aspects of the consent procedure that could be optimized in neonatal research (395). Additional attention should also go to parents with low educational attainment, for which we found evidence that they were more likely to overestimate the beneficial effects of trial participation (a concept called therapeutic misconception). Finally, we also noted that follow-up overall did not meet the expectations of parents. We suggest that future trials foresee a good follow-up plan and good communication about the planned follow-up in the context of the trial.

Overall, we think this work can improve the practice of clinical trials by providing a tool for standardization of adverse event reporting, by reassuring clinicians, research or review boards that are worried about the effect of trial participation on parents and by highlighting the needs of parents in neonatal clinical trial.

General conclusion

As anticipated, this thesis did not result in the identification of “the golden bullet” to prevent or cure all BPD. We think however we have advanced the ongoing search for novel therapies in several ways. Along several phases of the drug development process, we developed new methods and new hypotheses to boost future research that will help to improve the respiratory outcome of survivors of extremely preterm birth.



De humani corporis fabrica libri septem – Vesalius (1543)
The first known drawing of the human appendix.

Appendix

Summary
Samenvatting
Scientific acknowledgements and personal contribution
Conflict of interest statement
Curriculum vitae
Personal acknowledgement/Dankwoord

Summary

Despite advances in neonatology, bronchopulmonary dysplasia (BPD) remains a frequent and important consequence of preterm birth. Innovative therapeutic approaches are needed to improve the respiratory outcome of survivors of preterm birth. The drug discovery pipeline has not been particularly successful for BPD. Currently, budesonide mixed with surfactant, mesenchymal stem cells and IGF1 are promising candidates for further research, but many treatment candidates failed. In this thesis we aimed to search for novel treatment strategies for BPD and improve the research methods necessary to develop new therapies along different phases of the drug development pathway.

In a first set of chapters we aimed to improve the methods for explorative and preclinical research on BPD. Animals models remain the cornerstone of BPD research, however many research results in the past were not translatable to humans. We hypothesize that the lack of prematurity in many models plays a role in this difficult translation. In chapter 1 we demonstrated that prematurity alone, in the absence of hyperoxia, results in altered lung function and structure in preterm rabbit pups, in comparison to term control animals. Focusing more on the effect and mechanisms by which preterm birth disrupts normal lung development, instead of focusing on hyperoxia and mechanical ventilation could advance the search for new therapies. A second reason for the difficult translation of positive findings from animal studies could be insufficient use of measures to avoid bias. A classic read-out in BPD research is the mean linear intercept, a read-out for alveolar structure, which is often obtained in a suboptimal way, prone to bias. In chapter 2 we developed and validated a semi-automatic method for rapid and reproducible assessment of mean linear intercept.

In a second set of chapters we used a previously described preterm rabbit model, in which prematurity in combination with exposure hyperoxia results in a lung functional, structural and vascular phenotype reminiscent of BPD, to search for novel therapeutic strategies. We started with target identification, by transcriptome analysis, in chapter 3. 2217 gene transcripts were significantly dysregulated in the lungs of preterm rabbit pups exposed to hyperoxia for 7 days. We identified clusters of dysregulated genes around functions as inflammation, lung development, vascular development and metabolism of reactive oxygen species. Furthermore we identified novel therapeutic approaches by upstream regulator analysis.

One of these approaches was the use of simvastatin. In chapter 4, we evaluated the efficacy of simvastatin therapy on lung disease in preterm rabbits exposed to hyperoxia. We noticed a beneficial effect on lung function and vascular remodeling, which makes it a promising approach for further research. More knowledge on the exact working mechanism is needed, as we were unable to confirm the gene transcription changes predicted by the upstream regulator analysis. Furthermore, control pups treated with simvastatin depicted an unexpectedly high mortality which warrants caution.

A recent clinical trial showed beneficial effects of a repetitively administered surfactant plus budesonide mixture. The important dysregulation of many inflammatory mediators in the transcriptome analysis suggested that the preterm rabbit model is appropriate to investigate this strategy. In chapter 5, we demonstrated that the preterm rabbit model can be used for studies evaluating the effect of intratracheally administered BPD drugs, something that was previously only possible in preterm lambs and baboons. We demonstrated a good distribution of labeled surfactant or saline after intratracheal injection in spontaneously breathing rabbit pups. In chapter 6 we then assessed the efficacy of a mixture of surfactant plus budesonide. We could show that a single dose of 0,25mg/kg budesonide in surfactant was as (and even more) effective than a daily repetitive dosing regimen. This suggests future clinical trials should assess the efficacy of lower cumulative doses (or a single doses) of budesonide in surfactant.

In final set of chapters, we focused on improving the methods for clinical trials in newborns. Any promising therapy for BPD should undergo evaluation in clinical trials. While a difficult task in any population, clinical trials have additional specific challenges in neonates. Also for the availability of research tools, neonates remain an “orphan population”. While neonates experience relatively more adverse events, there is a lack of tools to standardize safety reporting in this population. In chapter 7 we developed, with the input of broad field of stakeholders, a consensus adverse event severity scale, that can make safety information more comparable across trials and centers.

Finally, clinical trials in neonates are ethically difficult. There is paucity of empirical data on the parental perspectives on neonatal clinical trial participation, which we assessed in chapter 8. We showed, with a well-designed survey, that parents are overall contented with their participation. Almost no parents reported anxiety, stress or guilt when looking back at trial participation. A relevant minority was not aware of typical trial characteristics such as equipoise, the possibility of side effects, the presence of a control

group, randomization and blinding. Contentment with follow-up was furthermore low. Research teams should ensure effective communication on trial characteristics and follow-up during consent procedures.

In conclusion, we advanced the ongoing search for novel therapies for BPD in several ways. Along several phases of the drug development process, we developed new methods and new hypotheses to boost future research that will help to improve the respiratory outcome of survivors of extremely preterm birth.

Samenvatting

Ondanks vooruitgang in neonatologie, blijft bronchopulmonale dysplasia (BPD) een frequent en belangrijk gevolg van prematuriteit. Nieuwe behandelingsstrategiën zijn nodig om de respiratoire outcome van ex-prematuren te verbeteren. De ontwikkeling van farmacologische behandelingen voor BPD was tot nu weinig succesvol. Op dit moment zijn budesonide-surfactant co-administratie, mesenchymale stamcellen en IGF1 beloftevolle kandidaten voor verder onderzoek, maar vele andere kandidaatmoleculen leidden niet tot succes. De doelstelling van deze thesis was het zoeken naar nieuwe behandelingsstrategiën voor BPD en het verbeteren van de onderzoeksmethodes die noodzakelijk zijn voor de verschillende fasen van geneesmiddelenontwikkeling.

In een eerste groep hoofdstukken poogden we de methoden voor exploratief en preklinisch onderzoek naar BPD te verbeteren. Diermodellen blijven de hoeksteen van het onderzoek naar BPD, maar heel wat onderzoeksresultaten waren in het verleden niet vertaalbaar naar mensen. Onze hypothese is dat het gebrek aan prematuriteit in veel diermodellen de translatie naar de klinische setting bemoeilijkt. In hoofdstuk 1 toonden we aan dat prematuriteit alleen, in de afwezigheid van hyperoxie, leidt tot een veranderde long functie en structuur in neonatale konijnen. We denken dat het verschuiven van de focus van het effect van hyperoxie en mechanische ventilatie naar het effect van prematuriteit, de zoektocht naar nieuwe behandelingen voor BPD zou kunnen bevorderen. Een tweede mogelijke reden voor de moeizame translatie van bevindingen uit dierexperimentele studies, zou wel eens het gebrek aan rigoreuze onderzoeksmethodes kunnen zijn, waarmee bias vermeden wordt. Een typische uitkomstmaat voor BPD onderzoek is “mean linear intercept”, een maat die de alveolaire structuur karakteriseert. Helaas wordt deze maat vaak op een suboptimale manier bepaald, die erg gevoelig is voor bias. In hoofdstuk 2 ontwikkelden en valideerden we een semi-automatische methode voor een snelle en reproduceerdbare meting van de “mean linear intercept”.

In een tweede groep hoofdstukken gebruikten we het eerder beschreven preterme konijnenmodel om te zoeken naar nieuwe behandelingsstrategiën. In dit model leidt een combinatie van prematuriteit en hyperoxie tot een functioneel, structureel en vasculair phenotype dat doet denken aan BPD. Een eerste stap was de identificatie van nieuwe behandelingsdoelwitten, door middel van transcriptoom analyse, in hoofdstuk 3. In totaal waren 2217 gen transcripten significant gedysreguleerd in de longen van preterme

konijnen pups die voor 7 dagen werden blootgesteld aan hyperoxie. We identificeerden clusters van gedysreguleerde genen met een functie in inflammatie, long ontwikkeling, vasculaire ontwikkeling en metabolisme van reactieve zuurstofradicalen. Verder leidde analyse van de regulatoren hogerop in de genexpressiecascade tot voorspellingen voor nieuwe behandelingstrategiën.

Een van deze nieuwe strategiën was het gebruik van simvastatine. In hoofdstuk 4 evalueerden we het effect van simvastatine op longziekte in preterme konijnen die werden blootgesteld aan hyperoxie. We bemerkten een gunstig effect op longfunctie en vasculaire remodelering, wat dit tot een interessante strategie maakt voor verder onderzoek. Meer kennis over het exacte werkingsmechanisme is nodig, omdat we ondanks het gunstige effect op het phenotype, de voorspelde gentranscriptieveranderingen uit de transcriptoom analyse, niet konden bevestigen. Tot slot zagen we in dieren behandeld met simvastatine ook een onverwachte mortaliteit, wat pleit voorzichtigheid tijdens de verdere ontwikkeling van dit geneesmiddel.

Een recente klinische trial toonde een gunstig effect aan van repetitieve toedieningen van een surfactant-budesonide mengsel, op de incidentie van BPD. De belangrijke genexpressieverstoring van veel inflammatoire mediatoren in de transcriptoom analyse, suggereerde dat het preterme konijnen model een goed model zou zijn om deze strategie verder te onderzoeken. In hoofdstuk 5 toonden we aan dit model kan gebruikt worden voor onderzoek naar intratracheaal toegediende medicijnen tegen BPD, iets wat voordien enkel mogelijk was in premature schapen en bavianen. Met een intratracheale injectie van gelabeld surfactant of saline in spontaan ademende konijnenpups, konden we een mooie distributie aantonen. In hoofdstuk 6, gingen we dan het effect van een mengsel van surfactant en budesonide na. We konden aantonen dat een enkele dosis van 0,25mg/kg budesonide in surfactant was even (en zelfs meer) effectief dan een dagelijks repetitief doseringsschema. Dit suggereert dat in toekomstige klinische studies de cumulatieve dosis budesonide best laag wordt gehouden.

In een laatste groep hoofdstukken focusten we op het verbeteren van de methodes van klinische onderzoek met pasgeborenen. Elke beloftevolle therapie voor BPD zal ooit een klinische evaluatie moeten ondergaan. Klinische trials zijn al een hele opgave in eender welke populatie, maar stellen onderzoekers voor specifieke uitdagen in pasgeborenen. Wat betreft de beschikbaarheid van onderzoeksinstrumenten blijven pasgeborenen een “weespopulatie”: hoewel neonaten bijvoorbeeld betrekkelijk meer

neveneffecten ondervinden van medicamenteuze behandelingen, zijn er geen geschikte instrumenten beschikbaar om deze veiligheidsevents op een gestandardizeerde manier te rapporteren. In hoofdstuk 7 ontwikkelden we, met de inbreng van een breed spectrum aan belanghebbenden, een consensus schaal om de ernst ("severity") van neveneffecten in te schatten. Deze schaal kan de veiligheidsinformatie beschikbaar uit verschillende studies en centra meer vergelijkbaar maken.

Tenslotte zijn klinische studies met pasgeborenen ook op ethisch vlak moeilijk. Er is een gebrek aan empirische data over de perspectieven van ouders op deelname aan een neonatal klinische studie. In hoofdstuk 8 toonden we, met een goed geconstrueerde vragenlijst, dat ouders over het algemeen tevreden zijn met hun eerdere deelname. Bijna geen enkele ouder rapporteerde angst, stress of schuldgevoelens wanneer hij/zij terugkeek op deelname aan de klinische studie. Een belangrijke minderheid was zich niet (meer) bewust van de typische karakteristieken van de klinische studie: zoals de potentiële evenwaardigheid van de vergeleken behandelingen, de mogelijkheid op neveneffecten, de aanwezigheid van een controle group, randomisering en blindering. De tevredenheid over de follow-up na de studie was verder eerder laag. Onderzoeksgroepen moeten zouden duidelijker moeten communiceren over de werkwijze van een klinische studie en de geplande opvolging tijdens een "informed consent" gesprek.

We concluderen dat we met deze thesis de zoektocht naar nieuwe behandelingsmethoden voor BPD op verschillende manieren hebben vooruitgebracht. Langsheen de verschillende fasen van het geneesmiddelenontwikkelingsproces, werkten we aan nieuwe methoden en hypothesen die verder onderzoek naar het verbeteren van de respiratoire toestand van exprematuren, kunnen vooruitstuwen.

Scientific acknowledgement and personal contribution

Chapter 1: Thomas Salaets designed and performed the experiments and wrote the manuscript. We acknowledge André Gie and Julio Jimenez for their help with performing these experiments. Derek de Winter and Yannick Regin helped with the analysis of histology data. Greetje Vande Velde and Janne Vignero were specifically involved in the imaging experiments. Karel Allegaert, Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 2: Thomas Salaets developed the script for the semi-automatic method, designed and performed the validation study, and wrote the manuscript. Bieke Tack and Andre Gie helped performing the validation study (second and third observer). Benjamin Pavie and Nikhil Sindhwani provided advice and assistance on the development of the script. Karel Allegaert, Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 3: Jute Richter designed and performed the animal experiment, with help from Julio Jimenez and Taro Nagatomo. Rita Van Bree provided technical assistance for mRNA extraction. Paul Brady performed the mRNA sequencing. Thomas Salaets analyzed the results in IPA. Jute Richter and Thomas Salaets wrote the manuscript together. Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 4: Thomas Salaets designed and performed the experiments, and wrote the manuscript. Julio Jimenez and André Gie are acknowledged for their help with performing the experiments. Bieke Tack and Derek de Winter analyzed some of the histological results (and Bieke Tack helped writing the manuscript). Flore Lesage designed and performed the qPCR experiments. Nathalie Berghen helped with the assessment of collagen content. Karel Allegaert, Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 5: Thomas Salaets designed and performed the experiments, and wrote the manuscript. Julio Jiminez and André Gie helped performing the experiments. Greetje Vande Velde, Oliver Gheysen and Michel Koole helped designing, performing and analyzing the microPET-CT experiments. Margo Aertgeerts analyzed the imaging experiments. Xabi Murgia, Costanza Casiraghi, Francesca Ricci, Fabrizio Salomone and Gino Villetti helped designing and supervising the work. Karel Allegaert, Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 6: Thomas Salaets and Andre Gie designed and performed the experiments, and wrote the manuscript. Costanza Casiraghi and Fabrizio Salomone helped designing and supervising the work. Karel Allegaert, Jan Deprest and Jaan Toelen supervised the work and manuscript writing.

Chapter 7: Thomas Salaets and Karel Allegaert designed and guided the development process and drafted the manuscript. Mark Turner, Jonathan M. Davis and Robert M. Ward helped supervise and guide the entire process. Sandra Beauman, Gerri Baer, Isamu Hokuto, Ronald Ariagno, Agnes Klein, Mary Short, Kelly Wade and Merran Thomson participated in every step of the development process. Judy Harrison linked the terminology to MedDRA, Terry Quinn created the concepts and links in the NCI Thesaurus. Eve Roberts designed and provided the case descriptions for the validation exercise. All authors were involved in reviewing and rewriting the manuscript. Furthermore, all participants to any of the steps in the consensus process are acknowledged in a specific section in chapter 7.

Chapter 8: Thomas Salaets designed and performed the study and wrote the manuscript. Emilie Lavrysen helped with data collection. Anne Smits, Sophie Vanhaesebrouck and Maissa Rayyan provided the data from their former study participants and provided advice on every step of the study. Els Ortibus provided the BSID data. Laurence Claes helped designing the survey and guided the statistical analysis. Karel Allegaert and Jaan Toelen supervised the work and manuscript writing.

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Conflict of interest statement

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Curriculum vitae

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STUDIES AND EDUCATION

- Secondary education: Latin-Science Sancta Maria Institute Aarschot 2007
- Bachelor in Medicine KULeuven 2010, summa cum laude
- Master in Medicine KULeuven 2014, magna cum laude
    - Master thesis "Searching for new treatment strategies for hyperoxia induced lung injury : Transcriptome analysis in the preterm rabbit model", promotor: Prof. Dr. J. Toelen
    - Extracurricular courses: Biomedical Development Cooperation (2010), Tropical Medicine (2011), Medical French (2012), Medical Sociology (2012), Additions in Electrocardiography (2014)
- Master-after-Master in Specialist Medicine, started in 2014, degree expected in 2021
    - Additional training: Neonatal Life Support (2014), Pediatric Life Support (2017), GRIP Roadshow Pediatric Clinical Pharmacology (2017).
- Doctoral school of Biomedical Sciences, program in 'Molecular and Stem Cell Medicine', started in 2015, degree expected in 2019
    - Additional training: Laboratory Animal Science: attest Felasa B (2015), Statistics for Biomedical Sciences: certificate (2016), Writings skills for Biomedical Researchers (2016), Workshop Tissue Sample Selection and Preparation (2016), Image Processing Workshop VIB (2016), I3H Program in Translational Medicine (certificate) (2018),
- PhD program, plan entitled "Towards new therapies for bronchopulmonary dysplasia"
    - Performing clinical and explorative research on bronchopulmonary dysplasia (newborn chronic lung disease).
    - Involved heavily in a collaboration with an industrial partner: Chiesi Pharmaceutici SpA.
    - Involved in research projects aiming at improving clinical research in neonatology.

INTERNSHIPS AND CLINICAL EXPERIENCE

- Internship rotations 2012-2013:

    - Nomination for prize Prof. J. De Groote, for students excelling in social skills
- Pre-specialization training in Pediatrics, 2013-2014 in UZLeuven, under supervision of Prof. Dr. K. De Boeck
- Residency training in Pediatrics
    - First year, 2014-2015 in Heilig Hartziekenhuis Lier, under supervision of Dr. K. Garmyn.
    - Second, third and fourth year, 2015-2018; part time training due to the combination with a PhD program. Rotations at the pediatric nephrology, allergology, neurology, hematology, rheumatology, immunology and endocrinology consultations.

SCIENTIFIC OUTPUT

Publications:

- **Salaets T**., Richter J., Brady P., Jimenez J., Nagatomo T., Deprest J., Toelen J. (2015). Transcriptome Analysis of the Preterm Rabbit Lung after Seven Days of Hyperoxic Exposure. *PLoS One,* 2015 Aug 28;10(8):e0136569.
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- **Salaets T.**, Gie A., Jimenez J., Aertgeerts M., Gheysens O., Vande Velde G., Koole M., Murgia X., Casiraghi C., Ricci F., Salomone F., Villetti G., Allegaert K., Deprest J., Toelen J. Local pulmonary drug delivery in the preterm rabbit: feasibility and efficacy of daily intratracheal injections. *American Journal of Physiology Lung Cellular Molecular Physiology*, 2019 Apr 1;316(4):L589-L597.
- **Salaets T.**, Turner M., Short M., Ward R., Hokuto I., Ariagno R., Klein A., Beauman S., Wade K., Thomson M., Roberts E., Harrison J., Quinn T., Baer G., Davis J., Allegaert K., for the International Neonatal Consortium Development of a neonatal adverse event severity scale through a Delphi

consensus approach. *Archives of Disease in Childhood*, doi:10.1136/archdischild-2019-317399 (2019).

Contributions to congresses/meetings:

- Poster presentation at FMF 2014, 29th of June - 3th of July 2014, Nice. **Salaets T**., Richter J., Brady P., Jimenez J., Nagatomo T., Deprest J., Toelen J. Transcriptome analysis in a hyperoxia-induced preterm rabbit model to identify novel theurapeutic targets.
- Poster presentation at PAS 2018, 5th-8th of May 2018, Toronto. **Salaets T.**, Tack B., Jimenez J., Lesage F., Toelen J. Simvastatin mitigates lung functional and vascular manifestations of hyperoxia in preterm rabbits.
- Oral presentation at SPIN 2018, 1st-2nd of June 2018, Lübeck. **Salaets T.**, Gie A., Jimenez J., Aertgeerts M., Gheysen O., Vande Velde G., Koole M., Murgia X., Casiraghi C., Ricci F., Salomone F., Villetti G., Allegaert K., Deprest J., Toelen J. Intratracheal instillation for localized drug delivery in the preterm rabbit: a feasibility and distribution study.
- Oral presentation at EAPS 2018, 30th of October-3rd of November 2018, Paris. **Salaets T.**, Allegaert K., Ward B., Turner M. Standardizing adverse event data: developing a generic severity scale.
- Poster presentation at PAS 2019, 27th-30th of April 2019, Baltimore. **Salaets T.**, Lavryssen E., Smits A., Claes L., Allegaert K. Parents of neonatal study participants report low levels of emotional distress at long term after trial participation.
- Oral presentation at jENS 2019, 17th-21st of September 2019, Maastricht. **Salaets T.**, Gie A., de Winter D., Allegaert K., Toelen J. Prematurity affects lung development in neonatal rabbits.
- Active participation to the INC (International Neonatal Consortium), a multi stakeholder platform to accelerate the development of safe and effective therapies for neonates: leading several group discussions on standardizing adverse event reporting at:
    - Second Annual Meeting 12th – 13th of September 2016, London
    - Third Annual Meeting 27th – 29th of March 2017, Washington DC
    - Fourth Annual Meeting 11th-12th of April 2018, Washington DC
    - Fifth Annual Meeting 1st-3rd of May 2019, Washington DC

REPRESENTATIONS AND MANAGEMENT EXPERIENCE

- Resident representative for Pediatrics at the faculty of Medicine
    - Member of LVGA (Leuven Association of Medical Specialists in Training)
    - Member of the permanent educational committee of the Manama in Specialist Medicine
- ABAP (Assisting Academic Personnel) representative KULeuven
    - Elected member of the departmental board and council of the department of Development and Regeneration

TEACHING

Tutorship of:

- Master thesis of Bieke Tack (Master in Medicine)
- Student research projects: Margo Aertgeerts, Emilie Lavrysen, Laurent Veekmans
- Visiting scholar: Derek de Winter
- Lab rotations, Master in Biomedical Sciences

EXTRAPROFESSIONAL ACTIVITIES

- Member and treasurer of VZW Langebosheem (2005-now).
- Elected member of city council Aarschot (2019-now)
- Member of directors board of VZW Kommaraf (2019-now)
- Voluntary pediatrician for Lumos (medical development cooperation): missions to Boko, Benin (2017, 2018).

Personal acknowledgement/Dankwoord

For most PhD's, and mine is not an exception, the opposite of Neil Armstrong's legendary quote seems to be valid. Despite all dreams and ambitions, they are a giant leap for the doctorandus, but only a tiny step for mankind. Science however is a team sport. Even giant leaps like the first walk on the moon are built on many small advances in physics and engineering (from probably thousands of PhD's). I can only hope that the work from this PhD will be a small step towards a giant leap for BPD. At this occasion, it is my turn to thank everyone who contributed small or large steps, to my personal giant leap: finishing this PhD.

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