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Modelling Bronchopulmonary Dysplasia in Animals: Arguments for the Preterm Rabbit Model — Source link

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TITLEPAGE

Modelling bronchopulmonary dysplasia in animals: arguments for the preterm rabbit model.

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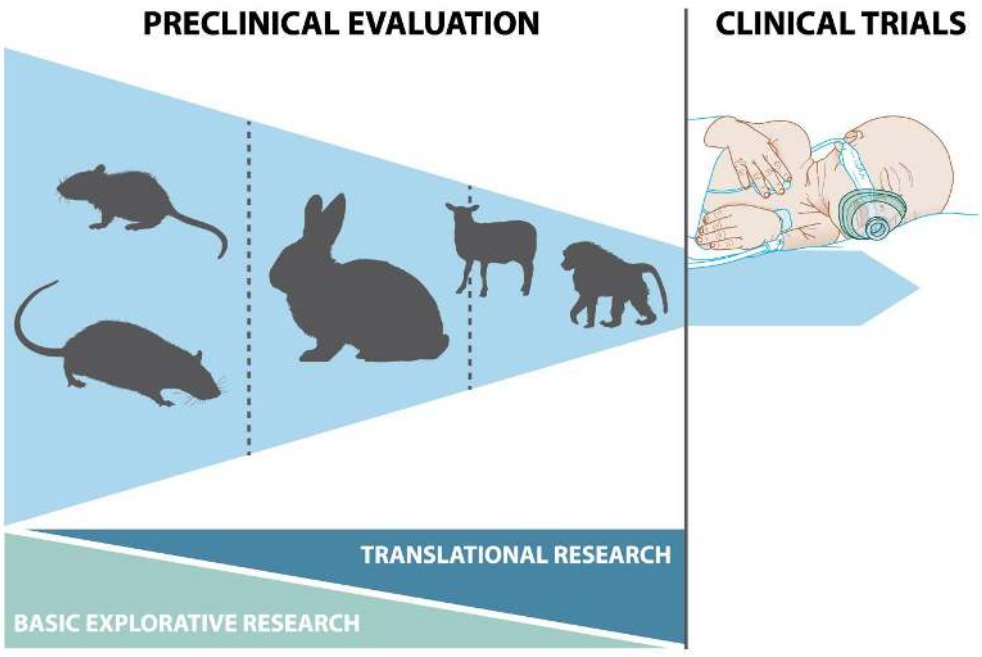
ABSTRACT

Bronchopulmonary dysplasia (BPD) remains a frequent and disabling consequence of preterm birth, despite the recent advances in neonatal intensive care. There is a need to further improve outcomes and many novel therapeutic or preventive strategies are therefore investigated in animal models. We discuss in this review the aspects of human BPD pathophysiology and phenotype, which ideally should be mimicked by an animal model for this disease. Prematurity remains the common denominator in the heterogeneous spectrum of human BPD, and preterm animal models thus have a clear translational advantage. Additional factors, like excessive oxygen, mechanical ventilation and infection, which frequently have been studied in animal models, can contribute to preterm lung injury however are not indispensable to develop BPD. The phenotype of human BPD is characterized by alveolar developmental arrest with extracellular matrix remodeling, signs of obstructive airway disease and pulmonary vascular disease. Many animal models mimic this phenotype and have their place in BPD research, but results should be interpreted bearing in mind the specific advantages and disadvantages of the model. Term mice and rats are well suited for basic explorative research on specific disease mechanisms, essential for the generation of new hypotheses, while the larger ventilated preterm baboons and lambs provide a good platform for the ultimate translation of these strategies towards clinical application. The preterm rabbit model seems a promising model as it the smallest model that includes a factor of prematurity and has a unique position between the small and large animal models.

GRAPHICAL ABSTRACT



KEYWORDS

bronchopulmonary dysplasia; animal models; rabbit; preclinical research; translational research; prematurity

MAIN TEXT

Introduction

Premature birth affects 11% of all pregnancies and is the leading cause of death in neonates worldwide [1]. Fifty years ago Northway was the first to describe the fibrotic changes in the lungs of preterm infants who could only be kept alive with aggressive mechanical ventilation and supraphysiological oxygen administration [2]. Even though advances in perinatal medicine have increased the survival of ever more premature infants, these were unable to reduce long-term respiratory morbidity. At present 45% of survivors of extremely preterm birth (<28 weeks of gestation) still develop bronchopulmonary dysplasia (BPD) [3]. This lung disease has disabling consequences throughout life as it is associated with respiratory morbidity (lung function abnormalities, episodes of wheezing and frequent hospital admissions), pulmonary hypertension (which is associated with a mortality rate up to 38% [4, 5]) and even abnormal neurological development [6, 7]. Also economically BPD remains an important problem [8]. As the current clinical care fails to prevent BPD, the development of effective preventive and therapeutic strategies remains imperative.

Jensen *et al.* have reviewed the evidence behind the current pharmacological strategies for prevention of BPD in 2015 [9]. There is high-quality evidence that vitamin A [10] and caffeine [11] reduce BPD, but their effect size is 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 [12, 13] and low dose regimens [14, 15] of steroids. However, the uncertainty about their safety remains as for instance inhaled budesonide resulted in a trend towards increased mortality [12]. Jensen *et al.* also reported on the currently ongoing clinical trials. Besides intratracheal stem cells, only few novel medicinal products are being investigated. Yet a recent 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, illustrates the potential benefits of exploring novel therapeutic strategies, as the researchers noticed a significant reduction in BPD (Ley *et al.*, abstract presented at the EAPS 2016 congress).

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 [18, 19] or tracheal aspirates, bronchoalveolar lavage fluid or exhaled air [20-22]. 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 [23-26]. Even though they are interesting for biomarker identification [27] and the generation of new hypotheses, studies on human material often do not prove causality. Therefore, researchers employ 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 [28] (recently illustrated by a clinical trial where the use of docosahexaenoic acid (DHA) supplements failed to reduce BPD [29, 30]). Therefore there is still a need to improve our models for this disease. In this review we will discuss the characteristics of an 'ideal BPD model'. We will first describe the factors contributing to the development of human BPD which can be used as insults in animal models, and we will then discuss the phenotype of the disease which should be mimicked. We will subsequently outline the characteristics and practicalities of existing animal models. Finally we will make the case for the preterm rabbit model, as this model provides a good balance between translational and practical considerations. In the last few years, many excellent reviews on animal models for BPD have been published [28, 31-38]. In this review we look at this subject from a different perspective: we focus on the utility of the different models (and more specifically the rabbit model) for preclinical drug evaluation.

Translating the pathophysiology of BPD into animal models

Bronchopulmonary dysplasia is a unique disease as it is not defined by its pathophysiology but by its treatment. This disorder is currently defined as 'the requirement for oxygen therapy on the 28th postnatal day or 36th week post conception'. As this is a very 'clinical definition', it is no wonder that it invokes increasing critique in this age of 'omics' and systems biology [39]. 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 [40]. This makes BPD, in contrast to for instance a genetic surfactant deficiency, particularly hard to mimic in an animal model. 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 [41]. 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) [42]. 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, incidences of BPD tend to vary between centers (depending on the definitions being used and the age limits of the study population) with the common relationship of increasing incidence of BPD with decreasing gestational age and birth weight [41, 43].

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) [44]. While in healthy pregnancies the saccular stage proceeds into alveolarization in utero, both pre- and postnatal factors disturb this developmental program in preterm birth (figure 2). 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 (figure 1).

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 tension and thus allowing 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)) [45]. 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 [40]. 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 2) [40]. A complete review of BPD related noxiae is beyond the scope of this text and we will limit ourselves to the major contributors.

Oxygen

Signs of oxidative injury are a frequently reported finding in samples of BPD neonates: e.g. protein and lipid peroxidation in exhaled gases and bronchoalveolar lavage fluid [51-53]. 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 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 20mmHg to the fetal lung through the vasculature. After birth, the lungs are filled with air, resulting in alveolar partial oxygen pressures of 100mmHg 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 [46, 47]. Animal data suggest that these high enzyme levels are the result of a preparatory process during the final part of gestation [48]. 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 [49-51], 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% [52-54]. 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 FiO_2 to which babies were exposed. This resulted in increased mortality, and a decreased ROP rate, but interestingly no further decrease in BPD prevalence was noted in comparison to the higher target group (90-94%) [55, 56]. These findings pare down the role of oxygen

supplementation as the most important contributor to the pathophysiology of today's 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 FiO_2 has been used to create a damage reminiscent of human BPD, making use of different timings and intensities [31-33, 36]. Recently a robust study compared different oxygen exposure protocols in neonatal mice, highlighting advantages and disadvantages of each protocol [57]. 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 [58-61]. 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 CPAP was the initial treatment modality, 43% of the patients required switch-over to mechanical ventilation at some point [62]. Mechanical ventilation thus remains a very frequent intervention, and ventilator induced lung injury is therefore thought to be an important contributor to BPD [63].

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 [64, 65]. 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 [63, 64]. 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 [66, 67]. Even in the absence of increased FiO_2 the stretch of lung tissue might also lead to the formation of ROS [68, 69].

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) and 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 [70]. 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 [71]. 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, develop BPD (18,5% of neonates born between 25 and 28 weeks of gestation in group of nCPAP successes) [62].

In baboons and lambs researchers have been able to combine extreme lung immaturity with mechanical ventilation to create experimental BPD [34, 38]. Both invasive and non-invasive strategies of respiratory management have been used successfully in these larger animal models [72, 73], 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 [74, 75].

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 [76, 77]. There also seems to be a relationship between malnutrition and BPD [78] or fluid overload and BPD [79]. Although controversial some authors also suggest a link between patent ductus arteriosus and BPD [80]. These clinically relevant risk factors have occasionally been studied in animal models as well [81-84].

ELBW infants (at risk of BPD) are currently also 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" [39, 41]), but maybe also in terms of altered physiology [85-87]. 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 for a preclinical BPD model.

Prenatal factors

There is increasing interest in prenatal biomarkers for the early prediction of BPD [27], 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 [28].

Prenatal infection or chorioamnionitis is a frequent cause of preterm birth and seems to be associated to BPD [88]. In utero inflammation might interfere with normal lung development, however its direct effect, independent of gestational age remains unclear [77]. 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 [89, 90].

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 [88, 91]. An interesting finding is also the association between placental vascularity and pulmonary vascular disease in BPD [92]. In animal models, this state of decreased angiogenesis has been mimicked by intra-amniotic injections with an antagonist of vascular endothelial growth factor (VEGF) [93].

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 [94]. In a Canadian cohort a significant increase in BPD was noted in preterm babies of which mothers smoked during pregnancy [95]. Maternal smoking has also been used as an insult in animal models, to create a BPD-like phenotype [96, 97].

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) [98]. 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 [99].

Mimicking the BPD phenotype in animal models

By using comparable insults, animal models should be able to mimic the phenotype of BPD. In this paragraph 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 (Lm)) in 22 BPD autopsy specimens, compared to 15 lungs of age-matched controls [100]. 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 [26]. 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) [24, 25, 101, 102]. Despite the gaps in knowledge on current 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 [103]. The most reported measure is probably the mean linear intercept (Lm), reflecting the mean length of a random line between to gas exchange surfaces. Increased Lm as shown by Husain *et al.* in BPD autopsy specimens has been reproduced in many animal models [57, 104-107]. Other none stereological measurements can complete the picture: radial alveolar count (RAC), septal crest counting and acute lung injury score [108-110]. 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 [111, 112]. Alternatively, direct 3D assessment of alveolar architecture by means of micro CT and subsequent automated analysis will gain importance in the future [113, 114].

Another interesting finding in the limited histological data, is the disorganization of the elastin fiber network [115]. Quantitatively more elastin is present, but the fibers occur more tortuous and less organized [116]. This is

particularly interesting because of elastin's key role in the process of secondary septation [117, 118], as a dysfunctional elastin matrix might explain the alveolar developmental arrest described above. Furthermore, because of elastin's function in tethering of the airways [119], 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 [120]. 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 [121]. 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 [122, 123]. Additionally higher functional residual capacities and residual volumes are measured with body plethysmography and helium dilution, suggesting hyperinflation and air trapping [120, 124, 125]. 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 [122, 124], 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 [126, 127], 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 [128]. Invasive forced ventilatory maneuvers (pressure-volume loops and recruitment maneuvers) are commonly used to look for decreased lung capacities and compliance in BPD models [129, 130], however do not provide information on airway obstruction. Forced oscillation is therefore often added, to estimate airway (and tissue) resistance [129, 130]. An alternative is to add adapted harnesses or negative pressure chambers, to measure forced expiratory volume and flow [131], however this technique has, by our knowledge not been applied in neonatal animals until now. Both techniques can also be performed after methacholine instillation, to assess the hyperreactivity of the airways [130]. 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 [107, 132]. On the other hand, there are non-invasive measurements of lung function of which plethysmography has been used in many animal models of BPD [133-135].

Pulmonary vascular disease

About 14% of BPD-infants has pulmonary hypertension, as diagnosed on ultrasound at 36 weeks post conception [4], 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 [4, 136], reflecting the close interaction between acinar and vascular development [104, 137, 138]. Clinical echocardiographic data demonstrate a high incidence of both early and late pulmonary hypertension in BPD and an association with BPD severity [4, 139]. 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 [102]. Autopsy and biopsy specimens from BPD infants also demonstrated a dysmorphic microvascular pattern with variable abnormalities in the pulmonary capillaries [101, 140].

It thus seems essential that an animal model for bronchopulmonary dysplasia 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 [141-143], rats [104, 144], rabbits [145], lambs [146] and baboons [106]. 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 [141, 142, 147, 148]. Good correlation between these surrogates and invasive measures by catheterization have been reported [145, 149]. Finally, also micro CT 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 [104, 141, 145].

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 [31, 33] (table 1). The animals are small (birth weight mice: 0.5–1.5 g and rats: 5-6g), 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 [150, 151]. At term both mice and rats are in the saccular phase of lung development, which is morphologically similar to human premature infants at 28-30 weeks gestation (figure 1). 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. Additionally these pups do not encounter the gas exchange limitations seen in premature infants. Rats and mice seem to have developed adaptive mechanisms to function normally, with structurally preterm lungs (e.g. they express sufficient levels of surfactant and antioxidant defense enzymes [48, 152]). 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 rats 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 [104]). 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). The animals can be delivered prematurely and have respiratory failure as seen in human preterms, necessitating mechanical ventilation or non-invasive support measures [72, 73]. Standard of care interventions (exogenous surfactant, antenatal steroids, titrated oxygen supplementation and caffeine) have been

added to the models. Their size (mean 382g for preterm baboons at day 125, mean 3kg 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 [34, 38]. 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) [34, 38, 157]. 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 should also be taken in to account 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 [90, 156, 158] (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 [153], to study respiratory stability during different ventilation modalities [154] and recently also for trials with an ex vivo uterine environment [155]. 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 [38]. 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).

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). 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) [32]. Many different insults have been used to mimic BPD in term rabbit models, including different hyperoxia protocols [159-161], short term ventilation [162] and chorioamnionitis [163]. 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 [105, 129]. Below we will discuss the preterm rabbit model in more detail.

The case for a 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 [164], as well as in other laboratory animal species [48]. 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 [165]. 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) [166, 167]. 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 [165, 168, 169]. 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) [170]. In later work they showed that the developmental arrest is preserved in less preterm pups (29 days of gestation), exposed to lower oxygen concentrations (FiO_2 80%), however mortality remained high [105]. Furthermore the additional effect of postnatal malnutrition on the pulmonary phenotype of the model was explored [83].

Our group developed a comparable preterm rabbit model for BPD (figure 3) [129]. 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 (FiO_2>95%) or normoxia (FiO_2 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.

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 [129, 170]. Signs of increased inflammation were also present [171]. 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 [129], 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 [145].

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 [172, 173].

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 expression (qPCR, RNA sequencing) can compensate partially [129]. 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 [129]. 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 [174, 175]. On the other hand, the mortality can also be considered as a translational advantage, as it illustrates the functional prematurity of the rabbit pups. Finally, the lack of prenatal pathology leading to prematurity in this cesarean section based model, 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 [174, 176]. 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 [177]. The size of the rabbit pups allows, besides intraperitoneal administration, for more translatable methods: either subcutaneous, intramuscular, intravenous or enteral administration by gavage. Recently we also optimized a method for localized intratracheal pulmonary delivery, which will importantly expand the applications of the model (unpublished work). In terms of metabolism and excretion, it is important to notice that there are significant developmental differences between the adult and (preterm) neonate [178]. 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 [179-181]. Equally, renal clearance of neonatal rabbits is very low, and increases with aging [182]. 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

[183]. 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) [184], making the rabbit a popular model for fetal toxicity studies [185]. Recent data also suggest functional similarities in placental drug transporter spectra between humans and rabbits [186]. Transplacental treatment strategies in the preterm rabbit model have been carried out with rosiglitazone (PPARγ-agonist) and omeprazole (inducer of CYP1A1) [174, 175].

Discussion

The purpose of this review is to discuss the boundaries and characteristics of 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. 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.

Yet 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 (mostly single and exaggerated) 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. 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 [39].

We will thus conclude that a perfect animal model for bronchopulmonary dysplasia does not exist. However all models have their place in BPD research, and for every research question there is one that is ideal (figure 4). 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 are 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 an extensive use, so only the most promising therapies can be tested out in these models. In this review we focused on the preterm rabbit model. 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.

LIST OF ABBREVIATIONS

BPD	bronchopulmonary dysplasia
CYP1A1	cytochrome P450 monooxygenase 1A1
DHA	docosahexaenoic acid
ELBW	extremely low birth weight
FEV	forced expiratory volume
FiO_2	fraction in inspired oxygen
HFV	high frequency ventilation
HFNV	high frequency nasal ventilation
IGF1	insulin-like growth factor 1
IUGR	intra-uterine growth retardation
Lm	mean linear intercept
(n)CPAP	(nasal) continuous positive airway pressure
(n)IPPV	(nasal) intermittent positive pressure ventilation
PAAT	pulmonary artery acceleration time
PAAT/PAET	pulmonary artery acceleration over ejection time ratio
PPARγ	peroxisome proliferator-activated receptor gamma
RAC	radial alveolar count
RDS	respiratory distress syndrome
ROS	reactive oxygen species

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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FIGURES

Figure 1: Mammalian lung development. *The upper pane shows the 5 stages of mammalian lung development. 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. The lower pane shows the timelines of the 5 phases in humans and experimental animals frequently used to mimick BPD. Adapted from [31, 33, 34, 167, 187-189]*

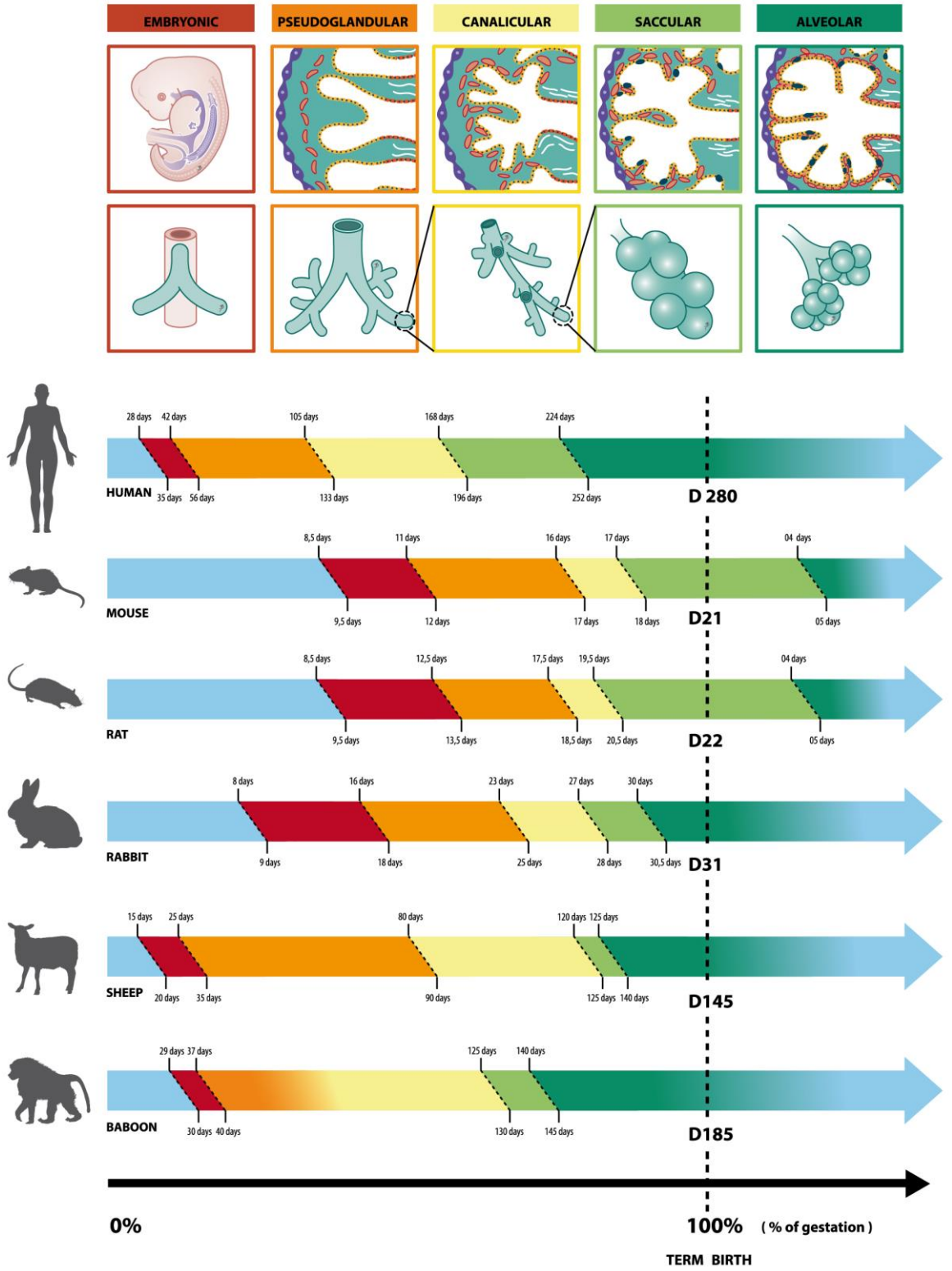

EMBRYONIC
PSEUDOGLANDULAR
CANALICULAR
SACCULAR
ALVEOLAR
HUMAN
28 days
42 days
105 days
168 days
224 days
35 days
56 days
133 days
196 days
252 days
D 280
MOUSE
8,5 days
11 days
16 days
17 days
04 days
9,5 days
12 days
17 days
18 days
05 days
D21
RAT
8,5 days
12,5 days
17,5 days
19,5 days
04 days
9,5 days
13,5 days
18,5 days
20,5 days
05 days
D22
RABBIT
8 days
16 days
23 days
27 days
30 days
9 days
18 days
25 days
28 days
30,5 days
D31
SHEEP
15 days
25 days
80 days
120 days
125 days
20 days
35 days
90 days
125 days
140 days
D145
BABOON
29 days
37 days
125 days
140 days
30 days
40 days
130 days
145 days
D185
0%
100%
(% of gestation)
TERM BIRTH

Figure 2: Pathophysiology of human bronchopulmonary dysplasia. *Schematic overview of the factors involved in chronic lung disease after preterm birth and schematic representation of BPD histology.*

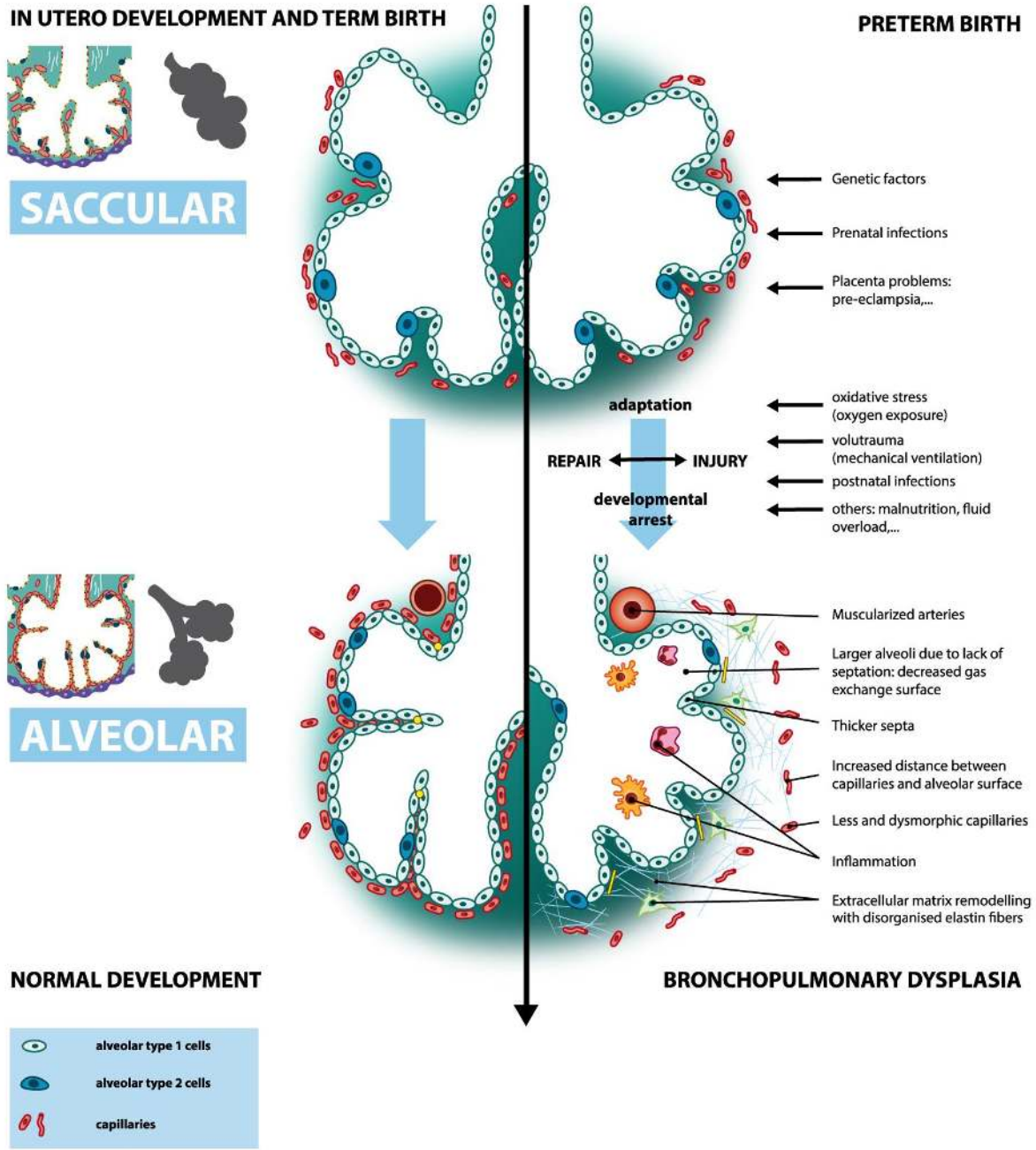


Figure 3: Methodology of an example of the preterm rabbit model. *Schematic representation of the preterm rabbit model as described in [129]*

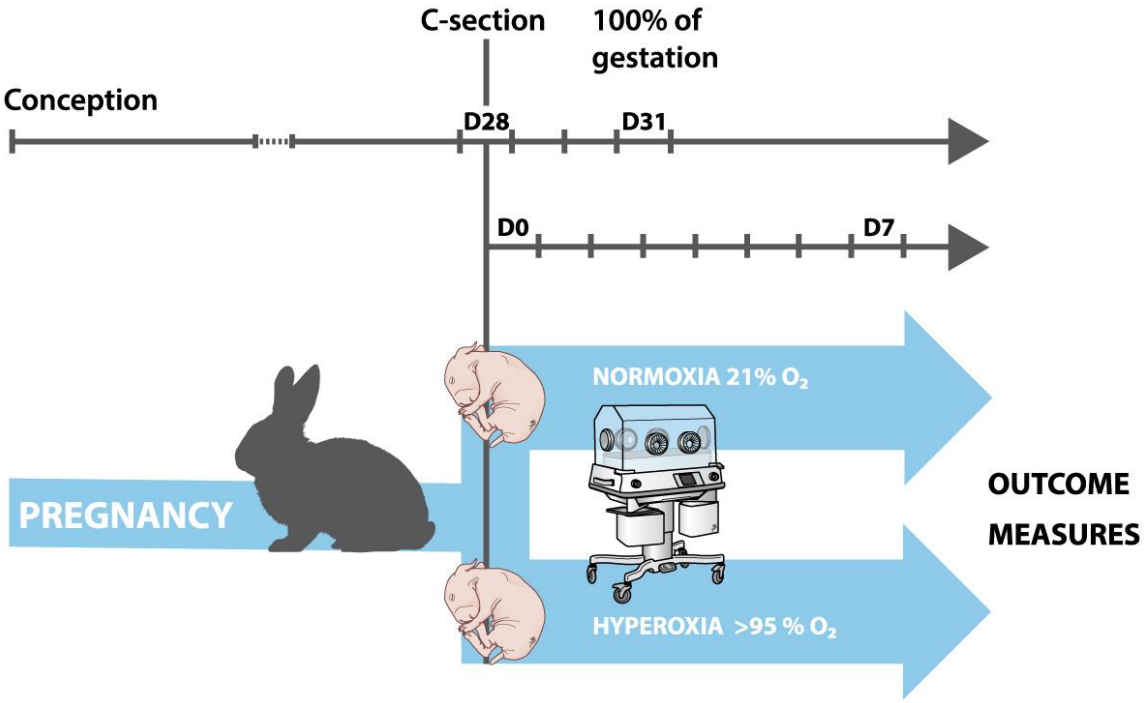
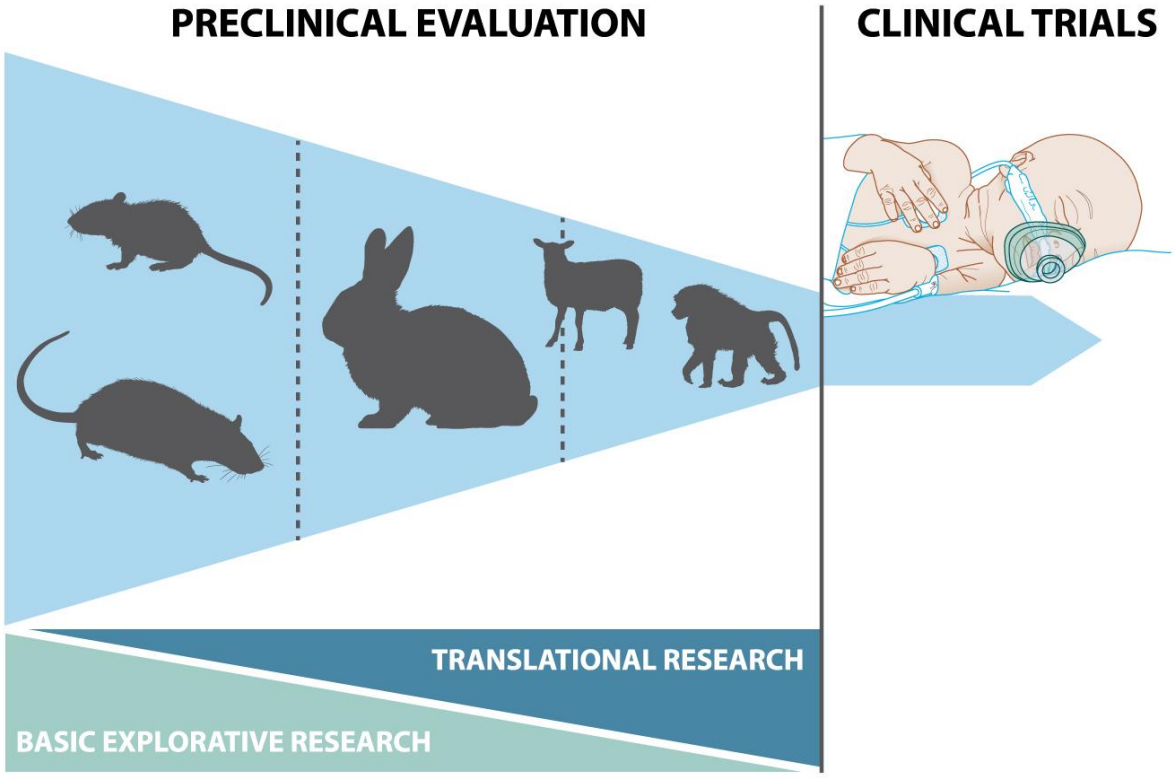


Figure 4: Place of the different animal models for BPD research. *Each animal for BPD has a unique set of practical and translational advantages and disadvantages, determining their place in BPD research, schematically represented in this figure.*



TABLES

Table 1: Animal models for BPD. Non-exhaustive summary of the factors used in the different animal models to mimic BPD pathophysiology. References: [31-34, 38, 74, 81, 93, 156, 158, 190, 191]

	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: HFNV - 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: CPAP, HFV - Oxygen (titrated) - Prenatal inflammation: Ureaplasma species - Antenatal steroids, postnatal surfactant