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Beta-3 Adrenoceptor (β3-AR) agonism is a promising strategy against bronchopulmonary dysplasia

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ABBREVIATIONS

α-SMA	*α-Smooth Muscle Actin*
A	*Adrenaline*
AD	*Alveolar Duct*
ADRP	*Adipose Differentiation-Related Protein*
AEC I	*Type I Alveolar Epithelial Cells*
AEC II	*Type II Alveolar Epithelial Cells*
Ang-1(2)	*Angiopoietin-1(2)*
AR	*Adrenergic Receptor*
AS	*Alveolar Sac*
β-ARK	*Beta-Adrenergic Receptor Kinase*
BAT	*Brown Adipose Tissue*
BHT	*Butylhydroxytoluene*
Bmp	*Bone Morphogenetic Protein*
BPD	*Bronchopulmonary Dysplasia*
BSA	*Bovine Serum Albumin*
cAMP	*cyclic Adenosine-Monophosphate*
CCPs	*Cardiopulmonary Progenitors*
CDP	*Continuous Distending Pressure*
CEPCs	*Circulating Early Endothelial Progenitors*
CTGF	*Connective Tissue Growth Factor*
DA	*Ductus Arteriosus*
DCM	*Dichloromethane*
DMEM	*Dulbecco's Modified Eagle Medium*
DPPC	*Dipalmitoyl-phosphatidylcholine*
E	*Embryonic Day*
ECL	*Enhanced Chemiluminescence*
ECM	*Extracellular Matrix*
EGF	*Epithelial Growth Factor*
EGFR	*Epithelial Growth Factor Receptor*
EMA	*European Medicine Agency*
eNOS	*Epithelial Nitric Oxide Synthase*
ENS	*Enteric Nervous System*
FBS	*Fetal Bovine Serum*
FDA	*Food and Drug Administration*
Fgf	*Fibroblast Growth Factor*
FiO2	*Fraction of Inspired Oxygen*
G-CSF	*Granulocyte Colony-Stimulating Factor*
GPCR	*G-Protein Coupled Receptor*
HBSs	*HIF-Binding Sites*
HIF-1	*Hypoxia Inducible Factor-1*
HREs	*Hypoxia-Responsive Elements*
IH	*Infantile Hemangioma*
iNOS	*Inducible Nitric Oxide Synthase*
IUGR	*Intrauterine Growth Restriction*
LIFs	*Lipofibroblasts*
LW/BW	*Lung and Body Weight Ratio*

MCP-1/CCL2	*Monocyte Chemoattractant Protein-1*
MIP	*Macrophage Inflammatory Protein*
MSCs	*Mesenchymal Stem Cells*
MYFs	*Myofibroblasts*
NA	*Noradrenaline*
NAFLD	*Non-Alcoholic Fatty Liver Disease*
NB	*Neuroblastoma*
NEC	*Necrotizing Enterocolitis*
NF-κB	*Nuclear Factor κ-Light-Chain Enhancer of Activated B Cells*
NHLBI	*National Heart, Lung and Blood Institute*
NICHD	*National Institute of Child Health and Human Development*
NK	*Natural Killer*
Nkx2.1	*Nk2 homeobox 1*
NLT	*Neutral Lipid Trafficking*
NO	*Nitric Oxide*
OAB	*Overactive Bladder*
OIR	*Oxygen-Induced Retinopathy*
P	*Postnatal*
PAF	*Paraformaldehyde*
PAS	*Periodic Acid-Schiff*
PBS	*Phosphate-Buffered Saline*
PDA	*Patent Ductus Arteriosus*
PDGFRα	*Platelet Derived Growth Factor Receptor alpha*
PGE2	*Prostaglandin E2*
PKC	*Protein Kinase C*
PMA	*Post-Menstrual Age*
PMSF	*Phenylmethylsulphonyl fluoride*
PPAR-γ	*Peroxisome Proliferator-Activated Receptor gamma*
PTHrP	*Parathyroid Hormone-Related Protein*
PTHrP-R	*Parathyroid Hormone-Related Protein Receptor*
PVDF	*Polyvinylidene Fluoride*
RA	*Retinoid Acid*
RB	*Respiratory Bronchioles*
RDS	*Respiratory Distress Syndrome*
ROI	*Regions of Interest*
ROP	*Retinopathy of Prematurity*
ROS	*Reactive Oxygen Species*
RT	*Room Temperature*
Shh	*Sonic Hedgehog*
SOX2	*SRY-Box Transcription Factor-2*
SP	*Surfactant Protein*
TB	*Terminal Bronchioles*
TEM	*Transmission Electronic Microscope*
TG	*Triglyceride*
TGF-α	*Transforming Growth Factor-α*
TGF-β	*Transforming Growth Factor-β*
TM	*Transmembrane*
TNF-α	*Tumor Necrosis Factor-α*
UCP-1	*Uncoupling Protein–1*
VEGF	*Vascular Endothelial Growth Factor*

VEGF-R2	*Vascular Endothelial Growth Factor Receptor-2*
WAT	*White Adipose Tissue*
WNT	*Wingless/Int*

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is the most common lung disease affecting premature newborns, especially those who undergo respiratory distress syndrome and need clinical lifesaving measures, such as mechanical ventilation and oxygen supplementation. Lung organogenesis begins in utero at low oxygen concentration (between 4% and 10%) and continues after birth, until adolescence. Premature birth exposes the underdeveloped lungs to ambient oxygen levels (21%), contributing to heightened oxidative stress that leads to impaired alveolarization, reduced vascularization, and excess collagen deposition. This condition can result in complications such as respiratory infections, pulmonary hypertension, and neurocognitive deficits. Among the various pathways implicated in the pathogenesis of BPD, the beta-3 adrenergic receptor (β3-AR) could represent a promising target for BPD treatment due to its oxygen-dependent regulation, its ability to reduce oxidative stress, and its protective effect against neonatal intestinal hyperoxia-induced damage. To explore this hypothesis, we evaluated the effect of BRL37344, a commonly used β3-AR agonist, in a rat BPD-like model. Sprague-Dawley rat pups were reared under normoxic (21%) or hyperoxic (95%) conditions for the first 2 weeks after birth and treated subcutaneously or not with BRL37344 at 1, 3, or 6 mg/kg. At the end of the experimental period, the animals were sacrificed, and the lungs were excised and collected for both histological and biochemical analyses. Hyperoxia increased oxidative stress levels, resulting in decreased pulmonary volume, impaired alveolarization and vascularization, hyperplasia of type II alveolar cells, and fibrosis, ultimately leading to a reduced survival rate. The administration of BRL37344 at 3 mg/kg effectively counteracted these alterations, thereby doubling the overall survival rate and protecting against the onset and the progression of the BPD-like disease. Our findings unveil a previously unrecognized role of the β3-AR in the onset/progression of BPD, paving the way for its pharmacological targeting as a new strategy for the prevention or treatment of this disease and other pulmonary disorders characterized by fibrosis.

1. INTRODUCTION

1.1 Bronchopulmonary dysplasia

Preterm newborns face a high risk of neonatal mortality and morbidities, both in the short-term and long-term (Siffel et al. 2021; Principi et al. 2018). The most common chronic lung disease occurring in preterm infants is Bronchopulmonary dysplasia (BPD) (Lecarpentier et al. 2019; Ahlfeld and Conway 2012). Indeed, preterm delivery results in an alteration of the normal lung developmental process, with a consequent failure in alveolarization. In addition, premature infants often require medical interventions such as mechanical ventilation and oxygen therapy, which, combined with other potential risk factors, elevate the risk of developing BPD (Siffel et al. 2021; Mathew 2020; Principi et al. 2018). BPD is associated with prolonged hospitalization, lifelong lung impairment, neurocognitive defects, increased susceptibility to early-onset chronic obstructive pulmonary disease, higher risk of infections during adulthood, and high healthcare costs (Siffel et al. 2021; Principi et al. 2018; Jensen and Schmidt 2014).

1.1.1 Definition of BPD

Defining BPD continues to be very challenging: through the last 50 years, BPD's definition has evolved, but to date, it is still based on therapy and not on pathology (Kalikkot Thekkeveedu et al. 2017; Jobe 2016). BPD was first characterized in 1967 by Northway and colleagues as a form of pulmonary damage observed in preterm infants with respiratory distress syndrome (RDS), primarily resulting from prolonged mechanical ventilation and oxygen therapy (Northway et al. 1967). Since then, there has been a relentless effort to understand the pathogenesis of this disease and to improve strategies for prevention and treatment. Currently, it has been established that the BPD to which Northway and co-workers refer is the "Old BPD". The "Old BPD" is the result of an aggressive mechanical ventilation (Hwang and Rehan 2018; Jensen and Schmidt 2014) and it is characterized by pulmonary structural alterations, severe fibrosis, airway injuries, inflammation, epithelial metaplasia and smooth muscle hypertrophy (Hwang and Rehan 2018; Jobe 2016; Jobe and Bancalari 2001). In the

subsequent years, therapeutic strategies for managing BPD evolved, with notable approaches including the administration of antenatal corticosteroids, surfactant therapy, and the adoption of gentler mechanical ventilation strategies (Mosca et al. 2011). The resulting increase in survival rates among the preterm infants leads to an evolution in the definition of BPD, as the need for oxygen at 36 weeks of postmenstrual age (PMA) (Shennan et al. 1988). Significant advances in therapeutic interventions have led to increased survival rates of very preterm infants (28-31 weeks of gestation) and extremely preterm infants (<28 weeks of gestation), who are more vulnerable to pulmonary morbidities like BPD (Mosca et al. 2011). That is why in 2000, the National Heart, Lung, and Blood Institute (NHLBI) proposed a more comprehensive definition for BPD, identifying the "New BPD" (Wang et al. 2019; Higgins et al. 2018). This definition classifies BPD based on severity levels: none, indicating less than 28 days of ventilation; mild, for infants who received oxygen for more than 28 days but are on room air at 36 weeks PMA; moderate, for newborns requiring ventilation strategies with less than 30% fraction of inspired oxygen (FiO2) beyond 36 weeks PMA; and severe, for cases where oxygen requirement exceeds 30% at 36 weeks PMA (Jobe and Bancalari 2001). The "New BPD" refers to a disruption in both the alveolar and pulmonary vascular development, rather than a disease due to oxygen therapy and mechanical ventilation, as for the "Old BPD". This condition leads to reduced fibrosis, more uniform inflammation, absence of smooth muscle hypertrophy and epithelial metaplasia, fewer but larger and simplified alveoli with thicker walls, and an abnormal vascular network. These changes collectively impair gas exchange (Hwang and Rehan 2018; Voynow 2017; Jobe and Bancalari 2001). However, the definition provided by NHLBI has some limitations: firstly, it does not provide information about long-term pulmonary outcomes; secondly, it does not account for the fact that BPD is a multifactorial disease; and thirdly, some newborns who undergo new ventilation strategies (like the nasal cannula) cannot be classified using this definition (Wang et al. 2019). To overcome this knowledge gap, in 2016, the National Institute of Child Health and Human Development (NICHD) defined BPD as a disease that occurs in infants born before 32 weeks of gestation, with persistent parenchymal lung alteration, that at 36 weeks PMA requires mechanical ventilation for more than three consecutive days, either invasive or non-invasive. Moreover, this definition uses different grades

for disease severity: I, II, and III, with Grade III being the most severe (Higgins et al. 2018). To date, this last definition is the most used.

1.1.2 Incidence of BPD

The advances in neonatal care allowed a great increase in the survival of premature infants, especially the extremely preterm ones, who are more susceptible to developing BPD (Siffel et al. 2021). Accordingly, while the incidence of other prematurity-related diseases, such as RDS, necrotizing enterocolitis (NEC), and retinopathy of prematurity (ROP), significantly decreased over the years, BPD incidence remained stable or even increased (Wang et al. 2019). Preterm birth rate varies across countries and ranges from 6% to 14% of pregnancies, and premature newborns can be classified based on the gestational age or the weight at birth. The gestational age classification identified extremely preterm infants, born <28 weeks of gestation, very preterm infants, between 28-32 weeks of gestation, moderate preterm infants, between 32-34 weeks of gestation, and late preterm babies, born between 34-37 weeks of gestation. The weight classification categorizes newborns as extremely low birth weight (those weighing <1000 g), very low birth weight (those weighing <1500 g), and low birth weight (those weighing <2500 g). The incidence of BPD inversely correlates with gestational age and birth weight, indicating that lower gestational ages and birth weights are associated with higher risks of developing BPD (Thébaud et al. 2019). A systematic review published in 2024 highlighted how the prevalence of BPD is dependent on the setup of the study, for instance, its inclusion criteria (whether it includes or excludes the extremely preterm newborns) (Moreira et al. 2024). However, this research analyzing data over more than 40 years showed that the incidence in the last three decades has remained stable and that there is significant variation among countries, although the differences between major continents are minimal (Moreira et al. 2024). According to global data, around 70% of extremely preterm or extremely low weight newborns, 20-25% of very preterm or very low weight infants, and 10% of moderate preterm babies are diagnosed with BPD (Jensen 2020).

1.1.3 Pathogenesis of BPD

BPD presents a multifactorial pathogenesis given the multitude of risk factors both intra- and extra-uterine (Sahni and Bhandari 2021; Mosca et al. 2011). Apart from premature delivery, which has the greatest impact, many risk factors contribute to the development of this disease. Predisposing factors can be categorized based on their origin as antenatal, natal, or postnatal (Kalikkot Thekkeveedu et al. 2017).

1.1.3.1 Prenatal risk factors

Genetic susceptibility. Some gene mutations seem to be associated with the development of BPD. However, establishing these correlations with certainty and translating this knowledge into clinical practice remains complex, and such applications are not yet used for treatment or prevention (Hwang and Rehan 2018). Moreover, a growing body of evidence—mainly from twin studies—indicates that BPD may have a genetic component (Kalikkot Thekkeveedu et al. 2017).

Intrauterine growth restriction (IUGR). IUGR is associated with an increased risk of developing BPD. It has been proposed that IUGR leads to reduced fetal lung development, which may predispose the fetus to various lung diseases, including BPD (Sahni and Bhandari 2021; Kalikkot Thekkeveedu et al. 2017).

Chorioamnionitis. Chorioamnionitis is an inflammation occurring in the membranes surrounding the fetus that can lead to an increase in fetal infection and inflammation, which often correlates with augmented inflammatory response (Thébaud et al. 2019; Hwang and Rehan 2018). Even if studies have not consistently demonstrated an association between chorioamnionitis and the development of BPD, it remains a potential risk factor that cannot be definitively excluded (Kalikkot Thekkeveedu et al. 2017). Indeed, chorioamnionitis plays a major role in triggering preterm labor and may expose the immature lungs to pro-inflammatory cytokines, two common features in BPD (Iliodromiti et al. 2013). It was demonstrated that more than 50% of the pregnancies that result in extremely preterm birth have histological evidence of chorioamnionitis (Kim et al. 2015). Moreover, a meta-analysis involving more than 13000 infants identified that histologically but not clinically diagnosed chorioamnionitis was associated with BPD (Hartling et al. 2012).

Lack of antenatal corticosteroids. In recent years, the preventive treatment of preterm high-risk pregnancies with corticosteroids has been a routine procedure (Jensen and Schmidt 2014). Studies showed that this strategy does not reduce the rate

of BPD, but it is not clear whether this is due to an actual lack of beneficial effect of the therapy or to insufficient statistical data for this specific outcome (Roberts et al. 2017; Carlo et al. 2011). Nevertheless, their use had drastically reduced the need for respiratory support, mechanical ventilation, and oxygen supplementation (Roberts 2006). Moreover, a meta-analysis study carried out in 2019 highlighted that the antenatal administration of corticosteroids significantly reduces the mortality of preterm newborns without any clear effect on neonatal morbidities (Blankenship et al. 2020).

Pregnancy-induced hypertensive disorders. Conditions such as gestational hypertension and preeclampsia can disturb the balance between pro- and anti-angiogenic factors, resulting in decreased vascularization and subsequent impairment of lung maturation. This process may contribute to the development of BPD or other pulmonary conditions (Sahni and Bhandari 2021; Kalikkot Thekkeveedu et al. 2017).

Maternal smoking. Tobacco smoking is strongly associated with preterm delivery, a strong risk factor for the progression of BPD (Sahni and Bhandari 2021; Kalikkot Thekkeveedu et al. 2017).

1.1.3.2 Natal risk factors

Gestational age and birth weight. The strongest predictors for BPD are prematurity and extremely low weight at birth (Thébaud et al. 2019; Kalikkot Thekkeveedu et al. 2017).

Gender. Male newborns are at increased risk of developing BPD (Jensen and Schmidt 2014). This feature is linked with the observation that male lung maturation is slightly delayed compared to female lung maturation, and that surfactant production follows lung development (Silveyra et al. 2021). Indeed, it seems that sexual hormones display a regulatory effect on lung physiology. In addition, male mice exposed to high oxygen levels show a reduction in the expression of vascular markers compared to females (Lingappan et al. 2016). Moderate and severe BPD are significantly more common in male newborns than in females (63.3% vs 36.7%). However, if the newborns are extremely preterm infants, these sex-related differences are not observed (Mathew 2020).

1.1.3.3 Postnatal risk factors

Hyperoxia and oxidative stress. Preterm newborns are at higher risk of developing hyperoxia-induced diseases, primarily because their antioxidant system is

still immature (Perrone et al. 2010). Moreover, the fetus grows and develops physiologically in the relatively hypoxic environment of the uterus, where oxygen levels range between 4% and 8%. Preterm labor exposes the neonate to ambient oxygen levels (approximately 21%) when organ systems, especially lungs, are still underdeveloped and insufficiently prepared for extrauterine life. The supraphysiologic oxygen levels in lung tissue, which cannot be offset by the immature antioxidant system, lead to the subsequent formation of reactive oxygen species (ROS). This imbalance favors the recruitment of a great number of inflammatory cells that exacerbate the inflammatory response. As a result, the lungs of the newborn are characterized by disruption of growth factor signaling, aberrant extracellular matrix assembly, impaired cell proliferation and vasculogenesis, and abnormal apoptosis, which together can result in lung development impairment, leading to BPD (Kalikkot Thekkeveedu et al. 2017; Alvira and Morty 2017).

Mechanical ventilation and supplemental oxygen. Although supplemental oxygen therapy is usually a life-saving measure for preterm infants to ensure adequate gas exchange and prevent lung collapse, if performed excessively and for a prolonged period, it can increase the risk of secondary lung injury, leading to increased ROS production, barotrauma, and volutrauma (Kalikkot Thekkeveedu et al. 2017; Mižíková et al. 2015). Two different types of respiratory support exist: invasive and non-invasive. The non-invasive support, through the use of continuous distending pressure (CDP) or nasal canula delivery, is first applied. If it is not enough, placement of an endotracheal tube will take place. Gestational age at time of ventilation can be a predictive parameter for the outcome: it was shown that extremely preterm newborns are more prone to develop stretch-related damage and impairment in alveolar maturation (Oakley et al. 2019; Shahzad et al. 2016). As the side effects of mechanical ventilation are well known, current focus has shifted toward optimizing its application to achieve a protective ventilation strategy. For example, the administration of high tidal volumes should be avoided to prevent alveolar overdistension. Additionally, if surfactant production is inadequate, the newborn should be treated with exogenous surfactant (Van Kaam 2024).

Sepsi and systemic inflammatory response. Study observations indicate that postnatal inflammation serves as a more significant risk factor for BPD compared to antenatal infections. The activation of the inflammatory cascade, characterized by

substantial recruitment of inflammatory cells such as macrophages and neutrophils, can trigger a secondary wave, and a substantial release of inflammatory cytokines. This amplification exacerbates tissue injury and endothelial dysfunction (Klinger et al. 2010; Lahra et al. 2009; Marshall et al. 1999). A growing body of evidence suggests that systemic inflammation leads to vascular permeability alteration with consequent alveolar injury and long-term impairment at the gas-exchange level (Kalikkot Thekkeveedu et al. 2017).

The airway microbiota. The airway microbiota is an important emerging field of interest; the fetal lung likely has its own microbiota from fetal life, as the placenta and amniotic fluid also contain microbiota. Lung microbiota can affect homeostasis and immune system development, potentially impacting inflammatory responses and tissue repair following injury (Surana and Kasper 2014). Recently, a decrease in lung microbiota diversity at birth has been associated with the development of BPD (Lal et al. 2016). Additionally, microbiome-produced microbial metabolites may influence lung development by modulating the inflammatory response (Marsland 2016).

Patent ductus arteriosus (PDA). Ductus arteriosus (DA) is only present in the fetus and allows blood flow from the pulmonary artery to the ascending aorta, bypassing the non-functional lungs. Its patency is driven by the low tension of oxygen characteristic of the uterine environment, and by the presence of high levels of endogenous vasodilators, such as prostaglandin E2 (PGE2) and nitric oxide (NO) (Pini et al. 2020). This ductus closes physiologically a few days after birth due to the rapid increase in oxygen tension. Patent ductus arteriosus (PDA) is a condition characterized by the failure of the ductus arteriosus to close after birth. This condition is commonly observed in infants with extremely low birth weight and is associated with an increased risk of lung injury, inflammation, and the development of BPD. This often results in a greater need for oxygen therapy (Kalikkot Thekkeveedu et al. 2017). Studies have highlighted that PDA does not necessarily increase the incidence of BPD, and the relationship between these two conditions is more likely an association rather than direct causation (Slaughter et al. 2017; Chock et al. 2014).

As BPD affects lung organogenesis and its anatomical structures, a description of lung anatomy and development has been included to improve understanding of the results and discussion related to these changes.

1.2 The anatomy of the respiratory system

The respiratory system is composed of organs and structures that facilitate the exchange of gases between the external environment and the organism. The primary organs include the lungs, which are paired organs: the right lung in humans has three lobes, whereas the left lung has two lobes and is slightly smaller. Within the respiratory system, the airways branch extensively, forming progressively smaller ducts until they reach the alveoli—the terminal structures of the respiratory tree—where gas exchange occurs. The alveoli serve as the functional units of this system.

The respiratory system is divided into upper and lower airways. The upper airways comprise nasal cavities, nasopharynx and oropharynx, and larynx; the lower airways are formed by trachea, bronchi (with their subsequent branching), and lungs. The upper airways, the trachea and a series of progressively narrowing bronchi (until the terminal bronchioles) constitute the conduction airways, which, as first, are essential to filter, heat and moisten the inspired air. The terminal bronchioles, the alveolar ducts and sacs, and the alveoli form the respiratory airways, and their main role is to offer a huge surface for gas exchange (there are approximately 300 million alveoli in human lungs, corresponding to 80-100 m^2 for gas exchange) (Pawlina and Ross 2021; Swarr and Morrisey 2015).

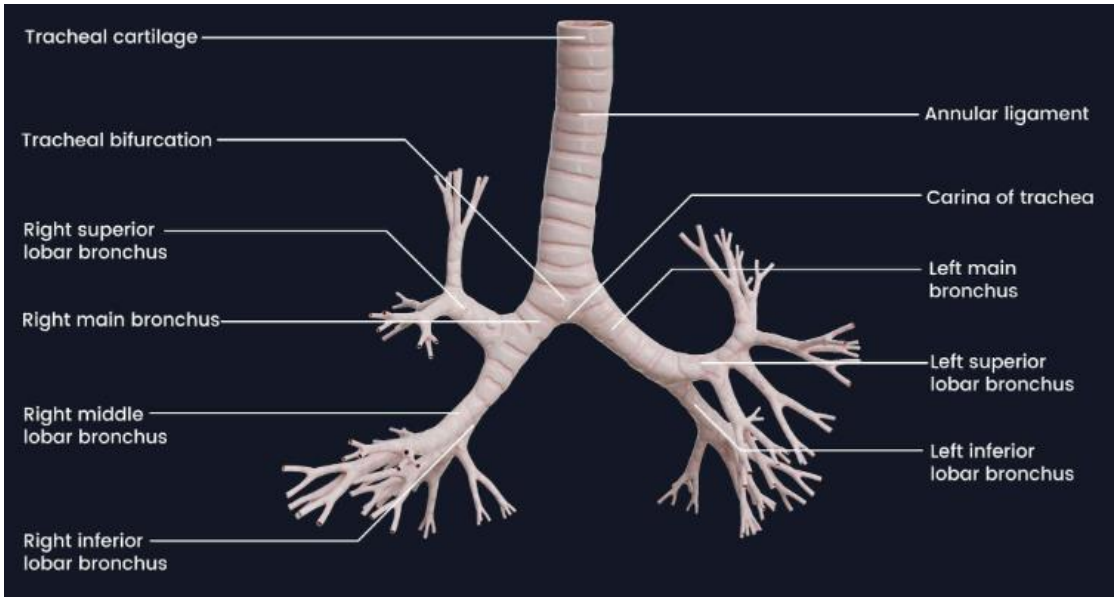

Fig.1 Schematic representation of human respiratory tree. *The human respiratory tree is characterized by the trachea and bronchi, which progressively branch into smaller ones. The terminal*

bronchioles lead to alveolar ducts, alveolar sacs and, finally, alveoli, where gases exchanges are mediated (Broaddus et al. 2021).

Considering lungs roles, it is possible to distinguish between respiratory and non-respiratory functions. Among the former, the central role of the lungs is to ensure adequate gas exchange with the external atmosphere, to obtain oxygen for cellular energy production and, simultaneously, to excrete carbon dioxide produced during cellular metabolism (Lalley 2013). Although the primary function is those mentioned above, studies showed that the non-respiratory ones are important too. While the inspiratory-expiratory process is underway, pathogens, microorganisms, and foreign particles can reach the lungs. Thus, the action of macrophages, which phagocytize those foreign particles, plays a vital role in the maintenance of lung homeostasis and defense against possible infections (Barkhordari et al. 2004). This goal is also facilitated by the presence of mucous secretion that traps the external particles, and thanks to the movement of cilia of the respiratory epithelium cells, helps to remove them. The presence of mucous is also essential to prevent dehydration of the epithelium in contact with air and to protect the respiratory surface from temperature changes. Additionally, the lungs enable the phonation and allow the olfactory function (Pawlina and Ross 2021), simplify the conversion of some inactive chemical precursors into their active forms (e.g. the conversion of Angiotensin I into Angiotensin II) (Jin et al. 2018), degrade and inactivate some vasoactive mediators (such as bradykinin), and present some cells, the Kulchitsky cells, that can secrete numerous factors, including catecholamines and hormones (Jin et al. 2018).

1.2.1 Anatomy of the lungs

The presence of a bilobed left lung and a trilobed right lung characterizes human lungs. The trachea bifurcates, giving rise to the primary bronchi, which penetrate the right and left lungs. Once within the pulmonary system, each main bronchus bifurcates into secondary bronchi: the right primary bronchus splits into three secondary bronchi, corresponding to the three lobes of the right lung, whereas the left primary bronchus divides into two, aligning with the two lobes of the left lung. This arrangement ensures that each lobar bronchus supplies a specific pulmonary lobe. The secondary bronchi branch into smaller bronchi called segmental bronchi, with 10 in the right lung and 8 in the left lung. This branching process occurs 22 times in total, creating airways with

progressively smaller diameters that end in the alveoli, the final part of the respiratory tree where gas exchange takes place. Until the bronchi reach outside the lungs, they maintain the same histological features trachea. Instead, once they enter the lungs, their morphology slightly changes.

1.2.1.1 Morphology of bronchi

In the primary bronchi, five distinct layers can be identified, which vary slightly as the diameter of the bronchi decreases with branching. Starting from the lumen side, the layers are as follows:

- The mucosa, composed of the respiratory epithelium and its lamina propria (fibrous connective tissue).
- The muscularis mucosae, characterized by a layer of smooth muscle.
- The submucosa, composed of thicker connective tissue than the lamina propria.
- The cartilage layer, composed of hyaline cartilage.
- The tunica adventitia, made of connective tissue.

The mucosa is characterized by a pseudostratified columnar ciliated respiratory epithelium, with cellular height decreasing concomitant with the reduction in bronchial diameter. Along this epithelium, the main cell types are ciliated columnar cells, goblet cells, and basal cells. Occasionally, and in very few quantities, brush cells and small granule cells are present. Ciliated cells are the most represented in the primary bronchi. They are characterized by the presence of apical membrane specializations, the cilia, and among their functions, they coordinate the movement of mucus (which traps pathogens and inhaled foreign particles) in the direction of the pharynx. This mechanism is essential to remove small particles inhaled during breathing. These cells present a considerable number of mitochondria, just below the apical side, providing energy for the coordinated movement of the cilia (Ganesan et al. 2013). Goblet cells are similar to those present in the intestinal epithelium: they are single-cell glands dispersed between ciliated cells, producing mucins. They are distinguishable at the optical microscope after both Hematoxylin-Eosin staining, due to their leakage of apical cilia and to their typical empty light area left by mucinogen granules extracted during the coloration process, and Periodic acid-Schiff (PAS) staining, mainly used dye to detect polysaccharides and muco-substances like mucins. Basal cells represent the staminal compartment of the respiratory epithelium and can be easily recognized

by their cuboidal shape with the alignment of nuclei near the basal lamina. Brush cells are columnar cells with short apical microvilli, and their basal side forms a synaptic contact with an afferent nerve ending, so they can be considered receptor cells. They play a role in both secreting surface fluid and aiding in breathing (Ganesan et al. 2013). Small granulate cells, or Kulchitsky cells, are pulmonary neuroendocrine cells. They are primarily present as single cells dispersed along the epithelium and are distinguishable from other cell types only through specific histological staining, such as silver impregnation. Their cytoplasm is quite extensive compared to the basal cells, and their nuclei are located in a relatively basal position.

The respiratory epithelium lies on a basal membrane. Transmission electron microscopy (TEM) analysis revealed that the membrane consists of two distinct layers: a basal lamina, located adjacent to the epithelium, and a reticular lamina. The basal lamina is produced by the epithelium and is further divided into two components: the lamina lucida and the lamina dense. The lamina lucida is primarily composed of membrane proteins associated with their glycocalyx. It is situated directly underneath the epithelium. The lamina dense mainly comprises tightly packed collagen type IV fibers. The basal lamina connects to the underlying reticular lamina via collagen type VII fibers, which encase the reticular collagen type III fibers characteristic of this region. The basal membrane rests upon the lamina propria, a loose connective tissue abundant in cells, collagen, and elastic fibers, as well as blood vessels, lymphatic vessels, and nerves. Among its functions, it is important to note its role in providing mechanical support, nourishing the overlying epithelium, facilitating lymphatic drainage, and offering immune defense against infections due to the presence of immune cells. Additionally, both the basal membrane and the lamina propria are thicker in the primary bronchi and progressively decrease in thickness as the bronchi diameter diminishes.

The muscularis mucosae is characterized by a continuous layer of smooth muscle organized in intertwined bundles. In the smaller bronchi, this layer is thinner, less organized, and can appear discontinuous.

The submucosal layer comprises a relatively loose connective tissue matrix, within which extensive lymphatic tissue and blood vessels are present. Additionally, in the submucosa of the primary bronchi, dislocated submucosal glands and adipose tissue are observed.

The typical cartilaginous rings of the trachea in the primary bronchi begin to be replaced by cartilaginous plaques with irregular shapes. These plaques are located around the entire circumference of the bronchi, thus giving them a cylindrical shape. As the bronchi branch and become smaller, the cartilaginous plaques decrease in number and size. When the bronchi reach about 1 mm in diameter, these plaques vanish, and the bronchi turn into bronchioles.

Adventitia is the outer layer and is made of a thicker connective tissue. This portion helps to maintain the bronchi in the correct position and contains blood and lymph vessels and nerves.

1.2.1.2 Morphology of bronchioles

Bronchioles have a pseudostratified columnar ciliated epithelium that, as their diameter decreases, becomes simple columnar ciliated epithelium. Neither subepithelial glands nor cartilaginous plaques are present in these ducts, even if small cartilaginous elements in correspondence to the branching spots could be present. Goblet cells are only present in the larger bronchioles. When the diameter of these air passages approaches approximately 0.5 mm, they are classified as terminal. Bronchioles are lined by a simple cuboidal epithelium mainly composed of ciliated cells, with some Clara cells dispersed among them and, occasionally, brush cells and Kulchitsky cells. Clara cells are not ciliated cells with a rounded apical side. If analyzed with TEM, these cells exhibit a well-developed endoplasmic reticulum, secretory granules of the serous type, and numerous cisterns of the smooth endoplasmic reticulum in the apical region. Clara cells release a surfactant that prevents the collapse of the cells surrounding the bronchiole ducts, performing a function analogous to that of alveolar cells. Moreover, Clara cells produce a protein called CC16, which is one of the most abundant proteins in lung secretions and functions as an anti-inflammatory and antioxidant agent in the respiratory system epithelium (*Fig.2*).

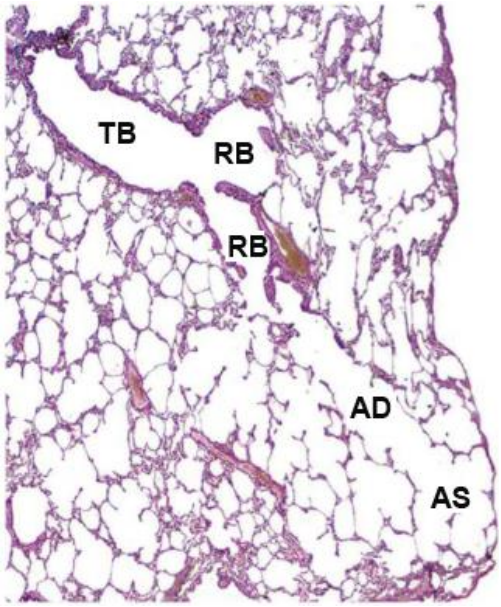

Fig.2 Structure of bronchioles in the respiratory tree observed with Hematoxylin/Eosin staining 120x. *Longitudinal section of a terminal bronchioles (TB) in the spot where it branches into two respiratory bronchioles (RB). The respiratory bronchioles give rise to the alveolar ducts (AD). The alveolar sacs (AS) are the spaces at the end of the AD (Pawlina and Ross 2021).*

1.2.1.3 Morphology of alveoli

The architecture of alveoli substantially enhances the surface area for gas exchange. Each alveolus is lined by an exceptionally thin epithelium and is interconnected within an alveolar sac—a confined space comprised of numerous alveoli. These alveolar sacs are mostly located at the end of an alveolar duct, which is an elongated airway, with almost no walls, only bordered by alveoli. A capillary network that brings blood close to the inflated air, promoting gas exchange, surrounds every alveolus. This feature explains the structure of the alveolar septum, which separates two alveoli and is composed of alveolar epithelium, alveolar capillaries, and connective tissue. The juxtaposition between alveoli and capillaries allows the generation of the blood-air barrier, which is 0.2-0.8 μm thick, permits gas exchanges, and consists of alveolar epithelium, its basal lamina, endothelial basal lamina, and endothelium. Sometimes the two basal laminae (the epithelial and the endothelial ones) fuse, leading to a reduction in the thickness of the barrier (*Fig.3*). The alveolar epithelium consists of two distinct cell types: type I and type II alveolar epithelial cells, designated as AEC I and AEC II, respectively. These pulmonary parenchymal cells have been shown to engage in significant crosstalk with their stromal counterparts, thereby facilitating proper and complete lung organogenesis and resulting in surfactant production (El

Agha and Thannickal 2023). Additionally, brush cells and alveolar macrophages may be present.

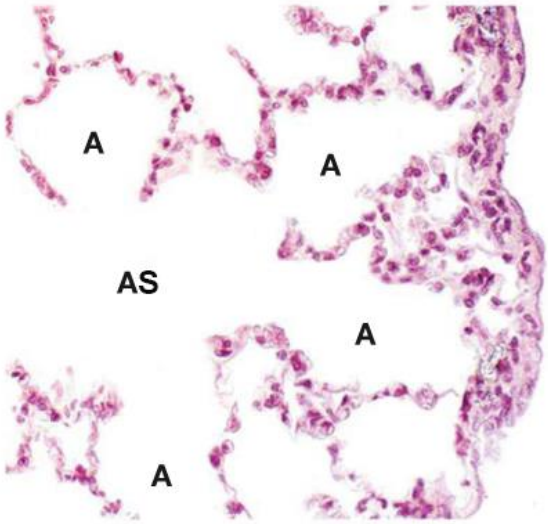

Fig.3 Structure of an alveolar sac surrounded by alveoli observed with Hematoxylin/Eosin staining, 360x*. This image shows an alveolar sac (AS) and its alveoli (A). The alveoli are surrounded and separated from each other by a thin layer of connective tissue, the intra-alveolar septum that contains the capillary network (Pawlina and Ross 2021).*

1.2.2 Alveolar epithelial cells

AEC I, also known as type I pneumocytes, represent only 40% of the total, but because they are squamous and flattened, they cover almost the entire surface of the alveoli (95%). The tight junctions between these cells create a physical barrier separating the intra-alveolar septum from the air inside its alveolus. These cells are terminally differentiated; thus, they cannot undergo mitosis. AEC II, even called type II pneumocytes, constitute about 60% of the alveolar cells, but because of their cuboidal shape, they cover only 5% of the total alveolar area. AEC II are dispersed among AEC I, even though most are localized near the junction points between different alveolar septae. AEC II are secretory cells with apical microvilli. When observed with TEM, they reveal secretory granules on the apical side, named lamellar bodies. These lamellar bodies are enriched with phospholipids, neutral lipids, and proteins, which are secreted through an exocytotic process, forming the surfactant. Furthermore, AEC II cells, unlike AEC I, can undergo mitosis, making them a progenitor population for lung repair: they are capable of long-term self-renewal and multipotent differentiation to generate AEC I (Parimon et al. 2020; Beers and Moodley 2017). The AEC II also

functions to transport sodium ions from the apical surface into the interstitium via the epithelial sodium channel. This process helps maintain a relatively dry alveolar space and plays a crucial role in regulating salt and water homeostasis. The high number of mitochondria observed in AEC II cells supports the energy demands of these activities (Ruaro et al. 2021).

1.2.3 Stromal cells

The specification of epithelial cell fate is known to be strongly influenced by mesenchymal signaling pathways. Nevertheless, since stromal cells are a heterogeneous group of mesenchymal-derived cells with numerous overlapping markers among different populations, sharing similar functional roles, and characterized by high plasticity, they are difficult to sort into distinct lineages or cell populations. Spatially and temporally, mesenchymal cells can be classified into many types, whose differentiation depends on their localization along the cranial-caudal axis during lung organogenesis, their association with lung structures, and their functional phenotypes. The different populations that define the mesenchyme are peribronchiolar and perivascular smooth muscle cells, myofibroblasts (MYFs), lipofibroblasts (LIFs), matrix-fibroblasts, pericytes, mesothelial cells, alveolar niche cells and mesenchymal stem cells (MSCs) (Riccetti et al. 2020). In recent years, the role of alveolar mesenchyme has gained increasing attention due to its impact on driving proliferation and differentiation of alveolar epithelium. Particularly, mesenchymal cells provide support by modulating ECM production, enabling elongation and narrowing of alveolar walls during secondary septation through the generation of contractile forces, and supplying paracrine stimuli that lead to epithelial and endothelial proliferation and differentiation (Ushakumary et al. 2021). During the last organogenesis phases and postnatal lung development, the two main significant mesenchymal populations are MYFs and LIFs (El Agha et al. 2017). MYFs are multifunctional cells that present ultrastructural characteristics between fibroblasts and smooth muscle cells (Thannickal et al. 2003). Indeed, they are muscle-like contracting cells expressing alpha-Smooth Muscle Actin (α-SMA). Their activation is vital for tissue repair and wound healing, and they are essential during secondary septation and pulmonary development since they produce elastin and other ECM components (Hecker et al. 2011). Aside from a few compartments that require localized contractile forces, such

as the uterine submucosa, intestinal lamina propria, and pulmonary septa, MYFs are not normally present in tissues. When they are, their activity is strictly controlled and regulated (Di Carlo and Peduto 2018). Instead, LIFs are lipid droplet-containing interstitial fibroblasts capable of accumulating neutral lipids. They are characterized by the expression of adipose differentiation-related protein (ADRP), which enables LIFs to take up, store, and eventually transfer triglycerides (TGs). They contain an abundance of glycogen and are localized centrally into the alveolar septum near AEC II, to whom they provide nutrients and lipids. This localization is crucial as soon as they are implied in surfactant production (El Agha and Thannickal 2023; Riccetti et al. 2020; Boros et al. 2002). LIFs contain 65% of TGs, 14% of cholesterol esters, 14% of phospholipids, and the remaining 7% of free fatty acids and cholesterol (McGowan and Torday 1997).

1.2.4 The Extracellular Matrix (ECM)

The pulmonary mesenchyme is a complex, integrated, and heterogeneous system. Based on the elastic theory, the extracellular matrix (ECM) plays an essential role in alveolarization, the last phase of lung development, providing structural support, regulating cell communications, favoring epithelial cells differentiation and migration, and supporting the thinning and the lengthening of secondary septa (Alvira and Morty 2017; Thibeault, Mabry, et al. 2003). Therefore, unsurprisingly, harmful stimuli such as inflammation, premature exposure to hyperoxia, and mechanical ventilation can disrupt the normal structure and function of the parenchyma, leading to typical BPD features, specifically increased and dysregulated matrix component deposition and their disturbed turnover and organization (Mathew 2020; Mižíková and Morty 2015; Ahlfeld and Conway 2012). The pulmonary mesenchyme is mainly composed of fibrous structural proteins produced by fibroblasts, among which type I and III collagen fibers (that account for about 50% of the total), elastin (about 18%), fibronectin and laminin, and is flanked by the ground substance, mainly composed of glycosaminoglycans, proteoglycans, and glycoproteins. Collagen and elastin provide most of the physiological characteristics to parenchyma and the structural framework for the alveolar walls (Suki et al. 2005), while fibronectin and laminin are indispensable to connect cells to the bigger matrix proteins (Mathew 2020; Mižíková and Morty 2015; Ahlfeld and Conway 2012). The lung ECM is characterized by a

highly dynamic composition during development from fetal to neonatal and adult, and the differentiation of its cellular populations and their migration is temporally and spatially regulated. For instance, in embryonic mice, there are all five forms of laminin γ, while in adults, only laminin γ3, γ4, and γ5 are present. Moreover, the fetal pulmonary ECM of mice contains more glycosaminoglycans and proteoglycans, and more collagen compared to that of adults (Zhou et al. 2018).

1.2.5 Pulmonary surfactant

The pulmonary surfactant is essential for human life, and its presence is required for breathing after birth (Ruaro et al. 2021; Schmiedl et al. 2005). Surfactant minimizes surface tension at the air-liquid interface within the alveoli, thereby preventing their collapse during expiration. Additionally, it performs an immune function (Han and Mallampalli 2015). As previously mentioned, the surfactant is produced by AEC II, stored in their lamellar bodies, and released through the exocytosis process within the alveoli lumen, where the walls absorb it. All these processes are finely regulated by several molecular signaling pathways, including cAMP (cyclic adenosine monophosphate), Protein Kinase C (PKC) activation, and mechanisms mediated by calcium (Hadrioui et al. 2020; Han and Mallampalli 2015; Mason 2006; Rooney 2001). Pulmonary surfactant primarily consists of lipids, accounting for approximately 90% of its composition, with proteins making up about 10%. This compositional profile is conserved across different species (Han and Mallampalli 2015). Regarding the lipid components, most are phospholipids, with only a small portion being neutral lipids, mainly cholesterol. The most represented phospholipids are phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol. About 70% of the lipidic portion is composed of phosphatidylcholine, which exists as dipalmitoyl-phosphatidylcholine (DPPC) (Possmayer et al. 2023; Han and Mallampalli 2015). Lipids are primarily responsible for reducing surface tension (Ruaro et al. 2021). Torday and colleagues proposed the first hypothesis that links LIFs with surfactant production by AEC II in 1995. They observed that LIFs begin to be present at embryonic day (E) 16 in rats: the TGs content increases between E17 and E21, just before the appearance of lamellar bodies in AEC II, and peaks around Postnatal day (P) 14. The abundance of LIFs follows a similar course, with a rapid decrease occurring just before weaning (McGowan and Torday 1997). Following *in vitro* studies, they confirmed that, contrary

to AEC II, LIFs are capable of TG uptake, which is an essential component of the lipidic portion in the generation of surfactant. Moreover, they demonstrated that LIFs are unable to release these TGs unless in the presence of AEC II. Consequently, some unknown mechanism should be at the basis of this process that connects lipid droplets of LIFs and surfactant production (Torday et al. 1995). In parallel with these considerations, it was demonstrated that lung stretching, resulting from respiratory processes, promotes surfactant production by enhancing the expression of PGE2 and Parathyroid Hormone-related Protein (PTHrP) in AEC II (Torday and Rehan 2002). PTHrP is a stretch-sensible hormone, expressed in the developing epithelium and persisting into the adult epithelium, capable of interacting with its receptor, PTHrP-R, expressed by mesenchymal cells, among which are LIFs. Loss or deletion of PTHrP is linked to the arrest of normal lung development, RDS, and an increased transdifferentiation of LIFs into MYFs, all features that correlate with an increased risk of developing BPD (Torday et al. 2003). Therefore, the typical stretching of AEC II during breathing processes induces the expression of PTHrP from these cells. PTHrP, via a paracrine mechanism, interacts with PTHrP receptors on LIFs, resulting in upregulated expression of ADRP, leptin, and peroxisome proliferator-activated receptor gamma (PPAR-γ). Under the stimulation of PGE2, ADRP promotes the uptake of circulating TGs from blood vessels and their storage into lipid droplets typical of LIFs. Leptin, by binding its receptor on AEC II, facilitates the transfer of these stored TGs to AEC II, with a complex mechanism called Neutral Lipid Trafficking (NLT), an essential pathway for surfactant production. The increase in the PPAR-γ pathway promotes the trans-differentiation of MYFs into LIFs (Lecarpentier et al. 2019; Rehan and Torday 2014; Rehan et al. 2006; Boros et al. 2002). These observations suggest that LIFs play a role in supporting surfactant synthesis from AEC II by trafficking towards them TGs through a complex regulatory mechanism, NLT (Torday and Rehan 2016).

Considering the protein constituents, four surfactant proteins (SP-A, SP-B, SP-C, and SP-D) are associated with the lipidic phase. SP-A, the most prevalent surfactant protein, along with SP-D, are hydrophilic proteins belonging to the collectin family. This classification elucidates their immunological function in promoting the clearance of bacterial and viral pathogens, as their C-terminal domains recognize and bind to non-self oligosaccharides present on exogenous microorganisms, thereby facilitating

opsonization and subsequent phagocytosis by macrophages (Han and Mallampalli 2015). In addition, SP-A and SP-D can directly bind to allergens and foreign particles, preventing specific IgE from recognizing them and thus decreasing allergen-induced histamine release (Schmiedl et al. 2005). Instead, SP-B and SP-C are hydrophobic proteins that are essential for supporting lipid function, helping to organize the thin surfactant film on the alveolar surface (Beers and Moodley 2017; Han and Mallampalli 2015). Both SP-B and SP-C are produced as immature pro-proteins and undergo extensive post-translational modifications during their trafficking toward the lamellar bodies (Schmiedl et al. 2005). These proteins mediate specific functions to produce an efficient surfactant, providing both functional and structural integrity to the active agent, maintaining its homeostasis, and stabilizing its components. Specifically, SP-B and SP-C allow the construction and fixation of the thin surface-active film (Schmiedl et al. 2005).

1.3 Lung organogenesis

Lung organogenesis is a highly complex process characterized by dynamic tissue interactions, growth factors, and signaling pathways that collaboratively drive the development of a fully functional lung. In human beings, the molecular pathways involved in the regulation of respiratory system organogenesis are likely conserved across the development of other organs, such as the kidneys and mammary glands. Histologically, it consists of five different stages: the embryonic, the pseudo-glandular, the canalicular, the saccular, and the alveolar, depending on time progression and morphological features (*Fig.4*) (Fanni et al. 2014; Ahlfeld and Conway 2012; Morrisey and Hogan 2010).

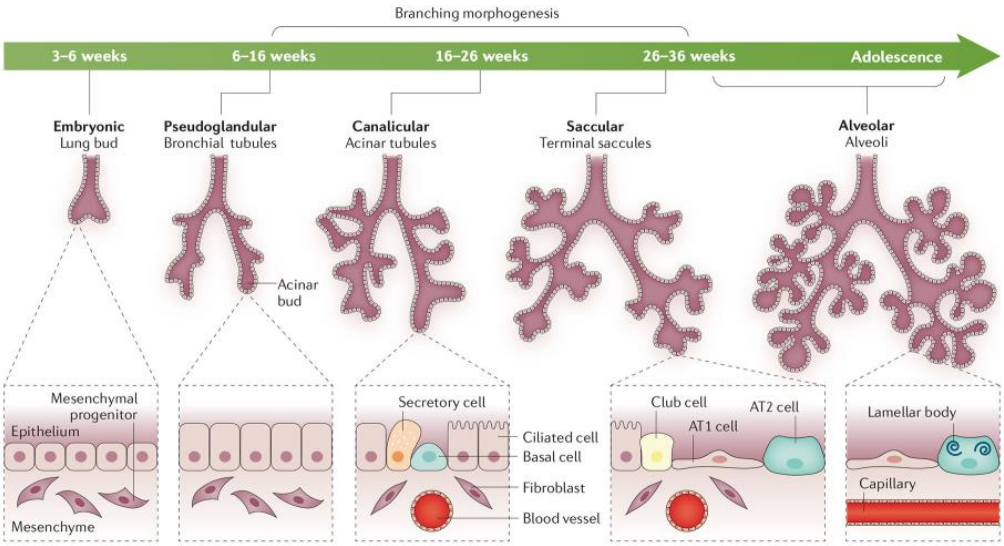

Fig.4 Schematic progression of human lung organogenesis*. In the embryonic period, two lung buds arise from the primitive foregut. During the pseudo-glandular phase, the branching morphogenesis begins, with the outgrowth of the branches in the surrounding mesenchyme. In the canalicular stage, the forming acinar buds develop to give rise to the alveoli for a second time. This phase also leads to the formation of the first immature capillary network. The saccular period is characterized by the presence of all the differentiated epithelial cell types and by the mesenchymal precursors. During the last phase, the alveolarization, the surface area for gas exchange increases significantly, and the process continues until young adulthood (Thébaud et al. 2019).*

Vertebrate lungs exhibit significant diversity in their anatomical structure, positioning within the body, ventilation mechanisms, integration with locomotor systems, and ontogenetic development (Duncker 2004). While mammals share the two main stages—branching and alveolar differentiation—there are notable differences among species (Warburton et al. 2010). For instance, in humans, the alveolar stage initiates in utero and persists into early adulthood. Conversely, in rodents, this process predominantly occurs postnatally. As a result, mouse and rat pups are born at the end of the saccular stage and are completely autonomous at birth. In contrast, human infants born prematurely during this stage face an increased risk of developing respiratory distress syndrome due to pulmonary immaturity (Berger and Bhandari 2014). Indeed, premature birth may alter the developmental processes, resulting in infants with underdeveloped lungs that are insufficiently prepared for extrauterine life. Therefore, elucidating the mechanisms underlying and governing pulmonary development is of paramount importance for treating the diseases associated with prematurity (Morrisey and Hogan 2010).

To improve understanding of lung organogenesis, a detailed overview of the events occurring during the five phases is provided, also comparing human and murine organogenesis. This comparison is particularly relevant because rodents are the most commonly used animals to model human pulmonary diseases (*Fig.5*).

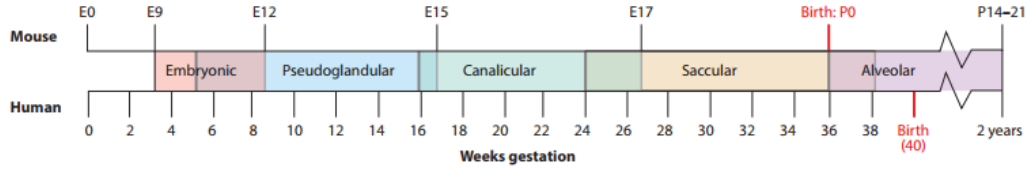

Fig.5 Gestation period in human and mouse. *The timeline shows a comparison between human and mouse period of gestation. The most important thing to notice is that in the mouse, the alveolarization process begins after birth, while in humans it starts during the 36th week of gestation (Swarr and Morrisey 2015).*

1.3.1 The embryonic stage (3-6 weeks of gestation)

The lungs first appear during the third week of gestation. Like many other organs, including the liver, esophagus, and thyroid, the respiratory system is specified in the anterior foregut endoderm, where the two primary lung buds (the future right and left lobes) appear (Swarr and Morrisey 2015). This explains the endodermic derivation of the respiratory system epithelium (Pawlina and Ross 2021). During this developmental period, the foregut tube differentiates into a dorsal duct, which contributes to the formation of the digestive system, and a ventral respiratory diverticulum, which develops into the trachea connected to the lung buds (Morrisey and Hogan 2010). In the meantime, the splanchnic mesoderm gives rise to the visceral pleura, and the somatic mesoderm to the parietal pleura (Schittny 2017).

Approximately on E9.0 in mice, which corresponds to 28 days in humans (*Fig.5*), a localized expression of the transcription factor Nk2 homeobox 1 (*Nkx2.1)* on the ventral side of the anterior foregut is identified. The expression of *Nkx2.1* is regulated by the coordinated network and crosstalk among different signaling pathways coming from the surrounding mesoderm and the notochord. The Wingless/Int (Wnt)-canonical pathway, activated by Wnt2 and Wnt2b ligands, promotes *Nkx2.1* expression, while reducing the presence of SRY-box transcription factor-2 (*Sox2)* at the distal side of the foregut endoderm (where the future esophagus will develop) (Swarr and Morrisey

2015; Goss et al. 2009). The loss of Wnt2, Wnt2b, or β-catenin results in the lack of *Nkx2.1* expression and an increase in *Sox2* presence, with consequent failure in esophagus-trachea separation. Moreover, a correct Bone morphogenetic protein (Bmp) signaling gradient is essential for properly directing lung development along the proximal-distal axis of the primitive foregut (Domyan et al. 2011). Bmp4 is present in the ventral mesoderm, while its antagonist, Noggin, expressed by the notochord, is located on the dorsal side. Inactivation of the Bmp signaling determines tracheal agenesis and retention of the branching area of the lungs. The ventral expression of Fibroblast Growth Factor (Fgf) further contributes to the early specification of lung development (*Fig.6*) (Morrisey and Hogan 2010; Warburton et al. 2010). Other polypeptides, such as epithelial growth factor (EGF), transforming growth factor-α (TGF-α), and amphiregulin play an essential role in directing fetal lung development, beyond epithelial repair after injury, by binding to the epithelial growth factor receptor (EGFR) (Kumar 2004; Warburton et al. 2000). EGFR belongs to the tyrosine kinase receptor family and presents a cysteine-rich extracellular domain for ligands' binding, a single α-helix transmembrane domain, an intracellular tyrosine kinase domain, and a C-terminal signaling domain (Finigan et al. 2012; Miettinen et al. 1997). EGFR stimulation favors lung branching morphogenesis, cytodifferentiation, epithelial and mesenchymal cell proliferation, and *Nkx2.1* and SP-C expression. As demonstrated by null mutant mice, abolishing EGFR signaling results in delayed structural and functional lung development, due to reduced branching morphogenesis and lower expression of both *Nkx2.1* and SP-C (Schmiedl et al. 2020; Warburton et al. 2000).

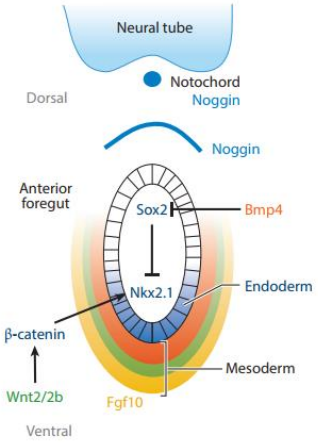

Fig.6 Fundamental signaling interactions for pulmonary endoderm development*. The specification of the lungs from the primitive anterior foregut is driven by several pathways, such as Wnt, Bmp, Fgf, and the Noggin signal (Swarr and Morrisey 2015).*

1.3.2 The pseudo-glandular stage (6-16 weeks of gestation)

As soon as the lung buds develop, a dynamic phase occurs, in which the lung outgrows and branches into the surrounding mesenchyme: this process is known as branching morphogenesis. Branching morphogenesis enables the formation of a highly branched bronchial tree, which is essential for maximizing the surface area needed for efficient gas exchange (Swarr and Morrisey 2015). The branching morphogenesis is almost conserved across different animal species and can be simplified into three cyclical steps: the formation of smaller branches from existing ones, then the outward expansion of the tips of these new branches, and finally, their bifurcation (Metzger et al. 2008).

The process of branching morphogenesis is meticulously controlled by a complex interplay of multiple signaling pathways. Some pathways are stimulatory, enhancing branching, while others are inhibitory, providing feedback mechanisms to prevent excessive growth. The Fgf10 signaling pathway, expressed in the mesoderm and acting on its receptors Fgfr1 and Fgfr2 in the epithelium, remains the primary mechanism driving lung development at this stage (Swarr and Morrisey 2015; Herriges and Morrisey 2014; Min et al. 1998). Loss of the Fgf pathway results in complete disruption of the branching process (Ohuchi et al. 2000; Sekine et al. 1999). The expression levels of Fgf genes are finely modulated by complex interactions between numerous signaling pathways, including retinoic acid (RA), Sonic hedgehog (Shh), Wnt, and BMP (Burns et al. 2004; Weaver et al. 1999; Park et al. 1998) (*Fig.7a*). The role of the Shh pathway in regulating Fgf signaling is intricate and dualistic: it downregulates Fgf10 expression in the distal epithelium (Lebeche et al. 1999), while at the very tip of the bud, it promotes an increase in Fgf expression (Chuang et al. 2003). Additionally, Wnt signaling is involved in regulating the branching process. The mesenchyme secretes many Wnt ligands, which activate the canonical Wnt pathway, leading to the nuclear localization of β-catenin, observed mostly in the distal epithelium (De Langhe et al. 2005; Okubo and Hogan 2004) (*Fig.7b*). Loss of this pathway at this stage results in impaired branching morphogenesis and reduced

epithelial differentiation (Shu et al. 2005; Mucenski et al. 2003). Furthermore, it is now clear that the interactions between the growing epithelium and the underlying ECM are crucial in regulating the branching phenomenon (Sakai et al. 2003). Some matrix proteins, such as fibronectin, accumulate at points where the branching starts, suggesting their importance in the process. It is well established that fibronectin interacts with cellular receptors, primarily the β1-integrin family. Recent research has shown that the loss of β1-integrin results in alterations to epithelial morphology, disruption of branching morphogenesis, and a loss of apical-basal cell polarity (Chen and Krasnow 2012).

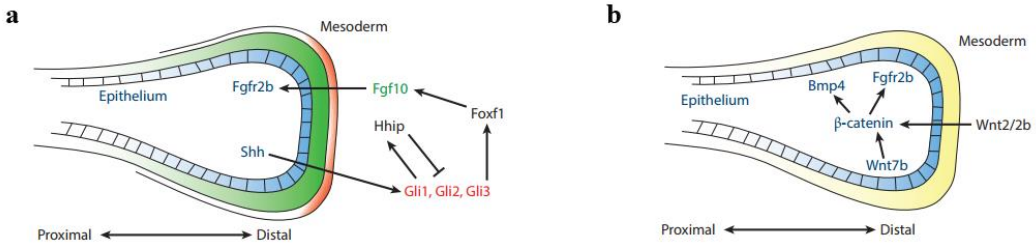

Fig.7 Fundamental signaling interactions for branching morphogenesis. *a) Signaling crosstalk in branching morphogenesis. b) Focus on the Wnt signaling in outgrowth and differentiation of lung epithelium (Swarr and Morrisey 2015).*

Around E12.5 in mice, coinciding with the period of lung branching, the lung endoderm begins to differentiate into distinct cell lineages along the proximal-distal axis. The Nkx2.1-positive endoderm generates two different progenitors, the $Sox2^{+}$ proximal progenitors and the $Sox9^{+}/Id2^{+}$ distal progenitors. These progenitor populations present distinct fates and are responsible for the development of all the epithelial cell types within the respiratory system (He et al. 2022; Swarr and Morrisey 2015). The Notch signaling pathway plays a key role in this differentiation process, enabling a proper balance between proximal and distal progenitor cells and maintaining the correct equilibrium among the various differentiated cell lineages derived from the proximal progenitors. Early in development, Notch inhibition causes a bias toward distal progenitor cells. Later in development, loss of Notch signaling leads to an increase in proximal progenitors (Tsao et al. 2009).

As shown in *Figure 8*, the proximal progenitors produce ciliated, secretory, airway neuroendocrine, mucosal, and basal cells. The leakage of Sox2 expression results in the loss of all these type cells.

The distal progenitors generate the alveolar epithelial lineages, AEC I and AEC II (Chao et al. 2016; Swarr and Morrisey 2015; Herriges and Morrisey 2014). Studies involving lineage tracing of $Sox9^{+}/Id2^{+}$ cells have demonstrated that, until E13.5 in mice, this population exhibits multipotency, capable of differentiating into both alveolar and airway epithelial cells. Post E13.5, approximately between E16.5 and E18.5, their differentiation potential becomes restricted to alveolar cell lineages (Hogan et al. 2014). To date, the molecular pathways that drive the formation of distal progenitors are poorly understood, even though it has been demonstrated that blocking both the Wnt and Bmp signaling causes a complete loss of this cell population (Mucenski et al. 2003). Moreover, the immature AEC II expresses EGF, which regulates their proliferation and differentiation (Warburton et al. 2000; Patel et al. 2000; Miettinen et al. 1997). Therefore, it is speculated that EGFR signaling may regulate communication between epithelial and mesenchymal cells to produce surfactant (Schmiedl et al. 2020; Kumar 2004). Late in gestation, EGFR inactivation leads to immaturity of AEC II, which can be observed by an increase in glycogen content and a decrease in lamellar bodies (Kumar 2004).

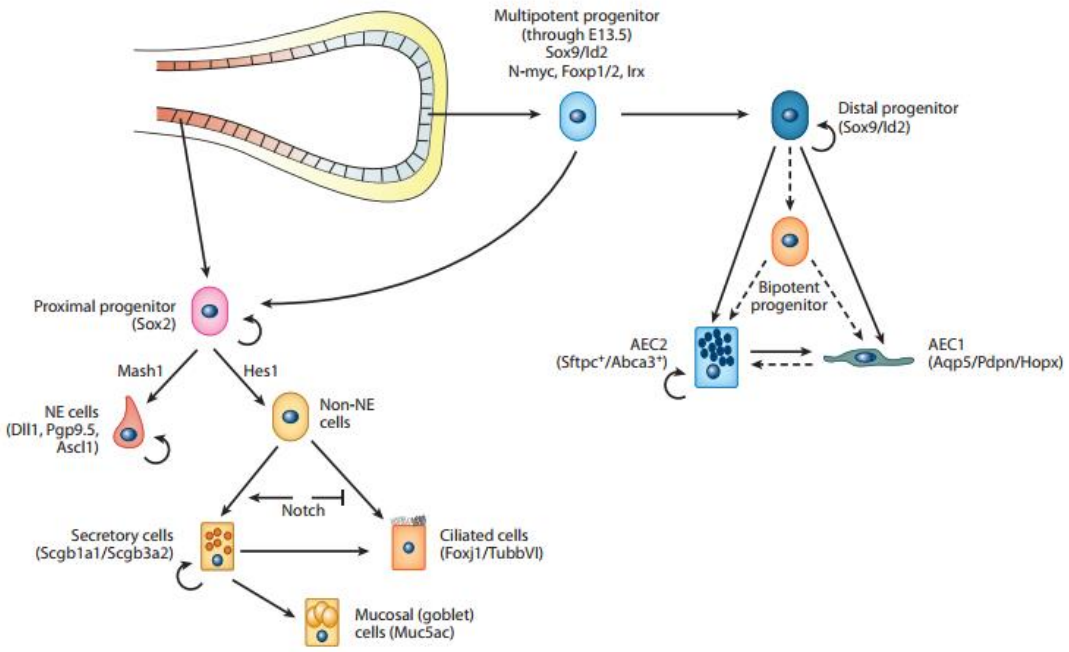

Fig.8 Endoderm progenitors in lung development. *Schematic representation of the cell fate of both proximal and distal cell progenitors of the lung epithelium. In brackets, the specific cellular markers for each subpopulation are highlighted (Swarr and Morrisey 2015).*

1.3.3 The canalicular stage (16-26 weeks of gestation)

At E16.5 in mice, a transition occurs in lung development, from a phase of branching morphogenesis to the initial stage of alveolar differentiation, which later leads to alveolarization (Morrisey and Hogan 2010). This intermediate stage may be because the genetic programs that drive the branching morphogenesis and the alveolarization are entirely different and cannot proceed simultaneously. In these weeks, the morphological distinction between conducting and respiratory airways is achieved (Schittny 2017), with the conducting airways of the respiratory system completely formed by 16-18 weeks of gestation (Groenman et al. 2005). At this point, the terminal bronchioles begin to branch into respiratory bronchioles. Each of these respiratory bronchioles leads to dilated terminal sacs (future primary alveoli) (Ahlfeld and Conway 2012).

The canalicular phase is characterized by an increase in the proliferation of the alveolar epithelial cells and the rapid expansion of the capillary network (Ahlfeld and Conway 2012). When the lung buds form from the anterior foregut, a vascular plexus, consisting of arteries, veins, and microvasculature, is present. Lung and vascular development proceed alongside (Woik and Kroll 2015; deMello et al. 1997). A significant increase in lung capillaries occurs during the canalicular period. They begin to be in close contact with the epithelium of the terminal sacs, generating the first, still immature, air-blood barrier (Stenmark and Abman 2005). The surrounding ECM results in elastin and poorly defined mesenchymal cell types (Morrisey and Hogan 2010). In contrast, this phase is characterized by the differentiation of the cuboidal epithelial cells of the future alveolar ducts into AEC I and AEC II. In humans, this is the period when surfactant can first be observed (around 22-24 weeks of gestation).

1.3.4 The saccular stage (26-36 weeks of gestation)

The saccular phase is essential in preparing the fetus for extrauterine life (Berger and Bhandari 2014). The terminal sacs formed at the end of each respiratory bronchiole continue to expand during a process called sacculation. These acini form clusters, and they keep growing, forming the so-called sacculi. Babies born before 28 weeks of gestation are at the highest risk of developing BPD, suggesting that any alteration at this organogenesis stage can be responsible for BPD pathogenesis (Ahlfeld and

Conway 2012). The walls of these forming sacculi are called primary septa and are close to the expanding vascular capillary network (Swarr and Morrisey 2015). Capillaries form a bilayer all around the epithelium of the alveolar septa (Stenmark and Abman 2005). The thinning of the mesenchyme decreases the distance between the blood vessels and the alveolar epithelial surface, thus preparing this area for gas exchange (Chao et al. 2016). MYFs arrived and started to produce and secrete elastic fiber and collagen fibrils, necessary for the subsequent alveolarization phase.

Between the end of the saccular and the onset of the alveolar stage, orchestrated changes in mesenchymal organization and differentiation are observed. The mesoderm-derived signals, such as Fgf10 and Wnt2, are essential in driving lung development of each phase, including mesenchymal cells specification (Goss et al. 2009; Weaver et al. 1999). Thus, the constant crosstalk between endoderm and mesoderm is essential for proper organogenesis. The mature lung contains many mesodermal derivatives, such as endothelial and mesothelial cells, vascular smooth muscle and airway smooth muscle cells, pericytes, alveolar fibroblast and LIFs, lymphatic cells (Chao et al. 2016; Herriges and Morrisey 2014). To date, the origins of many of these cell types remain poorly understood. A published study demonstrated that the majority of lung mesodermal cell types originate from $Wnt2^{+}$ cardiopulmonary progenitors (CPPs), which play a crucial role not only in cardiac development but also in certain aspects of lung formation (Herriges and Morrisey 2014). It should be noted that CPPs do not produce most of the endothelium, which appears to be derived from VE-$cadherin^{+}$ endothelial cells. The Hedgehog, Shh, and Bmp pathways seem to play a role in regulating the mesoderm derivatives (*Fig. 9*) (Herriges and Morrisey 2014).

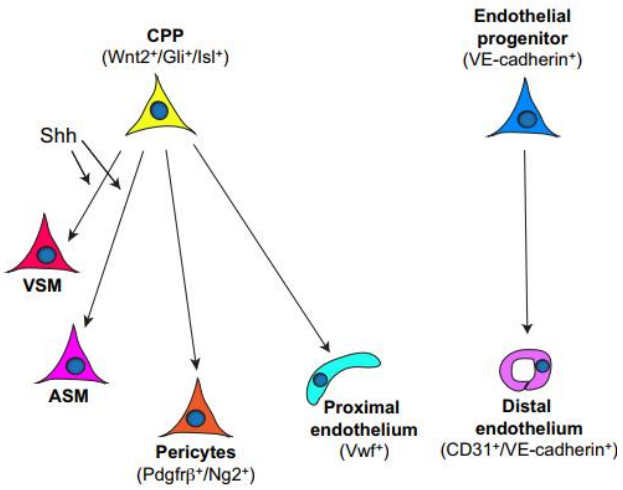

Fig.9 Mesenchymal progenitors in lung development. *Schematic representation of the cell fate of mesenchymal cell progenitors of the lungs. In brackets, the specific cellular markers for each subpopulation are shown (Herriges and Morrisey 2014).*

1.3.5 The alveolar stage (36th week of gestation – adolescence)

The alveolar stage is a process that happens in mice after birth (P0-P21), while in humans, it begins before birth and continues into early adulthood (Morrisey and Hogan 2010). Alveologenesis is the last stage of respiratory system development and has two phases: classical alveologenesis and continued alveologenesis (Rippa et al. 2021; Schittny 2017). This period is most commonly affected when lung diseases develop (Branchfield et al. 2016).

At the end of the saccular stage, the lung parenchyma still features immature, thick septa containing a double layer of capillaries (*Fig. 10a*). The classical alveologenesis phase is characterized by the formation of secondary septa from primary ones, which initially increase the alveolar surface area (Silva et al. 2015; Greer et al. 2014), subdividing the airspace into smaller regions called alveoli (J. Li et al. 2016). Septa formation requires coordination among several cell types: AEC I and AEC II must work together with other mesenchymal cells, mainly alveolar MYFs and LIFs (Nelson and Kugler 2024; Branchfield et al. 2016). Alveolar MYFs are contractile fibroblasts that express α-SMA and, due to their origin, Platelet-derived growth factor receptor alpha (PDGFRα) (Vila Ellis and Chen 2021). In contrast, LIFs are characterized by the expression of ADRP and contain lipid droplets in their cytoplasm, where they store neutral lipids because of their role in surfactant production (Torday and Rehan 2016; Tahedl et al. 2014; Rehan et al. 2006). During classical alveologenesis, alveolar MYFs are located at the very top of the primary septa (Li et al. 2015). They actively participate in secondary septa formation and receive stimuli from AEC I (Rippa et al. 2021). Specifically, their contractile activity is thought to drive alveoli formation (R. Li et al. 2020), and they are regarded as the primary producers of elastin (Branchfield et al. 2016; Boström et al. 1996), which ensures septal elongation and provides structural support (Rippa et al. 2021). Conversely, LIFs are located at the base of the developing secondary septa, are less associated with elastic fibers, and exhibit some morphological differences compared to MYFs, such as fewer organelles and a higher content of lipid droplets. These characteristics support their functions, mainly

surfactant production and cytoprotection against oxidative stress (Torday et al. 2001). Over recent years, a major role for ECM in the alveolarization process has been recognized, as ECM plays a vital role not only during lung development and the maintenance of normal lung function but also in lung repair after damage (Rippa et al. 2021). Collagen types I and III fibers are stiff and strong, providing structural integrity and tensile strength to the lung (Suki et al. 2005). Moreover, these fibers are crucial as load-bearing elements for alveolar walls and ducts (Rippa et al. 2021). On the other hand, elastic fibers, primarily produced by alveolar MYFs, are essential for maintaining lung elasticity (Ozsvar et al. 2021; Branchfield et al. 2016) and conferring tensile strength to the tissue (Suki et al. 2005). During septation, elastic fiber formation peaks (Rippa et al. 2021). Studies in mouse models have shown that postnatal inactivation of MYFs correlates with decreased elastic fiber production, leading to alveolarization arrest (Li et al. 2019). Additionally, PDGF-A null mice exhibit a complete absence of alveolar MYFs and elastic fibers (Boström et al. 1996). These authors also observed delayed terminal airway development, suggesting that elastin plays a role not only in alveolarization but also in the final phase of branching (Goodwin et al. 2019; H. Y. Kim et al. 2015). Other animal models, where MYFs failed to reach the tips of secondary septa and instead remained trapped in the surrounding parenchyma, showed abnormal and disorganized elastic fiber production with aberrant distribution, resulting in failed alveolarization (Hrycaj et al. 2018; Mandeville et al. 2006). These findings are similar to those observed in lung samples from patients diagnosed with BPD (Li et al. 2019).

Since the secondary septa contain a double-layered capillary network, gas exchange remains inefficient. It is during the terminal stages of classical alveologenesis that microvascular maturation initiates. Complete maturation is observed during the second phase of alveologenesis, known as continued alveologenesis. Throughout this maturation process, septa thinning is observed, accompanied by remodeling that involves the fusion of the two capillary layers into a single layer. Additionally, a notable reduction in interstitial tissue volume and apoptosis of MYFs is noted (Schittny 2017; Warburton et al. 2010). ECM maturation is also observed: remaining MYFs express reduced levels of α-SMA, and elastin fibers begin to form a complex, organized network (Branchfield et al., 2016) (*Fig. 10b*).

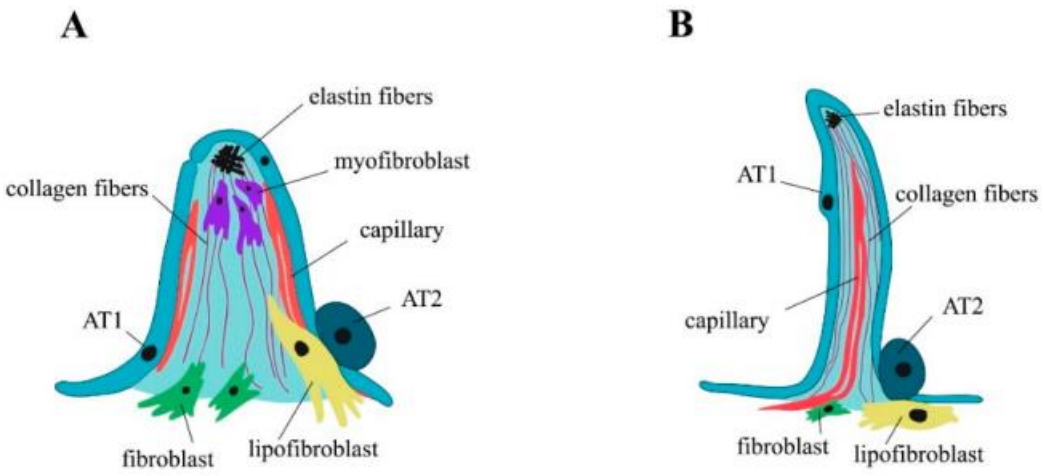

Fig.10 Secondary septa formation. *a) During the classical phase of alveologenesis, it is possible to observe thick, immature secondary septa characterized by the presence of a double capillary network. b) Throughout the continued alveolarization, thin mature secondary septa are present, with singular strand capillaries (Rippa et al. 2021)*

Since the formation of new alveoli requires a double-layered capillary network, it was postulated that alveoli cannot be formed after vascular maturation (Zeltner and Burri 1987; Burri 1975). However, emerging evidence over the years has shown that alveolarization can occur even after this process has concluded (Tschanz et al. 2014; Butler et al. 2012; Coxson et al. 2004; Massaro and Massaro 1996). It has been demonstrated that alveolarization continues until adolescence, where the newly formed septa initially present a bilayer capillary structure that undergoes maturation in a second stage (Rippa et al. 2021; Schittny 2017).

1.4 Signaling pathway alterations in BPD

From the perspective of the numerous risk factors predisposing to BPD, it is well known that more than one signaling pathway is altered in this disease. Furthermore, plenty of them interrelate, generating a complex framework that is difficult to tease apart into individual components.

1.4.1 ROS

In 1988, a new scientific theory emerged suggesting that during oxygen supplementation, ROS are generated at a rate exceeding the antioxidant capacity of premature newborns. This imbalance can make multiple organs susceptible to damage. Depending on the primary target, clinical outcomes may vary, with infants who can

develop BPD, ROP, or NEC, which, according to the referenced definition, likely share a common pathogenic mechanism. In preterm newborns, this harmful situation is exacerbated by the still immature antioxidant system, which usually develops during the third trimester of gestation, with consequent oxidative lung injury (Giusto et al. 2021; Perez et al. 2019). The main consequence of oxidative stress is the activation of the inflammatory system, leading to the recruitment of many mediators and the production of high levels of pro-inflammatory cytokines, resulting in exponential amplification of the inflammatory cascade. In addition, pro-inflammatory cytokines stimulate the upregulation of inducible nitric oxide synthase (iNOS), facilitating the production of NO. NO interacts with ROS, particularly superoxide radicals, resulting in the formation of a toxic metabolite that can cause tissue damage and impair surfactant function (Perrone et al. 2012). More generally, high ROS levels result in epithelial and endothelial cell death, pulmonary edema, impairment of mitochondrial function, and a decrease in lung resident and circulating progenitor cells (Alvira and Morty 2017). This scenario translates into an impairment of the normal septation and alveolar process, vascular leak, failure of the alveolar-capillary barrier, and breaking of the nuclear membrane with consequent DNA damage (Lius and Syafaah 2022; Sahni and Bhandari 2021; Shahzad et al. 2016). All these outcomes, in turn, trigger an inflammatory response that amply the damage, worsening the frame by attracting other macrophages and neutrophils (Lius and Syafaah 2022).

1.4.2 Vascular Endothelial Growth Factor (VEGF) and Angiopoietins (Ang)

One of the outcomes of the inflammatory process is an imbalance between pro- and anti-angiogenic factors, with consequent disruption of angiogenesis and vasculogenesis (Mathew 2020). Among the numerous vascular markers involved, Vascular Endothelial Growth Factor (VEGF) and its receptor (VEGF-R2) for the vasculogenesis, and Angiopoietins-1 (Ang-1) and Ang-2 for the angiogenesis processes are the pathways generally altered in BPD (Mathew 2020). VEGF is a powerful vascular mitogen marker physiologically expressed by the alveolar epithelium. Its expression peaks during the alveolarization phase, where it plays a crucial role in enabling vascular and parenchymal maturation and promoting surfactant production (Ahlfeld and Conway 2012). Moreover, aligned with the vascular

hypothesis of lung development, endothelial cell signaling plays a critical role in alveolar formation. This is consistent with experimental findings showing that administration of VEGF antagonists in an animal model of BPD leads to impaired alveologenesis (Oak and Hilgendorff 2017). Other studies carried out on animal models of BPD showed a reduction in lung VEGF levels and in VEGF-R2 on endothelial cells (Alvira and Morty 2017) and that VEGF gene therapy can improve animal survival by increasing the expression of alveolar Epithelial Nitric Oxide Synthase (eNOS), thus suggesting that, at least partially, these effects are NO-mediated (Mathew 2020). However, it is essential to highlight that VEGF levels must be precisely regulated for proper alveologenesis. Indeed, even elevated VEGF levels can result in alterations in morphology and the number of alveoli. It is known that the overexpression of VEGF in newborn mice is associated with a pathological phenotype, with simplified lung structures and a reduced area for gas exchange. These observations are in line with the failure of VEGF-therapy in completely reverting the damage and highlighted the necessity for a tight regulation of this molecular pathway (Oak and Hilgendorff 2017). Ang-1 is an angiogenic factor that binds to its Tyrosine Kinase Receptor, Tie2. This interaction promotes chemotactic and mitogenic effects on endothelial cells, inhibits apoptosis, and enhances the action of adhesion molecules at intercellular junctions. Additionally, Ang-1 exerts anti-inflammatory effects by suppressing pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Nuclear Factor κ-light-chain-enhancer of activated B cells (NF-κB) (Mathew 2020). Stated differently, Ang-1 stabilizes blood vessels and exerts a key role in the maintenance of the integrity of the mature vascular network. Furthermore, during lung organogenesis, Ang-1, along with other mediators such as VEGF, mediates vascular development (Mathew 2020; Stark et al. 2018). On the other hand, Ang-2, by interacting with Tie2, causes disruption at the endothelial cell level, counteracting Ang-1 actions (Stark et al. 2018). An imbalance between these two factors contributes to changes in the development of the alveolar vasculature and is a common feature of BPD (Mathew 2020).

1.4.3 Transforming Growth Factor-β (TGF-β)

Transforming Growth Factor-β is a superfamily encompassing TGF-β and Bmp factors, with the primary task of regulating cell survival, differentiation, and ECM

deposition. TGF-β consists of three ligands, TGF-β1, TGF-β2, and TGF-β3. All of them are present in the bronchiole subepithelial region during the late canalicular and saccular phases (Ahlfeld and Conway 2012). Moreover, they are localized in the alveolar area right after birth (Ahlfeld and Conway 2012). Although TGF-β expression is essential for lung organogenesis, high levels of this factor during alveologenesis are associated with the development of BDP in animal models. Preterm exposure to high oxygen concentrations upregulates TGF-β/Bmp signaling, leading to disruption of septation and alveologenesis, typical histopathological signs of BPD (Mathew 2020). Inflammation is also known as a trigger factor for the overexpression of TGF-β pathway (Oak and Hilgendorff 2017). Elevated levels of this molecular signaling result in an increase in the Wnt pathway, a decrease in PPAR-γ signaling, and remodeling of the ECM. This remodeling subsequently enhances the TGF-β pathway, creating a positive feedback loop. Upregulation of TGF-β is associated with impaired branching morphogenesis, altered cellular composition and differentiation, ongoing ECM remodeling, and increased transdifferentiation of LIFs into MYFs (Lecarpentier et al. 2019; Oak and Hilgendorff 2017).

1.4.4 Wingless/Int (Wnt) pathway and β-catenin

The Wingless/Int (Wnt) family is composed of 19 highly conserved glycoproteins that act as ligands mediating cascade signaling. These ligands can activate two different pathways: the canonical one, through a signaling that involves Frizzled receptors and β-catenin, or the non-canonical one, which is β-catenin independent (Moon et al. 2004). Considering the well-characterized canonical Wnt signaling pathway, it was observed that in the absence of Wnt ligands, the formation of a degradation complex promotes the phosphorylation of β-catenin, leading to its subsequent degradation (Angers and Moon 2009). When the binding between Wnt ligand and the Frizzled receptors occurs, the complex is degraded, and the β-catenin accumulates in the cytosol. Subsequently, β-catenin translocates into the nucleus, where it can exert its functions as a transcription factor, regulating nuclear transcription and cellular migration and adhesion (Angers and Moon 2009; Douglas et al. 2006; Moon et al. 2004). This signaling pathway plays a crucial role during lung development, in addition to maintaining lung homeostasis and repairing tissue damage. It is vital for the self-renewal and differentiation of progenitor cells, proper branching

morphogenesis, and the formation of peripheral airways. Notably, the deletion of β-catenin from the respiratory epithelium during the embryonic stage leads to unsuccessful lung morphogenesis (Li et al. 2020; Mathew 2020). Several studies have demonstrated the presence of canonical Wnt pathway components in the alveolar epithelium starting at the 7th week of gestation. Their expression peaks around the 17th week and then gradually declines after the 21st week. Various harmful events, such as pulmonary acute or chronic injury and inflammation, are known to impede the typical decrease in Wnt ligand expression (Lecarpentier et al. 2019). This phenomenon results in persistently elevated levels of these members even beyond the 21st week of gestation, leading to enhanced activation of the signaling pathway during both the saccular and alveolar developmental stages. Consequently, it causes alterations in lung development characterized by abnormal epithelial proliferation, fibrotic repair mechanisms, vascular leakage, ECM remodeling mediated by Connective Tissue Growth Factor (CTGF) and fibronectin—two downstream mediators of β-catenin signaling—impaired alveolarization, thickening of septa, and a metabolic shift toward aerobic glycolysis, commonly referred to as the Warburg effect in cancer biology (Mathew 2020; Lecarpentier et al. 2019).

1.4.5 Epidermal Growth Factor Receptor (EGFR)

Like many other pathways, EGFR signaling is involved not only during organogenesis but also reactivates in the case of pulmonary damage. EGFR is a receptor expressed in the airway epithelium of the lung during organogenesis and persists in adult life (Kumar 2004). In lung development, EGFR signaling plays a pivotal role in driving branching morphogenesis and both epithelial and mesenchymal cell differentiation (Kumar 2004). Its loss correlates with a decrease in surfactant production and interrupted alveolarization, lack of cell adhesion, and increased migration, which are typical features of BPD (Finigan et al. 2012; Li et al. 2012). Following pulmonary injury, its aberrant reactivation is associated with increased fibrosis and massive deposition of ECM components (Vallath et al. 2014; Kumar 2004). Hyperoxia exposure, a critical trigger for BPD onset, and the resulting ROS generation are known to alter numerous signaling pathways, including the EGFR (J. Li et al. 2016). Indeed, in both *in vitro* and *in vivo* experiments conducted using hyperoxia models of BPD, an increase in EGFR levels was observed, which was associated with impaired migration

of MYFs toward secondary septae tips. This strongly suggests that the EGFR pathway plays a role in the pathogenesis of BPD (Li et al. 2024; J. Li et al. 2016).

1.5 Cellular modifications in BPD

Tissue injury initiates a complex inflammatory response characterized by the recruitment, proliferation, and activation of various cell types, including neutrophils, macrophages, natural killer (NK) cells, MYFs, LIFs, and endothelial cells (Wynn and Vannella 2016). Together, these populations organized tissue repair (Di Carlo and Peduto 2018). Physiologically, tissue repair is a well-orchestrated process, and the damage is typically resolved quickly (Wynn and Vannella 2016). However, if the harmful stimulus continues, the healing response becomes dysregulated, and the process can develop towards pathological fibrosis or scarring, favoring BPD progression (Wang and Tsao 2020). The key players in this deranged injury resolution are MYFs, LIFs, macrophages, and monocytes.

1.5.1 MYFs and LIFs alterations

When tissue damage occurs, a physiological repair process begins. MYFs activate, producing matrix components, such as collagen, elastin, non-structural proteins, pro-inflammatory cytokines, chemokines, and growth factors. Moreover, thanks to their phenotype, they can generate contractile forces that help the healing process. Once the injury is resolved, MYFs undergo apoptosis. Failure in these physiological processes—characterized by the persistence of MYFs, a hallmark of BPD and other chronic pulmonary conditions—precipitates the development of pathological fibrosis and scarring. This progression is further associated with heightened inflammatory responses and an excessive accumulation of ECM components (Di Carlo and Peduto 2018). In BPD, alongside ECM remodeling, the abnormal presence of MYFs is associated with disrupted mesenchymal-epithelial communication (Torday and Rehan 2016). Specifically, hyperoxia-exposure exacerbated the transdifferentiation of LIFs into MYFs, which is also known to occur spontaneously (Boros et al. 2002). This means that LIFs rapidly lose their lipogenic phenotype in favor of a myogenic one. MYFs, unlike LIFs, are unable to maintain pulmonary epithelial cell growth and differentiation, resulting in failed alveolarization. They also cannot sustain AEC II for surfactant production (Torday and Rehan 2016), and cannot provide adequate

antioxidant protection (Boros et al. 2002). Even if physiologically LIFs can differentiate into MYFs, it has been shown that hyperoxia speeds up this transdifferentiation process (Torday et al. 2001). Rehan and colleagues elucidated the process underlying this increased conversion (Rehan and Torday 2003). The suggested mechanism indicated that hyperoxia disrupts PTHrP signaling. PTHrP, produced by AEC II, was identified as a key factor in mediating paracrine signals between developing lung fibroblasts and AEC II. Specifically, PTHrP causes fibroblasts to differentiate into LIFs, which then support alveolar health by providing substrate for surfactant production (Torday and Rehan 2002). Exposure to hyperoxia leads to a reduction in PTHrP expression (Rehan and Torday 2003). Since, under normoxic conditions, PTHrP promotes the expression of lipogenic phenotypes in fibroblasts, the researchers examined the levels of lipogenic markers such as ADRP and leptin in hyperoxia and found these to be significantly decreased (Torday et al. 2003). Given the role of ADRP in the uptake, storage, and transfer of TGs for surfactant production, the hyperoxia-mediated reduction of this marker correlates with impaired function of AEC II (Rehan and Torday 2003). Furthermore, hyperoxia impairs the migratory ability of MYFs, which fail to reach the tips of the developing secondary septa. They remain trapped within the parenchyma, making it impossible for the correct formation of secondary septa (Branchfield et al. 2016). Therefore, preterm exposure to high oxygen content may reduce the expression of PTHrP, favoring LIF transdifferentiation to MYF with consequent failure in alveolarization and the onset of BPD (Lecarpentier et al. 2019; Rehan and Torday 2014; Rehan et al. 2006).

1.5.2 Inflammatory cells

Excessive or prolonged inflammation can contribute to impaired lung development, increasing the risk of BPD (Sun et al. 2019). Furthermore, premature infants have an immature immune system characterized by fewer neutrophils and monocytes in the cord blood, which raises their susceptibility to infections (Basha et al. 2014; Palmeira et al. 2012). In utero, exposure to foreign antigens is limited; newborn defense relies mainly on innate immunity rather than adaptive immunity (Yu et al. 2018; Levy 2007; Newburg and Walker 2007). Circulating and resident myeloid immune cells, including monocytes and neutrophils, are the main players of innate immune response and can initiate inflammation by releasing cytokines and chemokines (Sokol and Luster 2015).

After lung epithelial injury and the release of pro-inflammatory cytokines, circulating neutrophils and monocytes— which quickly differentiate into macrophages—migrate into lung tissue (Qing et al. 2022; Heydarian et al. 2021; Misharin et al. 2017). BPD has been linked to higher levels of specific cytokines associated with macrophage activation, such as monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein (MIP), along with lower levels of anti-inflammatory cytokines, such as IL-10 (Sun et al. 2019; Köksal et al. 2012; Bose et al. 2008; Baier et al. 2004). Additionally, preterm infants exhibit impaired neutrophil function, marked by low expression of granulocyte colony-stimulating factor (G-CSF) (Liechty 1993).

1.5.2.1 Neutrophils in BPD

Neutrophils play a fundamental role in acute lung inflammation (Heydarian et al. 2022). They are produced in the bone marrow and released into the bloodstream, where they mediate the first line of immune defense, being recruited to the site of injury (Prame Kumar et al. 2018). Through phagocytosis and by releasing proteases and ROS, neutrophils eliminate pathogens (Nauseef and Borregaard 2014). In preterm newborns, the immature lungs are exposed to high oxygen levels, which promotes a ROS-dependent neutrophilic inflammatory response (Yildiz et al. 2015; Wen et al. 2013; Turunen et al. 2006) that, in turn, causes neutrophil activation and increased ROS production (Thomas et al. 2022; Wang and Dong 2018). Other neutrophil-mediated mechanisms may also facilitate BPD progression: upon activation, neutrophils promote the proliferation of smooth muscle cells, thereby enhancing ECM remodeling (Genschmer et al. 2019; Vargas et al. 2016; Yi et al. 2004). Furthermore, the neutrophil-mediated release of elastases and metalloproteinases increased ECM degradation, further contributing to BPD progression (Zhu et al. 2021; Xu et al. 2020).

1.5.2.2 Monocytes in BPD

Macrophages are the first immune sentinels, regulating the balance between immune cell defense against pathogens and tolerance to innocuous stimuli. Thus, they are indispensable in providing tissue homeostasis and repair (Puttur et al. 2019). In response to lung damage, circulating monocytes migrate into the alveolar space, resulting in an increase in pulmonary injury and remodeling (McQuattie-Pimentel et al. 2018). Indeed, recruitment of monocytes and monocyte-derived alveolar macrophages into the lungs is a typical feature of BPD (Kalymbetova et al. 2018). Macrophages can exist as M1 or M2 phenotypes, even if this does not represent a

discrete population; instead, they can evolve within the two phenotypes as the injury progresses (Hesketh et al. 2017). Pathological conditions favor macrophage activation toward the M1 phenotype: in this state, they are highly phagocytic and release pro-inflammatory cytokines and mediators that promote a localized inflammatory response (Wang and Tsao 2020; Hesketh et al. 2017). Subsequently, the activation shifts toward the M2 phenotype, which is typically anti-inflammatory. The local environment may influence this differentiation (Hesketh et al. 2017). Disturbance at any level in this process can determine an aberrant repair mechanism (Wynn and Vannella 2016). Despite the potential contribution to resolve inflammation, macrophage migration and activation tend to become chronic, leading to the development of BPD (Heydarian et al. 2022). The exact contribution of macrophages in the pathogenesis of BPD has not been identified yet. Still, it is clear that ROS production following preterm infant exposure to hyperoxia results in monocyte recruitment and macrophage differentiation (L. Li et al. 2016).

1.6 ECM modifications

Considering the role of ECM in promoting lung development (El Agha and Thannickal 2023; Thibeault, Mabry, et al. 2003), it is unsurprising that several lung disorders related to impaired organogenesis feature disrupted ECM (Mižíková and Morty 2015). In neonates with BPD, disorganized, tortuous, and thickened collagen fibers are identified (Thibeault, Mabry, et al. 2003; Thibeault et al. 2000). Moreover, in the parenchyma (Margraf et al. 1991; Nakamura et al. 1990) and in the vascular network (Thibeault, Truog, et al. 2003), elastin fibers presented an aberrant structure. These observations highlighted that ECM plays a pivotal role in achieving proper lung development (Mižíková and Morty 2015). Consistently, several animal models of BPD showed abnormalities in the collagen network, failing to correctly mature the gas-exchanging area (Kumarasamy et al. 2009; Thibeault, Mabry, et al. 2003; Kononov et al. 2001). In these models, collagen production is higher, and its fibers are thicker compared to physiological conditions, resulting in increased tissue rigidity (Zhou et al. 2018; Mižíková et al. 2015). These data are consistent with analyses performed on samples from patients, in which an increased number of collagen-positive cells and collagen fibers were observed (Kaarteenaho-Wiik et al. 2004; Shoemaker et al. 1984).

Apart from disorganization and thickness of collagen fibers, damage to the collagen network was ascertained in newborns diagnosed with BPD and treated with mechanical ventilation (Thibeault, Mabry, et al. 2003). The proposed mechanism suggests that positive-pressure ventilation induces alveolar expansion, which subsequently exerts mechanical stress on the adjacent ECM. This stress may disrupt the integrity of the collagen and elastin framework, thereby impairing the normal septation process necessary for alveolar development (Mižíková and Morty 2015). In mouse models, it has been observed that hyperoxia exposure is responsible for the increase in total lung collagen protein levels (Mižíková et al. 2015). Collectively, this data highlights that dysregulated collagen expression is involved in BPD (Mižíková and Morty 2015). Regarding elastin, its expression levels are tightly controlled during alveolarization, peaking during secondary septation and then rapidly decreasing once the process is complete (Richard A. Pierce et al. 2007; Mariani et al. 2002). The trigger for elastin expression, which is abnormally organized, characterizes pathological conditions. Aberrant elastin fibers have been described in the lung parenchyma of premature ventilated newborns (Bruce et al. 1992). The same disorganized structure was observed in animal models of BPD, where elastin's physiological localization is lost and instead appears disorganized in the walls of the developing septa (Mascaretti et al. 2009; Richard A Pierce et al. 2007; Bourbon et al. 2005; Albertine et al. 1999). This abnormal arrangement leads to impaired alveolarization, alveolar branching arrest and enlarged airspace with consequent reduction of the surface area available for gas exchange (Zhou et al. 2018; Oak and Hilgendorff 2017; Ahlfeld and Conway 2012). The pivotal crosstalk and balance between collagen and elastin, which physiologically provide stability to the ECM, are disrupted in both patients diagnosed with BPD and animal models of the disease (Mižíková and Morty 2015). Inappropriate ECM remodeling and vascular rearrangements are the main outcomes of this altered equilibrium, favoring the development of BPD (Mathew 2020).

1.7 Current therapeutic strategies

The most effective strategy to prevent BPD involves avoiding extremely premature birth, but due to its multifactorial etiology, treatment options are limited available. To date, the current therapy for BPD is limited to moderately active drugs such as steroids, surfactant, vitamin A, caffeine, and diuretics, which have been shown to be associated

with severe long-term neurodevelopmental adverse effects (Omar et al. 2022). Recognizing more viable treatment strategies to enhance pulmonary function and mitigate complications associated with BPD is crucial for elevating the quality of life in preterm infants. The constraints inherent in pharmacological interventions for BPD have catalyzed the exploration of alternative therapeutic modalities.

1.7.1 Corticosteroid therapy

Antenatal steroid therapy is a major advancement in perinatal care, leading to decreases in mortality, respiratory distress syndrome, intraventricular hemorrhage, and NEC. However, it does not lower the rates of BPD (Roberts et al. 2017).

Postnatal steroid therapy during the first two weeks of life has been used to reduce the risk of BPD and to facilitate early extubation in preterm newborns (Thébaud et al. 2019). Despite these positive outcomes, the use of corticosteroids is highly debated due to its association with plenty of severe adverse effects, such as hyperglycemia, hypertension, cardiomyopathies, growth failure, gut complications, increased probability of developing ROP, and neurodevelopmental delays (Principi et al. 2018; Jobe 2016). To address these limitations and enhance the risk/benefit profile of steroids, inhaled or intratracheal administration has been proposed as an alternative to systemic delivery, showing significant improvements (Hwang and Rehan 2018). Although promising, there remains uncertainty regarding the optimal dosage, and further testing in larger, multicenter studies is necessary.

1.7.2 Surfactant therapy

Exogenous surfactant therapy reduces the requirement for mechanical ventilation and oxygen supplementation and ameliorates the survival of preterm infants. Traditionally, surfactant has been administered using an endotracheal tube. Research indicates that extremely preterm infants often experience ongoing surfactant dysfunction, prompting exploration of late or repeated surfactant treatments. While the TOLSURF trial found no significant difference in 36-week survival rates between infants without BPD who received late surfactant and those who did not, there was a noticeable trend toward better outcomes at 40 weeks and a marked reduction in infants needing home

respiratory support within the first year (Keller et al. 2017; Ballard et al. 2016). However, there is no evidence of a reduction in the incidence of BPD (Hwang and Rehan 2018).

1.7.3 Vitamin A

Vitamin A, physiologically stored in the septal cells of alveoli, was found to be reduced in infants diagnosed with BPD. This deficiency is accompanied by disruption of epithelial cell integrity and reduction of alveolar septation. It has been demonstrated that the administration of vitamin A leads to a reduction in BPD and mortality at 36 weeks of life (Tyson et al. 1999). A meta-analysis indicated a borderline reduction in the incidence of BPD after the treatment with vitamin A therapy exclusively in infants weighing less than 1000 grams (Darlow 2007). Moreover, no differences were observed between extremely preterm newborns treated or not with vitamin A (Principi et al. 2018). Among the limitations, the administration of vitamin A does not reduce mortality, hospitalization period, and neurological outcomes. Furthermore, the optimal administration route has not yet been identified: so far, intramuscular injection is the most commonly used, but it carries an increased risk of sepsis and inflammation (Hwang and Rehan 2018).

1.7.4 Caffeine

Early caffeine therapy is commonly used in preterm infants for the treatment of apnea. Its employment before the third day of life is associated with a slight reduction in BPD incidence. Moreover, the administration of caffeine in preterm newborns is associated to a reduction in the need for corticosteroids, a diminished percentage of babies who undergo treatment for a PDA, low severity of ROP, earlier successful extubation and removal of oxygen supplementation, and improvement of motor and cognitive outcomes after 18 months of life (Jensen 2020; Principi et al. 2018). Nevertheless, important questions about stimulant administration remain, including their potential side effects on pro-inflammatory markers, the risk of NEC development, and a slight increase in mortality (Hwang and Rehan 2018).

1.7.5 Diuretics

Diuretics are often used “off-label” in different clinical settings to prevent or treat BPD. Their use, including how frequently and at what doses they are administered, varies greatly. A meta-analysis on the use of loop diuretics in infants with BPD showed short-term improvements in lung function and oxygen levels. However, the long-term effects remain unknown, and further research is needed to balance the benefits with potential risks of using diuretics for BPD prevention and treatment (Hwang and Rehan 2018).

1.8 Animal models in BPD

To better understand the mechanisms of BPD and develop effective new therapies, it is crucial to have animal models that reliably mimic some features seen in very preterm infants with BPD (O’Reilly and Thébaud 2014). The multifactorial etiology of this disease makes it challenging to generate a reliable animal model that mimics all the contributing factors of BPD. The first animal studies on BPD date back to the 1930s. Since then, many methods have been developed to create BPD-like features in animals (Berger and Bhandari 2014). For instance, between January 1, 2013, and June 30, 2015, there were 41 different protocols reported in the literature exclusively for mouse models (Silva et al. 2015). Exposure of neonatal pups to hyperoxia is extensively utilized as a small animal model of experimental BPD (O’Reilly and Thébaud 2014). The animal species used also varies depending on several factors, such as economic availability, research purpose, features of BPD, and ethical considerations. Commonly used species include rodents, lagomorphs, lambs, and non-human primates (Giusto et al. 2021).

1.8.1 Rodents

Mice and rats are the most commonly used animal models to mimic BPD, for several practical and anatomical reasons. First, rodents are physiologically born during the saccular phase of lung development, which is analogous to the preterm stage in human fetal development. This characteristic eliminates the need to induce preterm delivery in experimental models, representing a significant advantage. In human development, the saccular and alveolar stages occur in utero under low oxygen conditions

(approximately 4%). Preterm birth interrupts these processes, exposing immature lungs to the extrauterine environment with a higher oxygen tension of around 21%. This exposure results in the last stages of pulmonary development occurring under relatively hyperoxic conditions. To accurately model preterm human lung development, neonatal rodents—whose lung maturation in occurs in room air—must be subjected to elevated oxygen levels beyond atmospheric norms. Consequently, all rodent models of BPD, regardless of the experimental protocol, involve exposure to hyperoxia (O'Reilly and Thébaud 2014). Although rodents have an immature pulmonary structure at birth, their lungs are functionally mature, meaning they do not experience RDS and do not require the standard interventions needed for human infants. Moreover, while human preterm newborns often require the exogenous administration of surfactant to prevent alveolar collapse, rodents present a sufficient amount of mature surfactant at birth (Berger and Bhandari 2014; O'Reilly and Thébaud 2014).

From a practical point of view, rodents are readily available at low cost and with little concern from regulatory agencies compared with non-human primate animals. They exhibit a short estrous cycle lasting 4-5 days and a relatively brief gestation period—approximately 20 days for mice and 21-23 days for rats. Their reproductive cycle is predictable, allowing for up to 8 litters per year, with an average of 10 pups for mice and 11 for rats (Berger and Bhandari 2014; O'Reilly and Thébaud 2014).

The small size of rodent puppies is both a benefit and a drawback: on one hand, it makes them easier to breed and reduces the amount of pharmacological agents and, more generally, the materials needed during the trial period. On the other hand, it is a matter of concern for substance administration, because intravenous injections are not feasible, and the very thin skin of the newborn rodents makes it difficult to perform subcutaneous injections, favoring local trauma and necrosis if the administration needs to be repeated. In addition, the placement of a subcutaneous pomp is inapplicable (Nardiello et al. 2017).

Other characteristics that make rodents a good model to mimic BPD are the commercial availability of analysis products for both mice and rats, and the possibility of using transgenic mouse models. Moreover, considering that BPD has a multifactorial etiology, additional factors, such as chorioamnionitis, IUGR, inflammatory stimuli, and corticosteroid administration, can be included in the model

to better simulate the clinical outcomes (Berger and Bhandari 2014; O'Reilly and Thébaud 2014).

Despite the pros, rodent animal models for BPD present some limitations. The most impactful one is the lack of standardization among protocols. The differences can be related to beginning and lasting oxygen exposure and percentage of oxygen administration and generally vary according to the aim of the research. If the purpose is to evaluate new candidates for drug therapy, generally more severe procedures, with the administration of higher oxygen levels with early exposure, are employed; whereas if the goal is to investigate the morphological features of BPD, less invasive protocols are used. Each protocol presents some advantages and some disadvantages.

Regarding the duration and the start of hyperoxia exposition, there are several possibilities of exposition for pups, among which from birth to day 7 (Chen et al. 2007), 10, (De Visser et al. 2009; Franco-Montoya et al. 2009), 14 (Alphonse et al. 2011; Chang et al. 2009; Ladha et al. 2005) or 28 (Chen et al. 2007), followed or not by recovery period at room air, or after 1 to 4 postnatal day for 14 days (Kunig et al. 2005), or at two different oxygen concentration for 7 days each, followed or not by recovery time. Currently, the exposition for 14 days is the most accepted procedure, since it is the time point at which secondary septae formation is mostly completed (*Table 1*) (Nardiello et al. 2016).

Moreover, the oxygen concentration applied can vary. Although, to date, oxygen levels administered in the neonatal intensive care unit to preterm newborns do not exceed 40%, rodents are typically exposed to concentrations above 60%, regardless of the protocol used. While lower oxygen concentrations can help reduce lung injury in preterm infants, such levels in mice and rats may not accurately reproduce the hallmarks of BPD, complicating the assessment of potential drugs. This discrepancy is partly due to the more developed antioxidant system in rodents at birth compared to preterm infants. In most rodent protocols, oxygen levels above 90% are employed (Pierro et al. 2013; Hummler et al. 2013; Alapati et al. 2011; Vadivel et al. 2010; Thébaud et al. 2005). On one hand, this is associated with a very high mortality rate (around 60% if the treatment lasts for 14 days); on the other hand, this model best mimics all the key features of BPD, including impaired gas-exchange surface area and septal wall thickness (Nardiello et al. 2016). Specifically, this last feature is not observed if the exposure is set at 85% of oxygen. In this scenario, only simplified

alveolarization and a reduced vascular network are present, even if the survival rate increases. A very low mortality percentage characterizes exposure to 60% of oxygen, but lungs present heterogeneous morphological changes, with a low grade of fibrosis, slightly alveolar simplification, and almost absent vascular regression (*Table 1*) (Auten et al. 2009; Jankov et al. 2001; Buch et al. 2000).

Oxygen Exposure	Time Exposure	References
60%	P0-P14	(Auten et al. 2009; Jankov et al. 2001; Buch et al. 2000)
60%	P1-P14	(Belcastro et al. 2015; Ahlfeld et al. 2013)
75%	P2-P14 followed by room air to P22	(Kunig et al. 2005)
80%	P1-P7 followed by room air to P21	(Pham et al. 2014)
80%	P1-P28 followed by room air to P56	(Tibboel et al. 2015)
85%	P1-P7	(Koskinen et al. 2014; James et al. 2013)
85%	P2-P10	(Park et al. 2013)
85%/gradient	P1-P14 followed by gradient to 21% between P14-P28	(Rieger-Fackeldey et al. 2014)
90%	P0-P14	(Hummler et al. 2013; Alapati et al. 2011)
90%	P2-P10 followed by room air to P14	(Ramachandran et al. 2015)
95%	P0-P14	(McKenna et al. 2014)
95%/60%	P0-P7 at 95% followed by P7-P14 at 60%	(Chang et al. 2013)
>95%	P0-P10	(Franco-Montoya et al. 2009)
100%	P0-P10	(De Visser et al. 2009)
100%	Preterm birth (~E20)-P10	(Ter Horst et al. 2007; 2004)

***Table 1**. Overview of oxygen- and time-exposure in rodent models of experimental BPD*

Besides the lack of standardized protocols, there are other limitations associated with using rodents to mimic BPD. They do not require mechanical ventilation at birth, and mimicking this component is only possible for up to 24 hours, combined with mild hyperoxia (Bland et al. 2008; 2007). Moreover, different strains of mice and rats may respond differently to oxygen exposure, enhancing the context's complexity (Berger and Bhandari 2014; O'Reilly and Thébaud 2014).

1.8.2 Lagomorphs

Rabbits serve as a compromise between rodents and larger vertebrates since, like humans, alveolarization begins in utero. They can be delivered via cesarean section preterm, allowing the replication of pathological conditions related to premature birth in humans. Additionally, because they are larger than rodents, rabbits are suitable models for studying the harmful effects of mechanical ventilation (Nardiello et al. 2017). Also, in the rabbit model, the central stimulus used to induce BPD-like injury is hyperoxia. Rabbit models for BPD that implied the administration of high concentrations of oxygen present diminished lung function with architectural changes, surfactant impairment, altered antioxidant system, and inflammation. As for rodents, the main limitation is the lack of standardization between different protocols, with several conditions that can vary, including the percentage and time of oxygen exposure (Giusto et al. 2021). Other limitations of using this model include higher costs, limited availability of commercial analysis products, and a relative inability for term animals to mimic human anatomical and functional aspects of immaturity. Moreover, in the United States, the care, handling, and treatment of lagomorphs are strictly regulated, which complicates their employment (D'Angio and Ryan 2014).

1.8.3 Lambs

Large animals, such as lambs, present some advantages in the study of hyperoxia-induced pulmonary diseases. Since long-term morbidities accompany BPD, lambs enable the possibility to investigate those outcomes in an extended period and, contextually, to evaluate the long-term efficacy of treatment options (Giusto et al. 2021; Nardiello et al. 2017). Lung development in lambs, similar to humans and rabbits, occurs in utero and continues postnatally. To establish an appropriate model for BPD, lambs can be delivered prematurely, with subsequent exposure to hyperoxic conditions or mechanical ventilation (Albertine 2015). Despite those pros, lamb models face some disadvantages that are generally not surmountable. They are very expensive and require a dedicated structure and qualified personnel. Furthermore, they have a longer gestation period compared to rodents (150 vs 21 days), and their use is associated with relevant ethical issues (Giusto et al. 2021).

1.8.4 Non-human primates

The most easily translatable models to humans are non-human primates. Baboons can be prematurely delivered and intubated, and they allow follow-up for extended periods. Nevertheless, they are characterized by long gestation periods, about 168 days, are very expensive, require dedicated structures and qualified staff, are strictly regulated, and carry relevant ethical concerns. Usually, these aspects are not overcome in experimentation (Giusto et al. 2021).

1.9 Beta-3 adrenergic receptor (β3-AR)

1.9.1 The adrenergic system

The adrenergic system is a component of the autonomic nervous system, in which adrenergic receptors (ARs) are responsible for signal transduction. Through the binding with adrenergic neurotransmitters, noradrenaline (NA) and adrenaline (A), ARs exert their functions, maintaining homeostasis and producing fight-or-flight responses (Yang and Tao 2019). In humans, ARs were divided into two main classes, α- and β-, that in 1948 were categorized into three families based on localization, pharmacological properties, sequence homogeneity, and signaling pathways activated: α_1-AR, α_2-AR, and β-AR (Dongdem et al. 2024). These subfamilies exhibit varied affinity for NA, A, and ligands, as they are further categorized: for example, regarding β-ARs, β1-AR is equally sensitive to NA and A, while β2-AR and β3-AR are more responsive to A and NA, respectively (Samanta et al. 2024).

1.9.2 Discovery of the β3-AR

In 1967, β-ARs were classified into two subtypes, β1-AR and β2-AR (Lands et al. 1967). However, some of the effects mediated by β-ARs, such as the lipolytic responses in white adipose tissue (WAT), the smooth muscle relaxation in the rat colon, and the relaxation of the human urinary bladder (Dongdem et al. 2024), were impossible to attribute to either of the noted isoforms (Coman et al. 2009). The first evidence for the presence of a third isoform of β-ARs was provided in 1989 (Arch 1989). In the same year, the β3-AR was cloned by Emorine and coworkers (Emorine

et al. 1989), and it was accepted only a few years later as the third member of the β-ARs. Since its discovery, the β3-AR has garnered the attention of the scientific community due to its pharmacological properties, its broader tissue distribution than initially assumed, and its involvement in a multitude of physio-pathological conditions (Pini et al. 2020).

1.9.3 The *ADRB3* gene

In humans, the *ADRB3* gene is located on chromosome 8p11-12. β3-AR was then identified in numerous animal species, including rat (Granneman and Lahners 1991), mouse (Nahmias et al. 1991), dog (Sasaki et al. 1997), bovine, sheep, and goat (Forrest and Hickford 1999). The homology between the human *ADRB3* gene and those of other mammals is generally around 80-90% (Coman et al. 2009). β3-AR shares 51% and 46% identity with the sequences of β1-AR and β2-AR, respectively (Schena and Caplan 2019). This homology is primarily limited to the transmembrane region, which is implicated in ligand binding, and to the intracellular loops involved in interaction with a G-protein (Miyata et al. 1996). On the contrary, the domains of least overlap are the C-terminal portion and the third intracellular loop, where the main difference is the absence of the sites subjected to the regulatory mechanism typical of both β1-AR and β2-AR (Schena and Caplan 2019). Additionally, while the genes for β1-AR and β2-AR each have only one exon, the *ADRB3* gene has two, with one encoding the C-terminal tail, which explains the main differences at this level (Dongdem et al. 2024).

1.9.4 The protein structure of β3-AR

β3-ARs are transmembrane receptors that belong to the rhodopsin class A type of G-protein coupled receptors (GPCRs) (Dongdem et al. 2024). Like all GPCRs, β3-ARs are characterized by seven transmembrane (TM) segments, with three intracellular and three extracellular loops, an extracellular N-terminal domain that is glycosylated, necessary for ligand recognition and binding, and an intracellular C-terminal portion, crucial for signal transduction and receptor regulation. TM3, TM4, TM5, TM6 are vital for ligand binding, while TM2 and TM7 are essential for the interaction with the G-protein (Schena and Caplan 2019). After ligand interaction, the receptor undergoes

a conformational change that allows the interaction between the C-terminal tail and the G-protein. Generally, β3-AR interacts with a G_s-protein, although it is also possible that it binds to a G_i-protein. The interaction enables the activation of the G-protein, and the downstream signal cascade can be started (Dongdem et al. 2024) (*Fig.11*). Unlike β1-AR and β2-AR, which undergo classic β-arrestin-mediated desensitization, β3-AR lacks phosphorylation sites in its C-terminal domain, making it relatively resistant to desensitization (Okeke et al. 2019; Milano et al. 2018). This feature makes β3-AR an interesting option for chronic treatments. β1-AR and β2-AR, upon ligand binding, are recognized by β-Adrenergic Receptor Kinase (β-ARK). β-ARK phosphorylates these receptors on specific serine and threonine residues located at their C-terminal regions. This phosphorylation facilitates recognition by arrestin proteins, which subsequently promote receptor desensitization, decoupling from G proteins, and internalization via endocytic pathways (Dongdem et al. 2024). β3-AR, lacking the sites for phosphorylation, fails to undergo this desensitization process. Indeed, β3-AR has been shown to desensitize only after prolonged agonist-induced activation—hours or days, compared to minutes for β1-AR and β2-AR (Pasha et al. 2024) (*Fig. 11*). Several studies have debated how β3-AR becomes desensitized. The most supported theories involve a reduction in receptor mRNA levels after exposure to certain ligands, such as TNF-α (Liu et al. 2019; Granneman et al. 2005; Hadri et al. 1997)—a hypothesis still controversial (Okeke et al. 2019; Wachter and Gilbert 2012)—and a role for post-translational modifications (Dongdem et al. 2024).

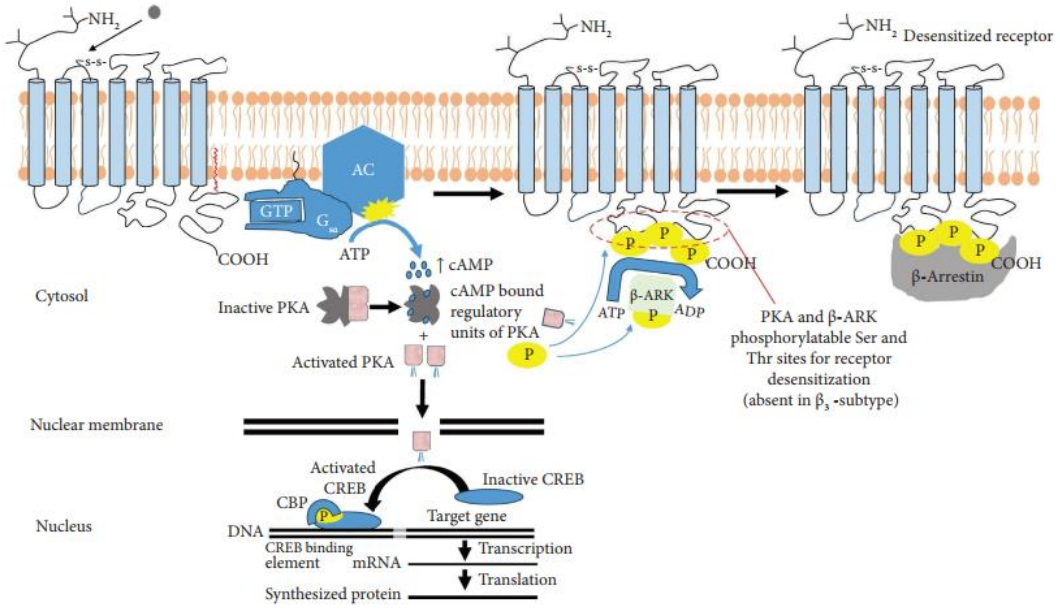

Fig.11 β-ARs activation and desensitization. *After ligand binding, a G-protein interacts with the receptor, leading to the transduction of a signal through a cascade of signaling. Activated β-ARs were recognized by β-ARK and phosphorylated, with the exception of the β3-AR, which lacks the phosphorylation sites, thus escaping the classical desensitization process (Dongdem et al. 2024).*

1.9.5 Oxygen-mediated regulation of β3-AR

It is well established that during intrauterine life, characterized by hypoxic conditions with oxygen levels ranging from 4% to 10%, β3-AR is expressed at high levels (Filippi, Pini, et al. 2022). At birth, when the neonate is exposed to ambient oxygen levels (21%), there is a significant reduction in β3-AR expression (Pini et al., 2020; Hynes et al., 2008; Resch et al., 2003). Despite its downregulation postnatally, β3-AR persists in specific tissues. Originally identified exclusively in adult adipose tissue (Cero et al., 2021), numerous studies have revealed its presence in other human tissues, including the myocardium (Michel et al. 2011; Dessy and Balligand 2010), pregnant myometrium (Rouget et al. 2005) and placenta (Calvani et al. 2020), bladder (Samanta et al. 2024; Liang et al. 2023), brain (Paermentier et al. 1989), retina, blood vessels (Melecchi et al. 2024; Filippi, Cammalleri, et al. 2022), and gut (Nardini et al. 2024; Filippi et al. 2023).

The upregulation of β3-AR expression under hypoxic conditions has been documented in both physiological processes, such as embryonic development, and pathological states, including cancer progression, potentially driven by a common underlying mechanism (Filippi, Pini, et al. 2022; Calvani et al. 2020; Dal Monte et al. 2019; Montoya et al. 2017; Chisholm et al. 2012; Lamkin et al. 2012; Perrone et al. 2008). Among several mechanisms that can be implicated in the hypoxia-mediated upregulation of β3-AR, the Hypoxia-Inducible Factor (HIF)-1 signaling pathway may play a key role. HIF-1 is an oxygen-sensitive transcription factor, highly conserved among different species, and almost ubiquitous (Shimoda and Semenza 2011). HIF-1 is a heterodimer, composed of HIF-1α and HIF-1β subunits. While HIF-1β is stable and ubiquitous, HIF-1α exists only at low oxygen concentration. In normoxic conditions, HIF-1α is hydroxylated by a prolyl-hydroxylase that consumes oxygen as a substrate. Once hydroxylated, HIF-1α is recognized by the proteosome and degraded. In hypoxic conditions, HIF-1α escapes the hydroxylation process, thus remaining available within the cell, and translocates into the nucleus, where, after dimerization

with HIF-1β, it acts as a transcription factor (Shimoda and Semenza 2011). Specifically, HIF-1 binds to the hypoxia-responsive elements (HREs), which are regulatory domains containing conserved HIF-binding sites (HBSs) (Amato et al. 2022). Considering that *VEGF* is one of the main target genes activated by HIF-1 (Amato et al. 2022), it is not surprising that HIF-1 plays a role during embryogenesis and has a crucial function during lung organogenesis, especially in the development of the pulmonary vasculature (Groenman et al. 2007). In a recent study, six HBSs to which HIF-1 can bind were identified on the ADRB3 mouse gene (Amato et al. 2022). Using an in silico approach, HBS#1, located in the promoter of the ADRB3 mouse gene, was proposed as the most likely binding site for HIF-1, which has been shown to effectively induce β3-AR expression under hypoxia (Amato et al. 2022).

Although a direct correlation between low oxygen levels and increased β3-AR expression has been established, the relationship between hyperoxia and a downregulation of receptor expression remains unconfirmed. Recent observations in the pediatric field suggest a link between these two conditions. The DA is an extracardiac fetal shunt that expresses β3-AR, which allows fetal circulation to bypass the immature pulmonary circulation. Physiologically, when the neonate begins spontaneous breathing at birth and oxygenated blood reaches the DA, this fetal shunt starts to close and typically closes completely within 72 hours after delivery. It was demonstrated that its closure is associated with a significant downregulation of the β3-AR expression. Consequently, researchers hypothesize that there may be a relationship between the postnatal increase in oxygen levels, the closure of the DA, and the subsequent decrease in β3-AR (Pini et al. 2020). Consistent with this observation, it has been demonstrated that the expression of the β3-AR is decreased during the hyperoxic phase of retinopathy of prematurity (Amato et al., 2022). Additionally, the number of colonic and ileal resident cells expressing β3-AR was significantly reduced in an experimental model of hyperoxia-induced intestinal damages. These reductions were associated with morphological alterations, indicating a potential role for β3-AR in intestinal development in relation to oxygen exposure (Nardini et al. 2024; Filippi et al. 2023). Nevertheless, further studies are still required to ascertain the correlation between hyperoxia and β3-AR downregulation conclusively.

1.9.6 β3-AR as therapeutic target

1.9.6.1 β3-AR in the adipose tissue

In human adipose tissue, the mRNA transcript of β3-AR was found in several stores of WAT in adults, in infant peri-renal brown adipose tissue (BAT), and, recently, in BAT deposits in adults (Cero et al. 2021; Finlin et al. 2021). Adrenergic activation promotes lipolysis and stimulates the expression of uncoupling protein 1 (UCP-1), which uses long-chain fatty acids from the intracellular lipid pools and circulating glucose as a source to generate heat (Cero et al. 2021). Over the past few years, researchers have aimed to pharmacologically target the β3-AR at the BAT level, as scientific evidence has shown an inverse relationship between active BAT and metabolic diseases such as obesity and type II diabetes (Orava et al. 2013; van Marken Lichtenbelt et al. 2009). Preclinical studies demonstrated that the stimulation with a selective β3-AR agonist leads to BAT activation, preventing fat accumulation and improving dyslipidemia and insulin sensitivity (Nahon et al. 2020; Berbée et al. 2015). Moreover, several clinical studies have shown that the activation of β3-AR with Mirabegron, a selective β3-AR agonist, increases the metabolic activity of BAT, the energy consumption, and the uptake of free fatty acids and glucose (O'Mara et al. 2020; Baskin et al. 2018; Finlin et al. 2018; Cypess et al. 2015). In a model of obese mice, the administration of Mirabegron results in significant body weight loss, reduced adiposity, and a marked increase in UCP-1 levels compared to untreated animals (Hao et al. 2019). Moreover, it was found that mutations to the *ADRB3* gene are associated with an increased risk of obesity, diabetes, and nonalcoholic fatty liver disease (NAFLD) in obese subjects (Clément et al. 1995; Widén et al. 1995; Walston et al. 1995). Despite these promising results and findings in rodents for the use of β3-AR agonists as a treatment for metabolic diseases, such as obesity and diabetes, human studies have mostly shown a lack of efficacy (Dwaib and Michel 2023). It could be due to the higher β3-AR expression in rodent BAT compared to human BAT and the greater selectivity of β3-AR agonists for rodent isoforms rather than human ones (Collins 2022).

1.9.6.2 β3-AR in the retina

In the retina, β3-AR is primarily localized to endothelial cells and mediates vasorelaxant and antiangiogenetic effects in the retinal blood vessels (Schena and Caplan 2019). Its expression increases in pathological engorged retinal tufts in

response to hypoxia, along with heightened HIF-1–mediated responses (Melecchi et al. 2024). The ischemic conditions promote the formation of new aberrant retinal vessels from existing ones and induce neuronal apoptosis (Casini et al. 2014), leading to diseases such as ROP, diabetic retinopathy (DR), and macular edema secondary to retinal vein occlusion (Campochiaro et al. 2006). Dal Monte and colleagues showed in a ROP model that BRL37344 prevented retinal vessels from sensing hypoxia by promoting revascularization of the central retina, counteracting β1/2-AR–induced abnormal neovessel growth, and supporting the resolution of ischemic damage (Melecchi et al. 2024).

1.9.6.3 β3-AR in the myocardium

β3-AR was detected in the human heart at both mRNA and protein levels, and interestingly, an upregulated expression was observed in cases of heart failure (Pasha et al. 2024). Its agonism shows considerable variability not only among different species but also depending on the specific cardiac region. In the ventricles, β3-AR activation generally results in a negative inotropic effect mediated by G_i-protein activation, leading to an increase in eNOS levels (Schena and Caplan 2019). Conversely, in the atrial myocardium, the data are heterogeneous: some investigations report the absence of negative inotropic effects, whereas others indicate an enhancement of L-type calcium channel activation, which may lead to positive inotropic effects. However, this phenomenon has not been definitively validated (Gauthier et al. 2000). Further research is needed to clarify these findings. Although more studies are required, the upregulation of β3-AR in some cardiac diseases, such as heart failure, along with its ability to reduce cardiac workload, makes this receptor a promising target for cardiovascular conditions (Balligand 2016).

1.9.6.4 β3-AR in the myometrium and pregnancy

In 2000, Bardou and colleagues demonstrated for the first time that β3-AR is expressed in the myometrium, and its activation exerts a myorelaxant effect (Bardou et al. 2000). A few years later, an increased β3-AR expression was demonstrated during pregnancy (Asif et al. 2023), confirming the potential use of selective β3-AR agonists as tocolytic agents. Additionally, β3-AR plays a pivotal role in germ cell function, being expressed in oocytes and spermatozoa (Čikoš et al. 2014; 2005) —where it influences motility (Adeoya-Osiguwa et al. 2006) —, and is involved in the development and maintenance of preimplantation embryos during the first week of gestation (Fujinaga and Scott

1997). β3-AR activation was also found to support metabolic adaptations and immune tolerance during pregnancy (Filippi, Pini, et al. 2022). Evidence suggests that β3-AR may play a role in fetoplacental circulation, as β3-AR agonism induces moderate vasodilation of the umbilical artery ring (Dennedy et al. 2002). Taken together, these data suggest a possible role of β3-AR in several processes implicated in the onset of pregnancy and in embryo development (Filippi, Pini, et al. 2022).

1.9.6.5 β3-AR in the urinary system

Along the urinary system, β3-AR has been identified in kidneys (Procino et al. 2016) and in the lower urinary tract (Michel and Vrydag 2006), including ureters (Shen et al. 2017), urethra (Limberg et al. 2010), prostate (Calmasini et al. 2015), and bladder (Otsuka et al. 2008). The presence of β3-AR in the human detrusor was first suggested in the 1970s and confirmed a few years later, when it was demonstrated that neither β1-AR nor β2-AR agonists could induce muscle relaxation, unlike the β3-AR agonist BRL37344 (Wuest et al. 2009; Igawa et al. 1999). The transcript (Igawa et al., 1999) and the protein (Otsuka et al., 2008) corresponding to β3-AR have been identified in both the urothelium and detrusor muscle, with the latter exhibiting a predominant expression of this receptor subtype (Yamaguchi, 2002). Activation of β3-AR mediates detrusor muscle relaxation, thereby enhancing bladder storage capacity. This pharmacological mechanism has led to the development and commercialization of a selective β3-AR agonist, Mirabegron, for the treatment of overactive bladder (OAB) syndrome. Additionally, Vibegron, another potent and selective β3-AR agonist, has successfully completed Phase III clinical trials (Yoshida et al., 2018) and has received approval in Japan for OAB treatment (Keam 2018).

Regarding the presence of the β3-AR in renal tissue, the available data are limited and sometimes conflicting. Several studies provide transcriptional and functional evidence supporting their expression (Rains et al., 2017; Kaufmann et al., 2015; Rojek et al., 2005), whereas other investigations have failed to detect the receptor in the kidney. Recent findings, however, indicate that β3-AR is expressed in vasopressin-sensitive segments of the nephron in murine models (Procino et al. 2016). Since the β3-AR KO mice were polyuric, a possible role for β3-AR in maintaining renal homeostasis (Procino et al. 2016) has been proposed. This suggests that β3-AR could be a target for issues related to impaired water or solute balance (Schena and Caplan 2019).

1.9.6.6 β3-AR in the intestinal tract

One of the sympathetic-mediated responses that could not be attributed to either β1-AR or β2-AR stimulation before the discovery of the third isoform, β3-AR, was related to smooth muscle relaxation in the rat's colon (Bianchetti and Manara 1990). To date, β3-AR expression has been identified in various tissues, including the gastrointestinal smooth muscle and enteric neurons (Cellek et al. 2007; Anthony et al. 1998). β3-AR agonism leads to relaxation of gastrointestinal smooth muscle (Koike et al. 1994) and reduces colon motility (Bardou et al. 2000), suggesting potential clinical applications (Sarma et al. 2003; De Ponti et al. 1996). Additionally, scientific evidence indicates that targeting β3-AR may help decrease inflammation-induced colitis (Vasina et al. 2008). Recently, my research team confirmed the presence of β3-AR on cholinergic neurons in the ileal and colonic enteric nervous system (ENS), and identified a possible correlation between β3-AR oxygen-mediated regulation and intestinal alterations (Nardini et al. 2025; Filippi et al. 2023). In a neonatal rat model of hyperoxia-induced damage, we observed that the high oxygen levels caused colonic morphological alterations, including changes to the mucosal architecture and ENS, directly associated with impaired organ development. These changes correlated with a reduction in the resident cells expressing β3-AR (Filippi et al., 2023), suggesting a potential role for this receptor in colonic maturation (Filippi et al. 2023). Indeed, the administration of BRL37344, a selective agonist for β3-AR, in the same animal model primarily enhanced the maturation process, thereby preventing morphological changes. In this model, we also observed that β3-AR agonism could prevent not only ileal mucosal and submucosal alterations but also the vascular regression induced by hyperoxia (Nardini et al. 2024). These findings highlight that targeting β3-AR pharmacologically may be a promising therapeutic option for addressing oxygen-induced alterations in preterm newborns.

1.9.6.7 β3-AR in cancer

Catecholamines are known to favor tumor progression through the interaction with β-ARs (Dal Monte et al. 2019; Calvani et al. 2018). In the recent past, research predominantly focused on the function of β2-AR in tumor progression, as existing studies identified β2-AR as the primary isoform mediating catecholamine signaling in oncogenesis (Eckerling et al. 2021; Filippi et al. 2015; Cole and Sood 2012). Only recently, a high expression of β3-AR has been found in several tumors (Filippi, Pini, et al. 2022; Montoya et al. 2017; Chisholm et al. 2012; Lamkin et al. 2012; Perrone et

al. 2008), and it has been correlated with increased progression, angiogenesis, and reactivity of the stromal compartment (Calvani et al. 2018). The role of β3-AR in cancer progression has been investigated in melanoma, both *in vitro* and *in vivo,* as well as on human biopsies. In the mouse melanoma cell line B16F10, the presence of all isoforms of β-ARs was demonstrated. However, exposure to hypoxia, a typical feature of the tumor microenvironment, is accompanied by a significant increase in β3-AR expression (Dal Monte et al. 2019). The administration of a selective β3-AR antagonist has been demonstrated to reduce tumoral progression and increase cell survival (Dal Monte, Casini, et al. 2013). The same antineoplastic properties were exerted by the β3-AR antagonism in a murine model of melanoma cancerogenesis, where it was able to reduce tumor mass and its vascular network (Dal Monte, Casini, et al. 2013). β3-AR activation in melanoma also promotes the formation of a tumor microenvironment that escapes immune system activation, thereby creating an immune-tolerant habitat for the tumor (Calvani et al. 2019). In this context, blocking β3-AR increases the number of NK and CD8+ cells and shifts polarized macrophages from M2 to M1, thereby enhancing immunocompetence in the area surrounding the tumor (Calvani et al. 2019). A similar effect of β3-AR antagonism was demonstrated in a murine model of neuroblastoma (NB)—the most common extracranial solid tumor of childhood—where β3-AR antagonist SR59230A increased the number of NK and $CD8^+$ cells, activating an immune reaction (Bruno et al. 2023). Although β3-AR antagonism shows potential as a therapeutic approach for neoplasms, particularly those exhibiting malignant and aggressive phenotypes, its clinical application remains constrained due to the absence of currently available drugs that function as selective antagonists of β3-AR.

1.9.7 Pharmacology of β3-AR

Since their discovery, β3-ARs have attracted the attention of the scientific community for their unique regulatory mechanisms, which make β3-ARs a promising pharmacological target for chronic treatments (Pini et al. 2020). Additionally, recently identified aspects have opened the door to new potential clinical applications. For instance, Mirabegron, a selective β3-AR agonist, has been approved for the treatment of OAB (Chapple et al. 2014). In recent years, some pharmacological criteria have been described to identify β3-ARs. Besides their binding to the two natural

catecholamines, NA and A, β3-ARs exhibit high affinity and potency for selective agonists, which were initially described as activators of lipolysis and thermogenesis in WAT and BAT (Ursino et al. 2009). Furthermore, β3-ARs exhibit partial agonist activity toward most common β1-AR and β2-AR antagonists (Kaumann 1996), show an atypical low affinity for conventional β-AR antagonists such as propranolol and nadolol (Ursino et al. 2009), and have a lower stereoselectivity index for both agonists and antagonists compared to β1-AR and β2-AR (De Ponti et al. 1996).

During the last twenty years, several efforts have been made to generate selective compounds for β3-AR. Most of them share the same backbone, composed of three portions: a left-hand side, which is typically an arylethanolamine or aryloxypropanolamine (Perrone et al. 2009), a linker with various structures, and a right-hand side that contains polar and/or ionizable moieties (Strosberg 1997).

1.9.7.1 Agonists

In vivo and *in vitro* studies have highlighted the significant interspecies variability and the heterogeneous pharmacological profile of β3-AR agonists: their potency and efficacy depend on the intrinsic properties of the molecule and on the tissue or cells where the response is evaluated (Ursino et al. 2009). β3-AR agonists can be classified into two generations of compounds. The first-generation class is mainly composed of phenylethanolamine, such as BRL37344 and SR58611A (*Fig.12*), and it is characterized by limited efficacy for the human β3-AR compared to the rodent gene (Wilson et al. 1996), with metabolites often associated with off-target effects (Brucker et al. 2022). The second-generation class mainly consists of aryloxypropanolamine, which was first introduced as β1-AR and β2-AR antagonists but can also act as β3-AR partial agonists, such as CGP12177A (*Fig.12*) (Ursino et al. 2009). This generation also includes L-755507, Mirabegron, and Vibegron (*Fig.12*) (Brucker et al. 2022). These compounds display greater efficacy for the human β3-AR and higher affinity compared to the other receptor isoforms (Ursino et al. 2009).

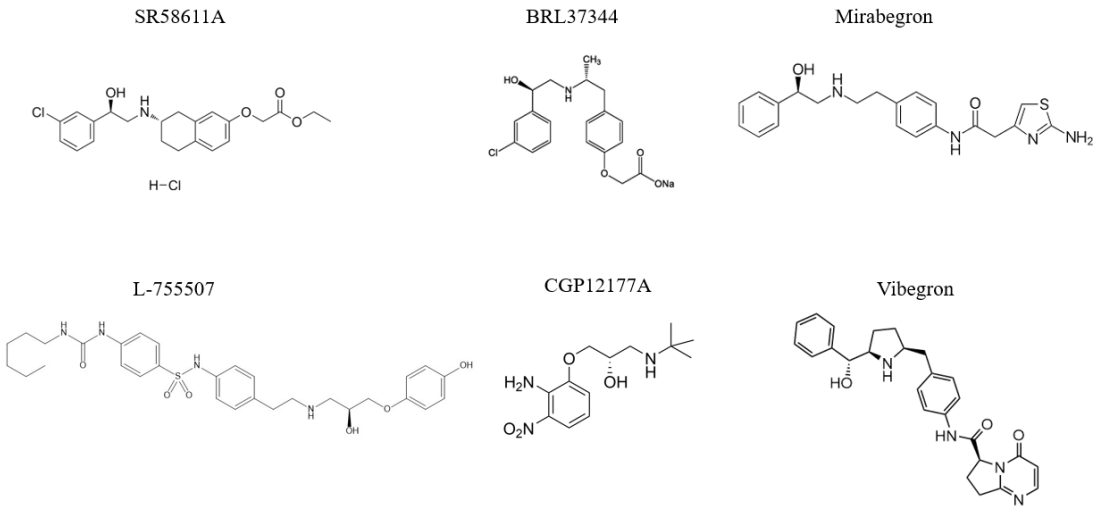

Fig.12 β3-AR agonists. *Chemical structure of first- and second-generation β3-AR agonists. Image modified from Brucker et al. 2022; Yamada et al. 2022.*

To date, some β3-AR agonists are included in clinical trials or have been approved: Mirabegron was approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2012 for treating OAB syndrome; GW427353 (Solabegron) is in Phase II for treating OAB syndrome (Ohlstein et al. 2012; Hicks et al. 2007); SR58611A reached Phase III for managing major depressive disorders, but that trial was later discontinued due to lack of efficacy in patients (Simiand et al. 1992).

1.9.7.2 Antagonists

The identification of the β3-AR subsequent to the discovery of the other two β-adrenergic receptor isoforms is primarily attributable to its lower binding affinity compared to β1-AR and β2-AR, which likely contributed to its later characterization. For instance, the K_i value for propranolol is 1.8, 0.8, and 186 nM for β1-AR, β2-AR, and β3-AR, respectively (Ursino et al. 2009). SR59230A, which belongs to the aryloxypropanolaminotetralins, is a potential β3-AR antagonist. However, this molecule, despite its affinity for rodent β3-AR, is less effective as a selective antagonist for the human receptor (Manara et al. 1996), and some findings have shown that it can exhibit agonist properties (*Fig.13*) (Hutchinson et al. 2005; Horinouchi and Koike 2001). Furthermore, studies indicate that SR59230A is non-selective, with a greater affinity for β1-AR and β2-AR than for β3-AR (Baker 2010; Vrydag and Michel 2007; Niclauß et al. 2006; Hoffmann et al. 2004). Two other selective antagonists for

β3-AR have been identified so far: L-748328 and L-748337, with the latter exhibiting 100-fold higher affinity for β3-AR compared to β1-AR and β2-AR, as well as for the rodent β3-AR, and it does not have agonist activity (*Fig.13*) (Candelore et al. 1999).

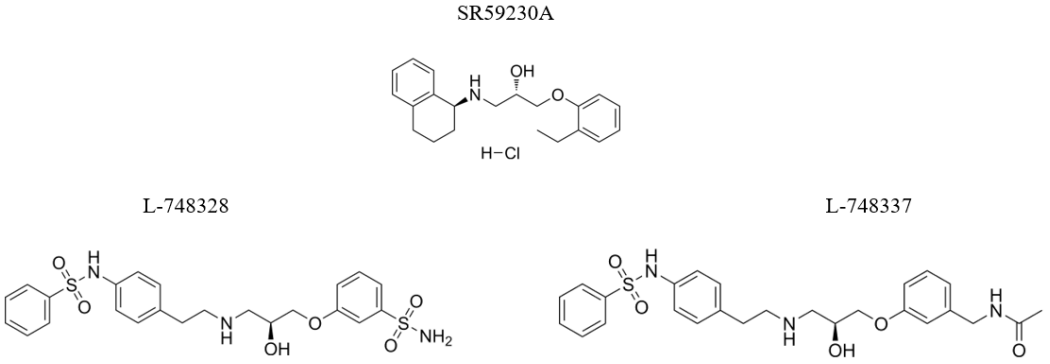

Fig.13 β3-AR antagonists. *Chemical structure β3-AR antagonists. Image modified from Brucker et al. 2022; Yamada et al. 2022.*

2. AIM OF THE STUDY

BPD is a chronic lung disease, particularly affecting preterm newborns who undergo mechanical ventilation and supplemental oxygen therapy. Although these clinical treatments are typically lifesaving, they can alter normal lung structure, potentially leading to BPD (Thébaud et al. 2019). Exposure of immature lungs to ambient and supplemental oxygen levels increases oxidative stress and inflammation, which may lead to changes in pulmonary tissue (Kalikkot Thekkeveedu et al. 2017). Apart from impairing lung function, newborns with BPD are at increased risk for both acute and chronic morbidities, including pulmonary infections, pulmonary hypertension, neurocognitive impairments, and developmental delays.

Although some treatments are currently available, such as corticosteroid administration, surfactant therapy, caffeine, vitamin A, and diuretics, none of them can fully achieve the therapeutic goal, and their adverse effects remain difficult to overcome (Hwang and Rehan 2018). In the absence of specific treatments for BPD, β3-AR has gained increasing interest as a pharmacological target (Dal Monte et al. 2013), because its activation has been shown to reduce oxidative stress (Bundgaard et al. 2017) and protect intestinal maturation against hyperoxia-induced damage (Nardini et al. 2025; 2024; Pini et al. 2020). Furthermore, β3-AR exhibits some unique pharmacological traits that make it a promising target for long-term treatment, as it is resistant to agonist-induced desensitization, unlike β1-AR and β2-AR (Schena and Caplan 2019).

This study aims to evaluate if treatment with the selective β3-AR agonist, BRL37344, effectively prevents or reduces hyperoxia-induced lung changes in an animal model of BPD.

3. MATERIALS AND METHODS

3.1 Animal and Experimental Design

The Ethical Committee of Florence University and the Italian Ministry of Health (Authorization N. 453/2021) approved this study. Pregnant Sprague–Dawley rats (Crl:CD(SD)), purchased from Charles River Laboratories (Wilmington, MA, USA), were individually housed in cages under constant environmental and nutritional conditions at 25 ± 2 ◦C. They were exposed to light/dark cycles every 12 hours and could access food and water without any restrictions. Promptly after delivery, puppies were pooled together and randomly distributed among the different experimental groups: they were allocated to either ambient oxygen levels (normoxia, 21%) or oxygen-enriched atmosphere (hyperoxia, 95%). Hyperoxia was maintained in a semi-sealable plexiglass animal chamber (BioSpherix, Parish, NY, USA), where oxygen levels were monitored using a ProOx P110 probe (BioSpherix). To avoid oxygen toxicity, the lactating dams were swapped between normoxia and hyperoxia every 24 hours. Specific experimental groups were subcutaneously treated with a selective β3-AR agonist BRL37344 (Merck, Darmstadt, Germany), administered from birth (P0) to day 14 (P14) twice a day. The litters were randomly divided into six groups: (1) normoxia (control group), where pups were reared with 21% oxygen and untreated; (2) normoxia with BRL37344 (3 mg/Kg), where animals were at 21% oxygen and treated with 3 mg/kg BRL37344; (3) hyperoxia (hyperoxia control group), where puppies were exposed to 95% oxygen and untreated; (4) hyperoxia with BRL37344 (1 mg/kg), where pups were reared to 95% oxygen and treated with 1 mg/kg BRL37344; (5) hyperoxia with BRL37344 (3 mg/kg), where animals were exposed to 95% oxygen and treated with 3 mg/kg BRL37344; (6) hyperoxia with BRL37344 (6 mg/kg), where neonatal rats were exposed to 95% oxygen and treated with 6 mg/kg BRL37344. Before the injections, body weights were measured. At the end of the experimental period, rats were sacrificed by the intraperitoneal injection of sodium pentobarbital (800 mg/kg) and lung specimens were closely collected, washed in 0.1 M phosphate-buffered saline (PBS) pH 7.4 and processed for morphologic and biochemical evaluations. Within the first week, all animals exposed to hyperoxia and treated with BRL37344 at 6 mg/kg died. Since the hyperoxic chamber was opened only to perform the administrations, so as to limit fluctuations in oxygen, there is no available data of the latter experimental group, except for mortality data.

3.2 Tissue Sampling

Lung specimens were collected in different ways depending on the analysis to be performed.

For morphological evaluations, firstly animals were bled by severing the inferior vena cava so as to prevent the abdominal cavity from filling with blood. Then, the heart-lung-trachea block was removed, cannulated, rinsed with 0.1 M PBS at pH 7.4, and fixed with 4% formalin solution at a constant fluid pressure of 25 cm for 10 minutes. Heart and each pulmonary lobe were then excised and assigned to specific analysis.

For biochemical and cytofluorimetric analyses, lungs were explanted from the abdomen of rats, washed in 0.1 M PBS at pH 7.4, each lobe was separated from the others, and they were immediately stored at -80°C.

3.3 5-epi-5-F_{2t}-IsoP Measurement

At P8 and P14, blood was collected in heparin-containing tubes, then centrifuged at 1500 x *g* for 10 minutes at room temperature to isolate plasma. Butylhydroxytoluene (BHT, 15 mg/mL in methanol, 1:100 sample volume ratio) was added to each sample to prevent lipid peroxidation, and samples were stored at -80°C until analysis. The plasma level of 5-*epi*-5-F_{2t}-IsoP was quantified with a Micro-Extraction by Packed Sorbent (MEPS) liquid chromatography–tandem mass spectrometry platform (MEPS-LC-MS/MS) as previously reported (Biagini et al. 2022).

3.4 Three-Dimensional Morphometric Analyses of the Lungs

The intermediate lobe of the right lung was treated using iDISCO+ protocol with eosin staining (Laurino et al. 2023). Concisely, samples were dehydrated using increasing subsequent percentage of methanol while gently shaking at room temperature; then stained with Eosin Y (Sigma Aldrich, Milan, Italy) for 24 hours. Eosin was removed and the samples were incubated in a solution composed of Dichloromethane (DCM) (270997 – 250 mL, Sigma Aldrich) and methanol for 3 hours at room temperature. After performing optical clearing with DiBenzyl Ether (Sigma Aldrich) incubation, samples were imaged using a high-resolution light-sheet microscope with real-time

autofocus (Silvestri et al. 2021). To achieve quantification, images were downscaled to an isotropic voxel size of 5x5x5 μm^3 and separated into blocks of 1 mm^3. Ilastik (Berg et al. 2019) was used to perform segmentation of cavities and septa. To isolate alveoli, we used a custom script written in Python. The segmented cavities were skeletonized and then represented as a tree. The 'leaves' (final branches) were isolated as the skeletons of the alveoli. Starting from them, the full alveolar shape was obtained by a watershed algorithm applied on the cavities mask with the alveolar skeleton as seed.

3.5 Flow Cytometric Analysis

Flow cytometry was performed on whole-lung single-cell suspension of at least 10 animals per group. Frozen lung samples were homogenized with the Multi Tissue Dissociation Kit 2 (#130-110-203, Miltenyi Biotec, Bergisch Gladbach, Cologne, Germany). Cells were isolated from the extracted total cell suspension and were subjected to flow cytometric analysis. Cells were immunolabeled with appropriated diluted combinations of FITC-conjugated antibodies for different cell lineage markers (*Table 2*). To investigate the presence of LIFs, HCS LipidTOX green (H34475, Invitrogen; Waltham, MA, USA) was used. Compensation beads were employed for antibody compensation analysis. The labeled cells were analyzed using a MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec). Data were processed using Flowlogic 8.7 software (Miltenyi Biotec).

ANTIGEN	FLUOROPHORE	CATALOG NUMBER	SOURCE
ACTA2	PE-Cy7	Bsm-33187M	Bioss Antibody
β3-AR	APC-Cy7	Bs-10921-R	Bioss Antibody
CD31	PE-Cy5	Bs-0195R	Bioss Antibody
CD34	AF594	Sc-7324	Santa Cruz Biotechnology
CD45	AF405	Sc-1178	Santa Cruz Biotechnology
CD133	AF647	Sc-365537	Santa Cruz Biotechnology
CD140a	Cy3	Bs-0231	Bioss Antibody
CD326	AF647	Bs-1513R	Bioss Antibody

HCS LipidTOX	AF488	H34475	Invitrogen
SP-B	AF488	Sc133143	Santa Cruz Biotechnology

Table 2. FITC-conjugated antibodies for Flow Cytometric Analysis.

3.6 Histological Evaluations

Lung specimens were collected as previously described, fixed overnight at 4°C in paraformaldehyde (PAF) and the left lung of each animal was used for histological evaluations. The samples were dehydrated by passages in alcohol of increasing concentration, diaphanized with Bio-clear (Bio-Optica, Milan, Italy), embedded in paraffin, and cut with a microtome in 5 μm thick sections. All sections were stained at once, to minimize artefactual staining differences.

Paraldehyde fuchsin

To evaluate the elastic fiber network, the slices were stained with paraldehyde fuchsin. Fifteen microphotographs from each sample were randomly captured at 10×, blinded to experimental groups, using a light Zeiss Axioskop microscope (Zeiss, Mannheim, Germany) equipped with 10×, 20×, 40×, and 100× objectives and a high-resolution camera (Leica DFC310 F× 1.4-megapixel, Leica Microsystems, Mannheim, Germany). The morphometric analyses were performed on fifteen regions of interest (ROIs) randomly taken for each section using ImageJ with Java8 software (NIH, Bethesda, ML, USA). The results were expressed as the positive area relative to the total area of the ROI.

Sirius red

To evaluate collagen content, the slices were stained with Sirius red. Twenty microphotographs from each sample were randomly captured at 20×, blinded to experimental groups, using a light Zeiss Axioskop microscope (Zeiss) equipped with 10×, 20×, 40×, and 100× objectives and a high-resolution camera (Leica Microsystems). The morphometric analyses were performed on twenty ROIs randomly taken for each section using ImageJ with Java8 software (NIH). The results were expressed as the positive area relative to the total area of the ROI.

Sudan black

To evaluate cytoplasmic lipid droplets content, the slices were stained with Sudan black. Since this staining is lipid-specific staining, the samples were not dehydrated in alcohol otherwise the lipid drops would have dissolved. The slices were rinsed in distilled water and then directly stained. Thirty microphotographs from each sample were randomly captured at 40×, blinded to experimental groups, using a light Zeiss Axioskop microscope (Zeiss) equipped with 10×, 20×, 40×, and 100× objectives and a high-resolution camera (Leica Microsystems). The morphometric analyses were performed on thirty ROIs randomly taken for each section using ImageJ with Java8 software (NIH). The results were expressed as the positive area relative to the total area of the ROI.

3.7 Hydroxyproline Assay

The accessory lobe of each animal was used to perform hydroxyproline assay after 12 hours lyophilization. Hydroxyproline assay Kit (MAK008, Merck, Millipore) was used according to manufacturer's guidelines.

3.8 Western Blot Analysis

The cranial lobe was lysed with the Tissuelyser LT system (Qiagen; Hilden, Germany) in appropriate volume of ice-cold Lysis Buffer (CellSignaling; Danvers, MA, USA), supplemented with phenylmethylsulphonyl fluoride (PMSF, Merck Millipore; 10 µL/mL). The supernatants obtained after centrifugation at 13,000 g for 15 minutes at 4°C were collected and the total amount of protein was quantified spectrophotometrically using Pierce BCA™ Protein Assay Kit (Invitrogen). 60 µg of total proteins were electrophoresed using BIO-RAD 4-20% Acrylamide Mini-Protean TGX Stain-Free protein Gels (Bio-Rad Laboratories; Hercules, CA, USA) and blotted onto polyvinylidene difluoride (PVDF) membranes via Trans Blot turbo system (Bio-Rad laboratories). After blocking with 5% w/V of Bovin Serum Albumin (BSA) (422351S, VWR, Milan, Italy) in PBS with 0.1% Tween for 1 hour at room temperature (RT), the membranes were incubated overnight at 4°C with the primary antibodies listed in *Table 3*. The bands were detected with peroxidase-labelled secondary antibodies (*Table 3*) using Immobilon ECL Ultra Western HRP solution (Merck Millipore) and visualized through ChemiDoc^{TM} (Bio-Rad Laboratories). The

densitometric analysis was performed by ImageJ software (NIH), and the values were normalized to a housekeeping (β-actin or tubulin).

ANTIGEN	SPECIES	CONCENTRATION	CATALOG NUMBER	SOURCE
β-actin	Mouse	1:4500	A2228	Sigma Aldrich
proSP-C	Rabbit	1:750	AB3786	Merck Millipore
TGF-β	Mouse	1:5000	MA1-21595	Invitrogen
Tubulin	Mouse	1:7500	T5201	Merck Millipore
VEGF-R2	Mouse	1:1000	Sc6251	Santa Cruz Biotec.
Peroxidase-conjugate AffinePure Goat anti-Rabbit IgG	Goat	1:7500	111-035-003	Jackson ImmunoResearch (Ely, UK)
Peroxidase-conjugate AffinePure Goat anti-Mouse IgG	Goat	1:7500	115-035-044	Jackson ImmunoResearch (Ely, UK)

Table 3. Primary and secondary antibodies for Western Blot analysis.

3.9 ELISA Assay

The accessory lobe of at least 10 animals per experimental group was homogenized in a specific Lysis Buffer (Abcam) for VEGF ELISA assay. After centrifugation at 13000 g, for 15 minutes, at 4°C, the supernatant of each sample was transferred to a new eppendorf. Using an ELISA kit for VEGF (Ab100787, Abcam), the VEGF levels in the lung samples were measured according to the manufacturer's indications.

3.10 Lung Myofibroblast Isolation and Transwell Migration Assay

Lung MYFs were isolated as previously described (Reese et al. 2025). On day 7, rats from all experimental groups were euthanized. MYFs were isolated through enzymatic digestion of accurately chopped lung tissue collected following bronchoalveolar lavage and vascular perfusion with PBS. Tissue specimens were digested in Dulbecco's Modified Eagle Medium (DMEM) (D5030-500, Merck, Millipore)

supplemented with 1 mg/ml collagenase I (scr301, Merck, Millipore), 2.5 mg/ml trypsin (392-0459, VWR), and 2 mg/ml DNase I (Merck, Millipore) at 37°C. After filtering the cells with a sterile gauze, they were centrifuged at 400 *g*, for 10 minutes. The pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and the cells were cultured for 12 hours.

For the transwell migration assay, 30000 cells were resuspended in DMEM supplemented with 0.5% FBS and seeded into the upper chamber of an 8 µm pore size insert. In the lower chamber, 600 µl of medium supplemented with 2% FBS were added. Following a 24-hour incubation, the cells in the upper compartment were gently removed, and those migrated to the lower compartment were fixed and stained using Diff-Quik dye kit (9990700; Epredia, Portsmouth, New Hempshire) Migration was quantified by counting five independent, non-overlapping, fields.

3.11 Immunofluorescence Analysis

The caudal lobe of the right lung was included in OCT compound (Leica Microsystem) and samples were frozen at -80°C until analysis. Slices of 5 µm were cut using a cryostat (Leica Microsystem). The immunofluorescence analysis was conducted to evaluate vascularization and to determine the number of AEC II: single immunofluorescent staining was performed using the primary antibodies rabbit anti-proSP-C (Abcam, Cambridge, UK) and goat anti-CD31 (R&D, McKinley Place NE, MN, USA), respectively. Rehydrated sections in PBS were unmasked in sodium citrate buffer (10 mM, pH 6) for 20 minutes at 90°C for antigen retrieval, washed in PBS, blocked with 1.5% BSA (VWR) in PBS to avoid non-specific binding. The sections analyzed for proSP-C were additionally permeabilized using 1.5% BSA in 0.1% Triton-X in PBS. Then, the slices were incubated at 4°C overnight with primary antibodies, diluted in PBS 1% BSA at 1:50 for CD31 and 1:2000 for proSP-C (*table 4*). The following day, once washed, the sections were treated with appropriate fluorochrome-conjugated secondary antibodies, diluted 1:750 in BSA 1% in PBS for 2 hours at RT (*table 4*). After the final washing step with PBS, the strips were transferred into a droplet of the anti-fade DAPI mounting medium (Fluoroshield™ with DAPI, Thermo Fisher Scientific, Waltham, MA, USA). Slice treated with omission of the primary antibody was used as the negative control.

The isolated MYFs were analyzed with a double immunostaining. Cells were fixed with 10% paraformaldehyde, permeabilized and blocked with 3% BSA in PBS containing 0.3% Triton-X for 1 hour at RT. Then, were incubated overnight at4°C with a mixture of two primary antibodies, goat anti-α-smooth muscle actin (Abcam) and rabbit anti-epidermal growth factor receptor (Cell signaling) (*table 4*). The subsequent day, the slices were treated with appropriate fluorochrome-conjugated secondary antibodies, diluted 1:750 in BSA 1% in PBS for 2 hours at RT (*table 4*). Then, the slides were mounted with the anti-fade aqueous medium, DAPI (Thermo Fisher Scientific). Also in this case, omission of the primary antibody was used as negative control.

The immunolabeled sections were observed under the Leica Stellaris 5 confocal microscope (Leica Microsystem), equipped with a Leica Plan Apo 20×/NA and 63×/1.43NA oil immersion objective (Leica Microsystem). Vascular network was assessed on five non-overlapping parenchymal z-stack images for each animal. The number of blood vessels (consider as sections of 20-50 µm in diameter) was measured in each ROI as previously described (Sammour et al. 2016). Digitalized z-stack composite mosaic images of the whole pulmonary slice were examined to count the number of AEC II, by considering as positive cells marked by proSP-C, using ImageJ Software (NIH). The results were expressed as the percentage of positive proSP-C cells relative to the lung parenchymal surface. A total of two slices per rat for at least 10 animals per group were considered.

ANTIGEN	SPECIES	CONCENTRATION	CATALOG NUMBER	SOURCE
α-SMA	Goat	1:400	Ab21027	Abcam
CD31	Goat	1:50	AF3628	R&D
EGFR	Rabbit	1:50	Cs4267	Cell Signaling
ProSP-C	Rabbit	1:2000	Ab3786	Abcam
AlexaFluor 488 AffiniPure Donkey anti-Goat IgG (H+L)	Donkey	1:175	705-545-003	Jackson ImmunoResearch (Ely, UK)
AlexaFluor 488 AffiniPure	Donkey	1:175	711-545-152	Jackson ImmunoResearch

Donkey anti-Rabbit IgG (H+L)				
AlexaFluor 594 AffiniPure Donkey anti-Rabbit IgG (H+L)	Donkey	1:175	711-585-152	Jackson ImmunoResearch

Table 4. Primary and secondary antibodies for Immunofluorescence analysis.

3.12 Transmission Electron Microscopy Analysis

A small part of the right lung cranial lobe was collected and fixed in Karnovsky's mixture at +4 ◦C, overnight. Subsequently, samples were fixed in 1% OsO4 in phosphate buffer for 2 hours, RT, dehydrated by passages in acetone of increasing concentration, passed in propylene oxide, and embedded in epoxy resin (Epon 812, Sigma-Aldrich). Ultrathin sections (70 nm thick) were stained with UranyLess (Electron Microscopy Sciences, Hatfield, PA, USA) and alkaline bismuth subnitrate solutions and then examined under a JEM-1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV. A MegaView III high-resolution digital camera equipped with AnalySIS 2.2 imaging software (Soft Imaging System, Muenster, Germany) was used to acquire the photomicrographs. Lung samples were observed and acquired at 6000× for qualitative evaluations.

3.13 Data analysis and Statistical Test

Data are expressed as mean ± S.D. of at least 10 animals per group. Statistical analysis was performed using a one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison test. Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA) and $p<0.05$ was considered significant ($p<0.05$: *; $p<0.001$: ***).

4. RESULTS

4.1 Survival rate

Consistent with the findings reported in the literature (O'Reilly and Thébaud 2014), our model of severe BPD is characterized by a high mortality rate in the control group of animals exposed to hyperoxia. The treatment with BRL37344 at both 1 mg/Kg and 3 mg/Kg significantly increases the overall survival rate, while the 6 mg/Kg dose resulted in the death of all the puppies within the first week, hindering the possibility of performing any analysis on this group (*Fig 14*).

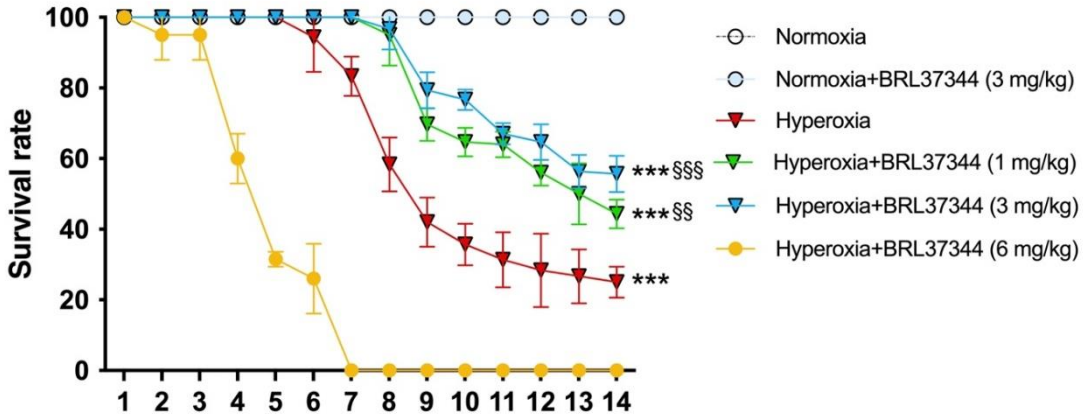

Fig.14 Survival rate. *The administration of BRL37344 at both 1 mg/Kg and 3 mg/Kg significantly increased the survival rate of hyperoxia-exposed rats. The highest dose, 6 mg/Kg, resulted in the death of all the puppies. Values are expressed as mean ± S.D. *** $p < 0.001$ versus normoxia; §§ $p < 0.01$ and §§§ $p < 0.001$ versus hyperoxia.*

4.2 Plasma levels of 5-epi-5-F_{2t}-IsoP

F2-isoprostanes (F2-IsoPs) are products of lipid peroxidation—specifically arachidonic acid peroxidation—by free radicals. 5-epi-5-F_{2t}-IsoP, the most abundant product formed, is commonly used as a biomarker to detect the presence of oxidative stress in numerous diseases (Durand et al. 2001). Since BPD is associated with increased oxidative stress levels and β3-AR is known to decrease ROS amounts, we assessed whether treatment with BRL37344 could lower this biomarker at P8 and P14. Our data showed a significant increase in 5-epi-5-F_{2t}-IsoP in the untreated hyperoxia-exposed rats compared to the normoxic animals, both at P8 and P14 (*Fig. 15*, Panels A and B). The administration of BRL37344 at 1 mg/kg was ineffective at both

evaluated time points (*Fig. 15*, Panels A and B). Conversely, 3 mg/kg of BRL37344 entirely abrogated the alteration observed at P8 and induced a slight, non-significant reduction in 5-epi-5-F2t-IsoP levels at P14 (*Fig. 15*, Panels A and B). As expected, no significant differences were observed among normoxia-reared pups, regardless of β3-AR agonist treatment (*Fig.15*, Panels A and B).

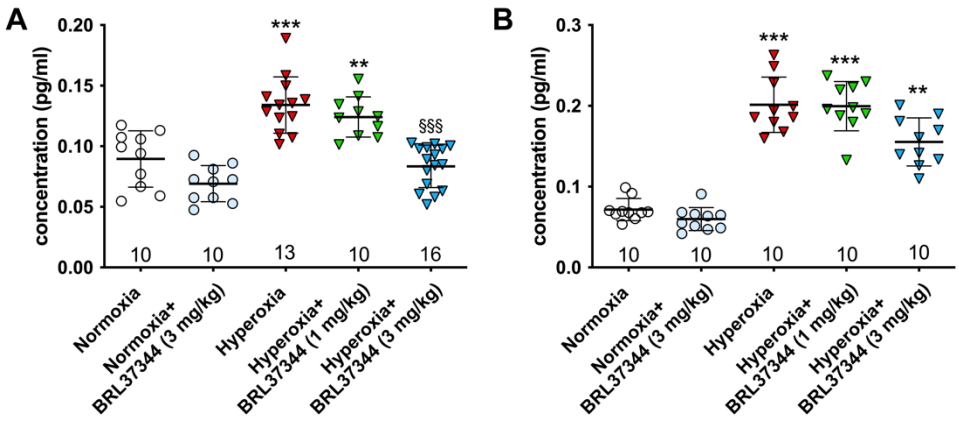

Fig.15 Plasma levels of 5-epi-5-F2t-IsoP dosage. *A marked increase in the plasma levels of 5-epi-5-F2t-IsoP was detected at both time points, P8 (Panel A) and P14 (Panel B). Whereas the treatment with BRL37344 at 1 mg/Kg dose was ineffective regardless of the time point considered, the administration of 3 mg/Kg of BRL37344 completely prevented the increase at P8, but not at P14. As expected, no differences were measured between the control and the treated group in normoxic conditions. Values are expressed as mean ± S.D. ** $p < 0.01$ and *** $p < 0.001$ versus normoxia; §§§ $p < 0.001$ versus hyperoxia.*

4.3 Histological assessment of the protective effect of BRL37344 on hyperoxia-exposed lungs

It is well known that hyperoxia exposure deeply impairs the alveolar phases of lung development, resulting in a significant decrease in lung volume and hindered alveolarization (Thébaud et al. 2019). Our study confirmed that the hyperoxia-exposed control group had a significant decrease in the lung-to-body weight ratio (LW/BW) compared to unexposed pups. The treatment with BRL37344 at 3 mg/Kg completely prevents this change, while the 1 mg/Kg dose was ineffective (*Fig. 16*, Panel A). Three-dimensional morphometric analyses also revealed a significant increase in the alveolar-to-volume surface area ratio and a decrease in alveolar volumetric density (*Fig. 16*, Panels B-D) in rats reared in hyperoxia compared to those raised at room air, demonstrating the detrimental effect of high oxygen concentration on alveologenesis.

Although the normalization of these alterations to baseline levels observed in normoxia-exposed rats was not entirely achieved, administration of BRL37344 at a dose of 3 mg/kg significantly attenuated the histopathological damage. Conversely, a lower dose of 1 mg/kg did not demonstrate significant protective effects (*Fig. 16*, Panels B-D). Animals raised in normoxia and treated with the agonist showed no difference in LW/BW ratio and alveolar volumetric density, suggesting that its protective effect mainly functions to counteract hyperoxia-induced injury by preserving lung morphology (*Fig. 16*, Panels B-D).

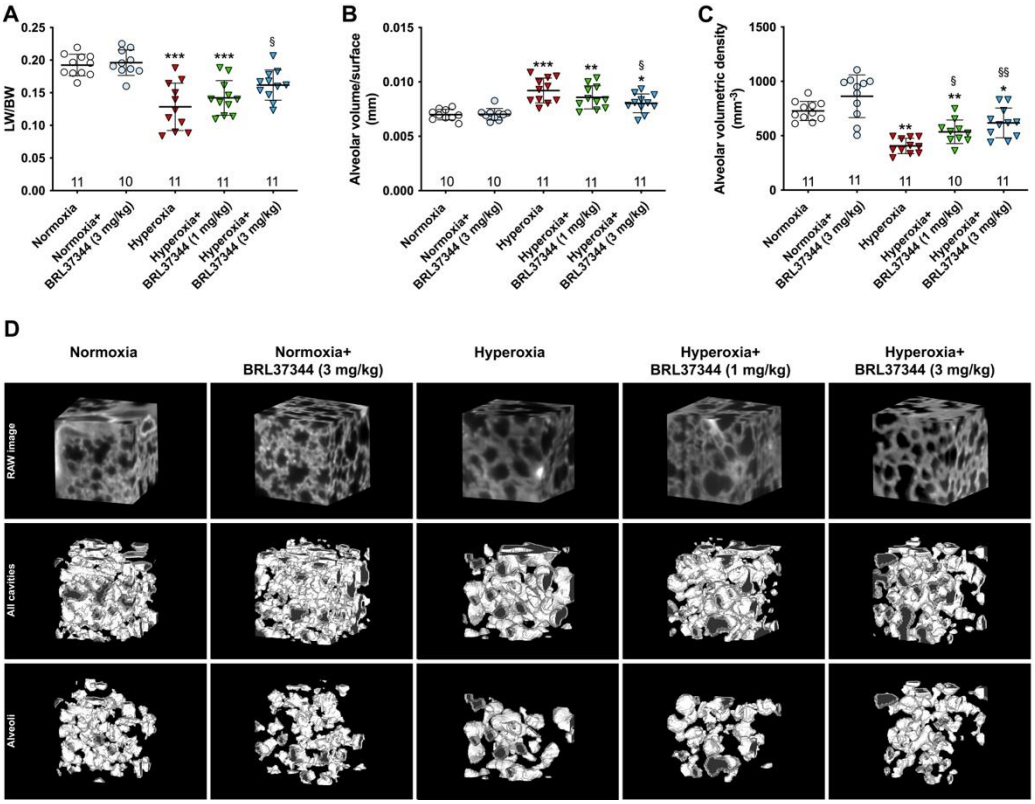

Fig.16 Histological assessment of the protective effect of BRL37344 on hyperoxia-exposed lungs. *(A) Exposure to hyperoxia significantly reduced the lung weight to body weight ratio compared to the normoxic controls. The administration of BRL37344 completely prevented this alteration. (B, C, D) The morphometric evaluations of the volume of the three-dimensional terminal airspaces highlighted an alveolar impairment in the hyperoxic animals, partially counteract by 3 mg/Kg BRL37344. Values are expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus normoxia; § $p < 0.05$ and §§ $p < 0.01$ versus hyperoxia.*

4.4 β3-AR expression of pulmonary cells

To better understand the role of β3-AR in lung development and evaluate its potential role in protection against elevated oxygen levels, we attempted to determine which cell lines express this receptor. For this purpose, flow cytometric analysis was performed on a whole-lung single-cell suspension. Since it is well known that inflammatory cells constitutively express β3-AR, these cells were excluded using the pan-hematopoietic marker CD45. Subsequently, the remaining $CD45^-$ cells were gated using three different protocols: for $CD326^-$, $CD31^-$, and $PDFGR\alpha^+$ to identify fibroblasts; for $SP\text{-}B^+$ to detect AEC II; and for $CD31^+$, $CD34^+$, and $CD133^+$ to identify circulating early endothelial progenitors (CEPCs). The fibroblast population was then further gated for $LipidTox^+$ and for $ACTA2^+$ to discriminate LIFs and MYFs, respectively. Each of these populations was further examined for the presence of β3-AR. This analysis provides the first identification of β3-AR expression on $PDGFR\alpha^+$ fibroblasts, including both LIFs and MYFs, as well as on CEPCs. In contrast, expression of β3-AR was not detected on AEC II (*Fig. 17*).

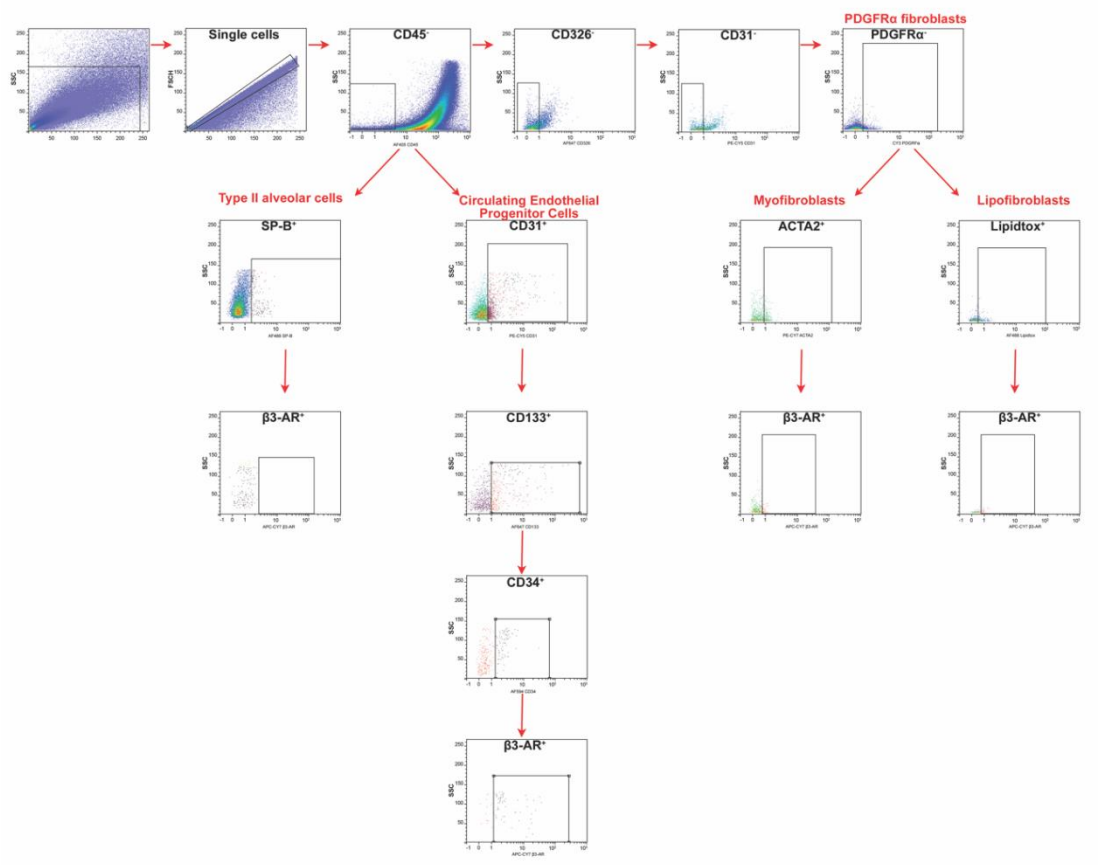

Fig.17 Evaluation of β3-AR on pulmonary cells. *Flow cytometric analysis of lung single-cell suspension demonstrated the presence of β3-AR on LIFs, MYFs, and CEPCs. Conversely, AEC II does not express the receptor.*

4.5 Effects of BRL37344 on myofibroblasts

MYFs play a crucial role in lung alveolarization, as they migrate to the tips of septae where they release elastic fibers necessary for the formation of secondary septa (Rippa et al. 2021). Through flow cytometry, we demonstrated that exposure to hyperoxia led to MYFs hyperplasia (*Fig. 18*, Panel A), and that the administration of BRL37344 at 3 mg/kg significantly prevented this alteration. Conversely, the treatment with the 1 mg/kg dose was ineffective (*Fig. 18*, Panel A). Since it is known that exposure to high oxygen concentrations is associated with a switch from a physiological role in lung development to the onset of fibrosis, we sought to investigate whether the administration of BRL37344 can retain MYFs function. First, we evaluated the elastic fiber network through morphometric analysis of lung sections stained with paraldehyde fuchsin (*Fig. 18*, Panels B and D). This analysis showed that the elastic fiber content was significantly compromised in the hyperoxia-exposed rats compared to that of the pups reared in normoxia (*Fig. 18*, Panels B and D). While the dose of 1 mg/Kg was unable to reverse this phenotype, the administration of BRL37344 at 3 mg/Kg resulted in a partial maintenance of the network (*Fig. 18*, Panels B and D). We secondly evaluated the extent of pulmonary fibrosis through morphometric assessments and biochemical assays. Analysis of lung sections stained with Sirius red, a specific marker for collagen detection, alongside hydroxyproline quantification in pulmonary tissue homogenates, revealed that untreated hyperoxia-exposed pups exhibited significantly elevated levels of both fibrosis markers compared to animals maintained under normoxic conditions. Notably, administration of BRL37344 demonstrated anti-fibrotic effects, showing a dose-dependent reduction of hyperoxia-induced fibrosis (*Fig. 18*, Panels C, E, and F). In accordance with these findings, Western blot analysis of TGF-β—a key profibrotic cytokine—revealed a significant increase of this marker in untreated hyperoxia-exposed animals compared to normoxic controls. The treatment with BRL37344 at both tested doses exerted a significant protective effect, although it did not completely reverse the alterations observed (*Fig. 18*, Panel G). We then assessed the migratory ability of isolated MYFs. Using the

transwell migration assay, we quantitatively demonstrated that hyperoxia negatively affected the migratory ability of MYFs (*Fig. 18*, Panels H and L). Administration of BRL37344 at 3 mg/Kg partially counteracted the oxygen-induced impairment, indicating a potential cytoprotective effect. Conversely, under normoxic conditions, no statistically significant differences in MYFs migration were observed between the treatment and control groups (*Fig. 18*, Panel H). Considering the importance of the epidermal growth factor receptor (EGFR) in mediating MYFs migration (Li et al. 2024; J. Li et al. 2016), we analyzed its expression in lung MYFs isolated from different experimental groups. The marked increase in EGFR levels in the hyperoxic control animals was partially counteracted by the administration of BRL37344 at 3 mg/Kg, while no differences were observed in the normoxic pups (*Fig. 18*, Panel M).

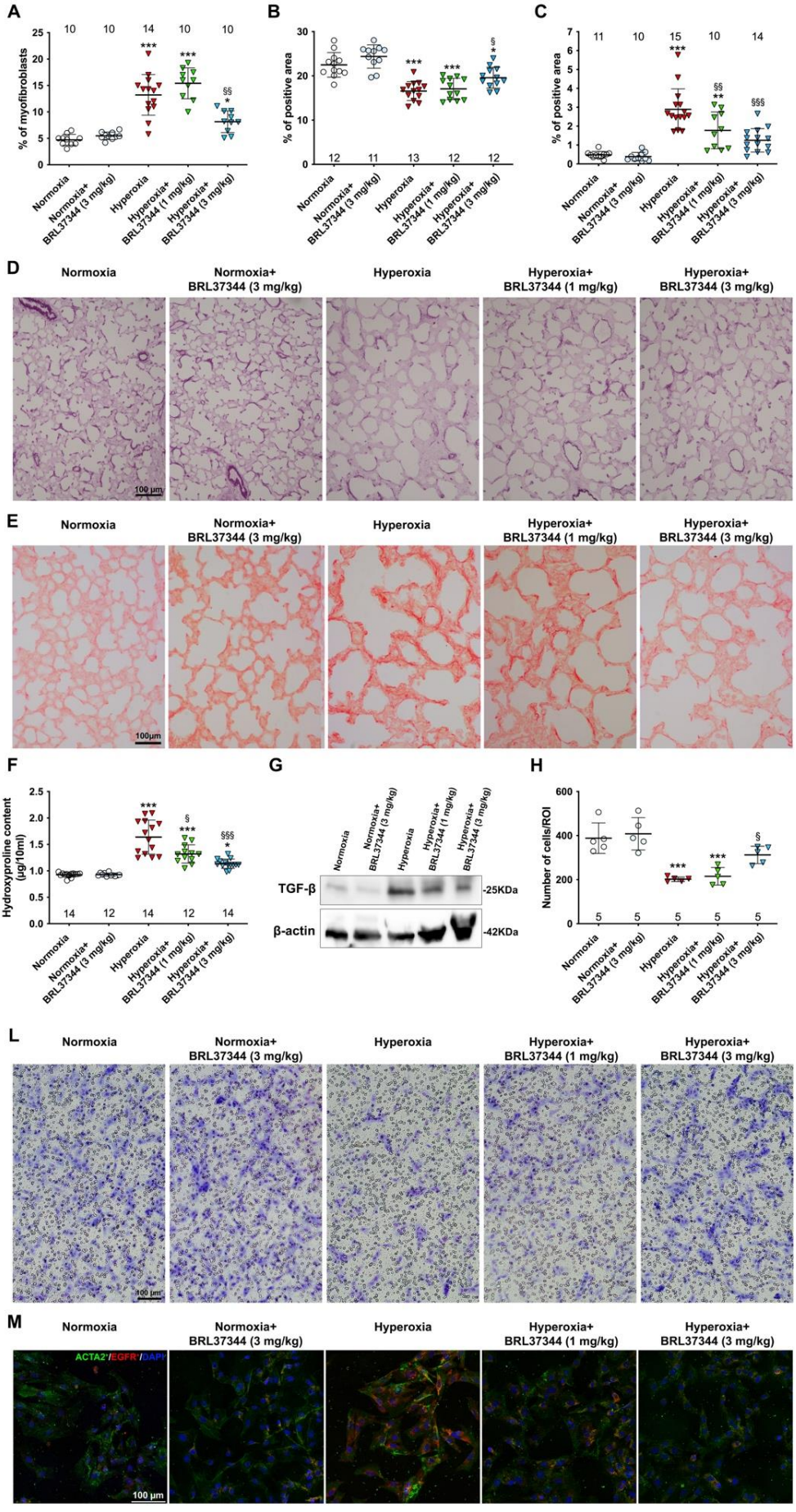
A
% of myofibroblasts
B
% of positive area
C
% of positive area
Normoxia
Normoxia+ BRL37344 (3 mg/kg)
Hyperoxia
Hyperoxia+ BRL37344 (1 mg/kg)
Hyperoxia+ BRL37344 (3 mg/kg)
D
E
100 μm
100μm
F
Hydroxyproline content (μg/10ml)
G
TGF-β
β-actin
-25KDa
-42KDa
H
Number of cells/ROI
L
M
ACTA2+/EGFR+/DAPI+

Fig.18 Effect of BRL37344 on MYFs. *(A) Flow cytometric analysis of MYFs on lung single-cell suspension. (B, D) Morphometric evaluation of slices stained with paraldehyde-fuchsin. Hyperoxia significantly impaired the elastin network, which, conversely, is well organized in both control and treated normoxic groups. 3 mg/Kg BRL37344 effectively counteracted this alteration, while the 1 mg/Kg dose was ineffective. Variation in lung collagen depositions was evaluated by (C, E) densitometric analysis of Sirius red-stained sections, (F), and through a hydroxyproline assay. Hyperoxia exposure mediated an increase in collagen deposition, which was prevented in a dose-dependent manner by the treatment with BRL37344 at 3 mg/Kg. (G) TGF-β levels were investigated with Western Blot analysis: hyperoxia exposure led to a notable increase in TGF-β amounts, which both doses of BRL37344 are able to restore to normoxic levels. (H) Quantification and (L) representative images of the transwell migration assay. While in the hyperoxia control group, migration of MYFs is hindered, BRL37344 at 3 mg/Kg partially prevented this impairment. (M) Representative images of isolated MYFs immunolabelled for ACTA2 (green) and EGFR (red). Nuclei are stained with DAPI (blue). Compared with normoxic samples, MYFs from pups exposed to hyperoxia showed increased EGFR expression. A partially protective effect was exerted by treatment with BRL37344 at 3 mg/Kg, while the 1 mg/Kg dose was ineffective. In none of the parameters considered were any differences observed between the control group and the normoxia-treated group. Scale bars = 100 μm. Values are expressed as mean ± S.D. * $p < 0.05$ and *** $p < 0.001$ versus normoxia; § $p < 0.05$, §§ $p < 0.01$ and §§§ $p < 0.001$ versus hyperoxia.*

4.6 Effects of BRL37344 on lipofibroblasts

LIFs play a crucial role in synthesizing the lipidic component of pulmonary surfactant and transferring it to adjacent AEC II cells (Torday and Rehan 2016). Using flow cytometry analysis, we demonstrated that hyperoxia increased the number of LIFs (*Fig. 19*, Panel A). The administration of BRL37344 at 3 mg/kg completely prevented this hyperplasia, while the lower dose of 1 mg/kg was ineffective. No differences were observed under normoxia (*Fig. 19*, Panel A). To assess the impact of hyperoxia on the ability of LIFs to accumulate lipid droplets in their cytoplasm, we further performed histochemical and ultrastructural analyses. Morphometric analysis of lung slices stained with Sudan black, a lipid-specific dye, showed that exposure to high oxygen levels caused a significant reduction in the number of lipid droplets within the cytoplasm. BRL37344 at 3 mg/Kg was partially able to counteract this alteration (*Fig. 19*, Panels B and C). Through ultrastructural analysis, we confirmed previous findings, highlighting few cytoplasmic lipid droplets in pups exposed to hyperoxia. Treatment with BRL37344 at 3 mg/Kg prevented this change, showing the number of lipid droplets similar to the normoxic controls (*Fig19*, Panel D).

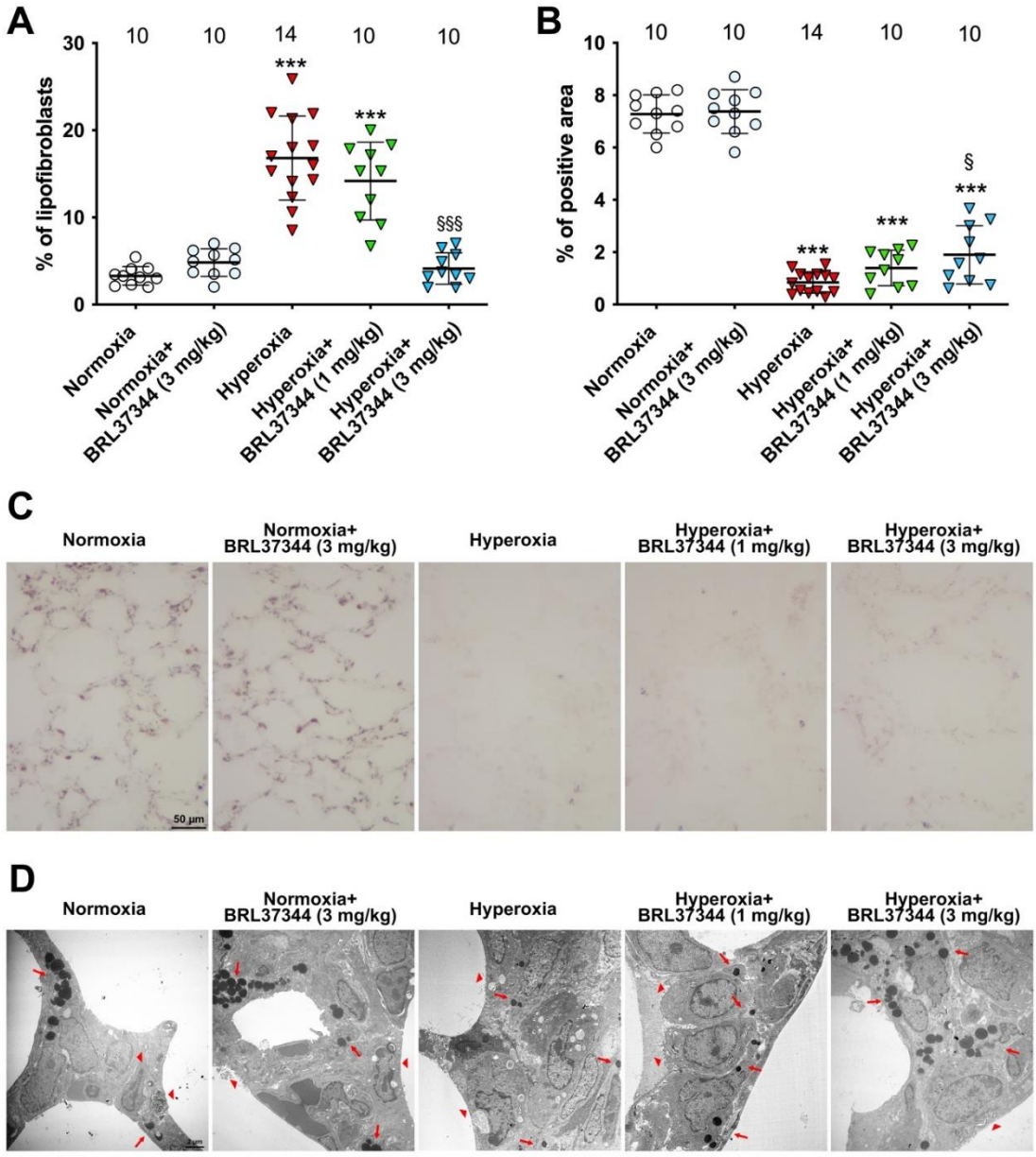

Fig.19 Effect of BRL37344 on LIFs. *(A) Flow cytometric analysis of LIFs on lung single-cell suspension. (B, C) Morphometric evaluation of slices stained with Sudan black for the identification of lipid droplets within the cytoplasm of LIFs. Scale bar = 50 µm. (D) Representative images obtained by TEM of the alveolar region of the right upper lung lobe. Scale bar = 2 µm. (A) Flow cytometry highlighted an increased number of LIFs in the hyperoxic group, (B-D) characterized by scarce lipid droplets. The administration of BRL37344 at 3 mg/Kg was able to counteract LIFs hyperplasia (A) and was partially effective in restoring the number of lipid drops (B-D). The dose of 1 mg/Kg was ineffective, and no changes were observed between the control and the BRL37344-treated groups under normoxia (A-D). TEM analysis (D) revealed that sometimes, LIFs with scant lipid bodies (red arrowheads) are close to AEC II (red arrows). Values are expressed as mean ± S.D. *** $p < 0.001$ versus normoxia; § $p < 0.05$ and §§§ $p < 0.001$ versus hyperoxia.*

4.7 Effects of BRL37344 on alveolar type II epithelial cells

The flow cytometric analysis demonstrated AEC II do not express β3-AR. Nevertheless, the interaction between AEC II and LIFs—cells that express β3-AR—plays a pivotal role in surfactant synthesis. Disruptions to this signaling axis have been associated with abnormal pulmonary development and impaired surfactant production (Torday and Rehan 2016). Thus, we decided to extend our research to AEC II.

Lung tissue sections were immunolabeled for the precursor to surfactant protein C (proSP-C), an AEC II marker, and subjected to morphometric analysis. Results indicated that, in the hyperoxia control group, there was observed hyperplasia of AEC II cells (*Fig. 20*, Panels A and B), a histopathological hallmark of BPD (Dankhara et al. 2023; Fehrenbach 2001). This hyperplastic response is attributed to oxygen-induced lung injury. The 3 mg/kg dose of BRL37344 effectively counteracted this alteration, even though the lung architecture did not fully return to the normoxic condition (*Fig. 20*, Panels A and B). The administration of BRL37344 at 1 mg/Kg was ineffective (*Fig.20*, Panels A and B). The indirect protective effect of β3-AR targeting on preserving the function of AEC II was evaluated using Western blot analyses to assess the levels of proSP-C and PTHrP. Regardless of whether they were treated with BRL37344 or not, no significant differences were observed in the hyperoxic groups regarding proSP-C levels (*Fig.20*, Panel C). Conversely, BRL37344 at 3 mg/kg partially prevented the reduction in PTHrP levels caused by hyperoxia exposure (*Fig.20*, Panel D). These findings suggest a potential indirect role of β3-AR agonism in maintaining AEC II function after high oxygen exposure.

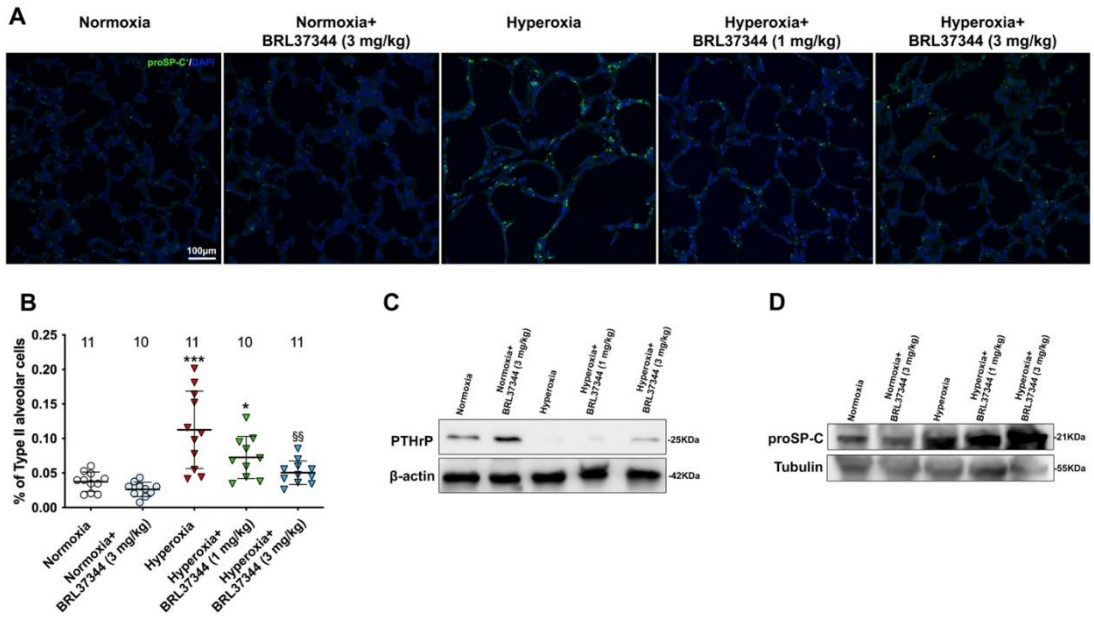

Fig.20 Effect of BRL37344 on AEC II. *(A, B) Representative images and morphometric analysis of the immunofluorescent expression of proSP-C (green) in lung sections. Nuclei are counterstained with DAPI (blue). Scale bar = 100 µm. The treatment with BRL37344 at 3 mg/Kg effectively counteracted the hyperoxia-mediated hyperplasia of AEC II. (C, D) Representative Western blot images of lung lysates. Hyperoxia exposure led to a reduction of PTHrP expression and an increase in proSP-C levels. The administration of BRL37344 at 3 mg/Kg partially prevented the PTHrP alteration, while it was ineffective in restoring the modifications observed for proSP-C. β-actin or tubulin was used as a loading control. Values are expressed as mean ± S.D. * $p < 0.05$ and *** $p < 0.001$ versus normoxia; §§ $p < 0.01$ versus hyperoxia.*

4.8 Effects of BRL37344 on endothelial cells and circulating endothelial progenitor cells (CEPCs)

Since the flow cytometric analysis revealed that β3-AR is expressed on lung endothelial cells and on CEPCs, we evaluated the influence of the treatment with BRL37344 on pulmonary vasculature. Blood vessel density was quantified through the analysis of endothelial CD31 immunostaining in lung tissue sections, and the number of CEPCs was evaluated using flow cytometry. Exposure to hyperoxia resulted in a significant reduction in pulmonary vascular density (*Fig. 21*, Panels A and B). Treatment with both doses of BRL37344 effectively prevented this alteration, although the vascular levels did not fully revert to those observed under normoxic conditions (*Fig.21*, Panels A and B). Additionally, the partially preserved vascular network in the pups exposed to hyperoxia and treated with BRL37344 was associated with a

significant increase in the number of CEPCs, exceeding the levels observed in all other experimental groups (*Fig.21*, Panel C). Considering that CEPCs are deeply involved in angiogenesis, this finding further underscores the potential role of β3-AR agonism in supporting vascular development. No differences were observed in the parameter considered under normoxic conditions, indicating that BRL37344 does not affect the lung vascular network on its own (*Fig.21*, Panels A-C).

Because β3-AR agonism has only a partial effect in counteracting hyperoxia-mediated vascular regression, we investigated the canonical signaling pathways involved in vascularization. It is known that hyperoxia impairs lung vasculature development by disrupting the VEGF signaling pathway (Mathew 2020; Alvira and Morty 2017). Consistently, we observed that hyperoxia significantly lowered VEGF levels using an ELISA assay (*Fig. 21*, Panel D). The administration of BRL37344 at 3 mg/Kg partially reverted this modification, while the lower dose 1 mg/Kg was ineffective (*Fig. 21*, Panel D). Through Western blot analysis, we also assessed the levels of VEGF-R2, which is known to be the main VEGF receptor involved in the pathogenesis of BPD (O'Reilly and Thébaud 2014). We observed a significant reduction of VEGF-R2 levels under hyperoxic conditions, which was prevented by the treatment with BRL37344 at 3 mg/kg but not at 1 mg/Kg (*Fig.21*, Panel E).

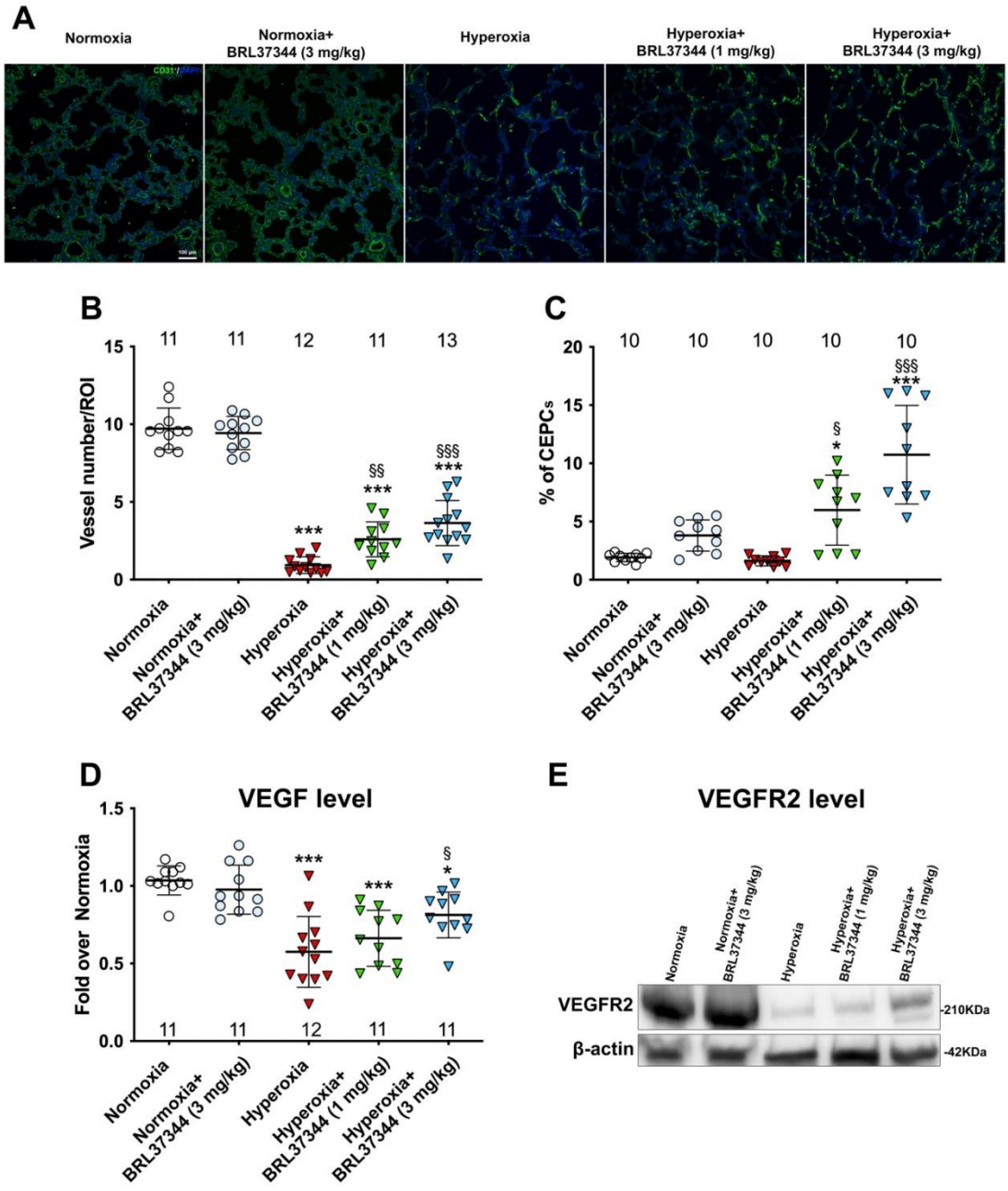

Fig.20 Effect of BRL37344 on endothelial cells and CEPCs. *(A) Representative images of lung sections immunolabelled for CD31 (green), a marker of endothelial cells. Nuclei are counterstained with DAPI (blue). Scale bar = 100 µm. (B) Vessel density morphometric analysis. The administration of BRL37344 at both doses partially mitigated the reduction in vascular density observed in the hyperoxia control group. (C) The number of CEPCs significantly increased after the treatment with BRL37344 compared to all the other experimental conditions. (D) ELISA assay of VEGF lung levels. Hyperoxia exposure mediated a reduction in VEGF levels compared to the normoxic conditions. B3-AR agonism with 3 mg/Kg of BRL37344, but not at 1 mg/Kg, effectively mitigated this reduction. (E) Representative Western blot image of lung lysates from all the experimental groups. Hyperoxia mediated a decrease in VEGF-R2 levels, which are partially restored by the administration of*

*BRL37344 3 mg/Kg. β-actin was used as a loading control. Values are expressed as mean ± S.D. Values are expressed as mean ± S.D. * $p < 0.05$ and *** $p < 0.001$ versus normoxia; § $p < 0.05$, §§ $p < 0.01$ and §§§ $p < 0.001$ versus hyperoxia.*

5. DISCUSSION

This study provides first evidence for the role of β3-AR in a rat model of hyperoxia-induced BPD-like disease, closely mimicking the authentic BPD of preterm infants. Our findings demonstrate that the treatment with BRL37344, a commonly used β3-AR agonist, significantly preserved the lung structural integrity, thereby increasing animal survival.

The first relevant finding of our study is that treatment with BRL37344 nearly doubled the pups' survival rate. We exploited an animal model of severe BPD, characterized by a low survival rate, achieved by exposing rat pups to very high oxygen concentrations (95%) during the first 14 days after birth. Lower oxygen concentrations, such as those below 60%, are more similar to those currently employed in clinical practice for the care of preterm human neonates. However, previous studies indicate that such concentrations may not lead to substantial lung damage in rat pups, likely due to their particularly potent antioxidant systems during early neonatal life (O'Reilly and Thébaud 2014). Conversely, exposure to 95% oxygen closely simulates the lung damage observed in preterm infants with BPD, albeit significantly increasing the mortality rates.

The second relevant finding of our study indicates that treatment with BRL37344 at the 3 mg/kg dose led to a significant reduction in oxidative stress induced by neonatal hyperoxia at P8. However, this beneficial effect was not observed at P14. To assess oxidative stress, we measured the plasma concentrations of 5-*epi*-5-F_{2t}-IsoP, a byproduct of AA peroxidation and a reliable oxidative stress marker (Nechuta et al. 2014) present in higher concentrations than other isoprostanes. In a previous study on an animal model of hyperoxia-induced bowel damage, carried out under a lower 85% oxygen concentration, we demonstrated that treatment with 3 mg/kg BRL37344 on P14 effectively reduced the levels of 5-*epi*-5-F_{2t}-IsoP increased by hyperoxia (Nardini et al. 2024). However, in our current investigation, hyperoxic pups at P14 exhibited increased levels of 5-*epi*-5-F_{2t}-IsoP, regardless of the treatment. This discrepancy suggests that the protective effects of BRL37344 against oxidative stress under the extreme hyperoxic conditions characteristic of the BPD model-where oxygen levels reach as high as 95%—may not persist beyond the first week of the experimental period. Notably, at P8, a time point selected because it corresponds to the peak of

alveologenesis under normal conditions (Branchfield et al. 2016), the β3-AR agonist demonstrated its effectiveness in providing protection.

The third significant finding of our study is that the administration of BRL37344 effectively prevented the structural lung alterations induced by hyperoxia exposure, thereby preserving overall lung morphology. A remarkable increase in the lung-to-body weight ratio was observed following treatment with this β3-AR agonist, suggesting preserved pulmonary development. The 3D morphometric analysis also revealed a partially preserved ratio of alveolar volume-to-surface area, accompanied by an increase in alveolar volume density in the BRL37344-treated pups, indicating improved alveolarization and preserved surface area of the respiratory epithelium. The distinctive pulmonary localization of β3-AR may explain the observed protective effects of BRL37344 against hyperoxia-induced lung damage. Until now, β3-AR has only been identified in bronchial epithelial cells (Bossard et al. 2011). Here, we first report that this receptor is also expressed by MYFs and LIFs, which are known to play crucial roles in alveologenesis (Endale et al. 2017), as well as by CEPCs, which are involved in angiogenesis (Qi et al. 2013). In an interesting contrast, we found that β3-AR is absent in AEC II, which are a critical component of the alveolar epithelium, as they are not only the source of surfactant but also serve as a stem cell reservoir during repair events (Fehrenbach 2001). Alveologenesis is a complex biphasic process primarily driven by MYFs (Branchfield et al. 2016), consisting of a first wave where new septal walls are formed, and a second wave where septae are lifted off existing walls (Mund et al. 2008). During the initial phase, MYFs migrate to the forming septae (Li et al. 2024) and lay down a matrix of elastic fibers, which are continuously remodeled to facilitate the septal formation and stabilize the newly formed structures. At P14, when the first wave ends, MYFs decrease their expression of α-SMA, while the elastin content peaks due to the deposition of an intricate network of thin elastic fibers within the interalveolar stroma (Branchefield). Hyperoxia exposure is known to alter several signaling pathways that regulate MYFs function, leading them to shift from normal alveolar wall build-up to fibrosis (Nelson and Kugler 2024; Branchfield et al. 2016; Berger and Bhandari 2014). Among them, the EGFR signaling pathway, which is activated by ROS (J. Li et al. 2016), is recognized as being critically involved in the migratory capability of MYFs and is overexpressed in animal models of BPD-like disease and in patients with BPD (Li et al. 2024). Moreover, abnormal activation

of EGFR is known for its disruptive effects on normal lung morphogenesis and its promotion of lung fibrosis (Vallath et al. 2014). Therefore, our findings indicate that β3-AR agonism may help preserve alveologenesis by maintaining normal MYFs function. We demonstrated that cultured MYFs isolated from untreated rats exposed to hyperoxia exhibited EGFR overexpression and a diminished migratory ability. Additionally, our findings indicate that, despite MYFs hyperplasia, the hyperoxia-exposed pups showed a reduction of elastic fibers accompanied by an increase in collagen production at P14. It is conceivable that the hyperoxia-deranged MYFs, while attempting to sustain alveologenesis, cannot properly reach the septal tips because of their impaired migratory abilities, and hence persist in the lung interstitium. This results in fibrosis rather than the normal maturation of lung tissue. In stark contrast, the cultured MYFs isolated from hyperoxic rats treated with BRL37344 at 3 mg/kg exhibited EGFR expression levels and migratory capabilities similar to those isolated from the normoxic controls. Furthermore, after administration of 3 mg/kg BRL37344, we observed a reduced number of MYFs compared to the untreated hyperoxic pups, along with a preserved elastic fiber network and reduced collagen deposition. A reduction in the profibrotic cytokine TGF-β was also observed. Taken together, our findings demonstrated that β3-AR agonism plays a pivotal role in preserving MYFs function against the adverse effects of hyperoxia during alveologenesis and exerts a marked antifibrotic effect. By preserving the EGFR pathway of MYFs, β3-AR agonism maintains their ability to migrate to septal tips, directing them toward the remodeling of the elastic fiber network, which is crucial for lung maturation, while preventing excess collagen deposition and fibrosis. Moreover, it is conceivable that BRL37344 effectively inhibited the well-documented transdifferentiation of LIFs into MYFs induced by hyperoxia (Torday and Rehan 2016), although further experiments are required to clarify this aspect.

The presence of β3-AR on LIFs may also help explain the protective effect of BRL37344 against hyperoxia-induced lung damage. LIFs are a specialized fibroblast subpopulation characterized by their ability to accumulate neutral lipids within cytoplasmic droplets (Torday and Rehan 2016; Tahedl et al. 2014). They are present in the alveolar septa and support the synthesis of surfactant in AEC II nearby by releasing TGs through a complex regulatory mechanism known as Neutral Lipid Trafficking (NLT) (Torday et al. 1995). Although newborn rats are surfactant-

sufficient (Berger and Bhandari 2014), it is known that hyperoxia disrupts the crosstalk between LIFs and AEC II, leading to NLT alteration (Lecarpentier et al. 2019) and defective surfactant production and effectiveness. In this context, in the untreated hyperoxia-exposed pups, we observed an increased number of LIFs characterized by significantly fewer cytoplasmic lipid droplets compared to the normoxic controls. This suggests that hyperoxia impairs the LIFs' ability to accumulate neutral lipids, thereby adversely affecting surfactant production and quality. The observed hyperplasia of LIFs could be attributed to a compensatory response to breathing distress and elevated ROS levels, given their ability to enhance resilience against oxidative stress (Torday and Rehan 2016; Tahedl et al. 2014). Notably, the hyperoxia-exposed pups also exhibited AEC II hyperplasia, an attempt to compensate for surfactant deficiency and alveolar damage (Carvallo and Stevenson 2022; Honda et al. 2003). In these animals, besides an abnormal amount of AEC II, Western blot analysis revealed almost undetectable levels of PTHrP, the key paracrine protein involved in crosstalk between these cells and LIFs, which is necessary for surfactant production (Torday and Rehan 2016). Conversely, the hyperoxic rats treated with 3 mg/kg BRL37344 exhibited no signs of LIFs and AEC II hyperplasia, a nearly preserved content of cytoplasmic lipid droplets in LIFs, and an increased PTHrP level similar to that of normoxic pups. These findings strongly indicate that β3-AR agonism exerts a protective effect on NLT and surfactant production. Interestingly, despite these promising results, we observed an increased level of proSP-C in all the hyperoxic pups examined. Possibly, given that β3-AR is expressed on LIFs but not on AEC II, its agonism might better preserve the lipid component of surfactant while being less effective on the protein fraction. This could help explain the observed increase in proSP-C levels irrespective of the BRL37344 treatment.

Another remarkable result from this study is that BRL37344 at 3 mg/kg partially prevented the hyperoxia-induced impairment in vascularization, a common characteristic of BPD. Vascular development is primarily regulated by VEGF signaling, whose dysregulation is a crucial factor in the pathogenesis of BPD (O'Reilly and Thébaud 2014; Thébaud et al. 2005) and other conditions of prematurity, such as ROP and NEC (Klerk et al. 2021; Dal Monte, Filippi, et al. 2013). Furthermore, the role of VEGF signaling in the lungs encompasses not only the maintenance of vascularization but also alveologenesis, which is shown to be significantly dependent

on angiogenesis (O'Reilly and Thébaud 2014). While there are some discrepancies regarding the impact of hyperoxia on VEGF levels, most studies reported diminished lung VEGF levels in pups exposed to hyperoxia after 9 days of age (Meller and Bhandari 2012). Additionally, pharmacological inhibition of VEGF signaling during the first two weeks post-birth has been demonstrated to reduce the number of lung capillaries (Galambos et al. 2002), while its exogenous administration alleviates hyperoxia-induced lung injury in BPD-like animal models (Kunig et al. 2005). Furthermore, in these models, a downregulation of VEGFR2 expression was found (Thébaud et al. 2005). Our findings demonstrate that β3-AR agonism counteracts the downregulation of both VEGF and VEGFR2 induced by hyperoxia, thus partially preventing vascularization impairment. We previously observed a similar, and even more remarkable, effect in a model of hyperoxia-induced gut injury, where the administration of 3 mg/kg BRL37344 completely reverted the negative impact of high oxygen levels on vascular growth (Nardini et al. 2024). Moreover, in a model of retinopathy of ROP, BRL37344 was shown to facilitate the revascularization of the central retina, restoring physiological condition and counteracting the hyperoxia-induced damage (Melecchi et al. 2024).

The cytofluorimetric analysis conducted in our study provided additional evidence supporting the expression of β3-AR by CEPCs. This observation aligns with previous findings obtained through immunofluorescent analysis in samples derived from infantile hemangioma (IH) (Bassi et al. 2022). CEPCs are essential players in the process of vascularization, and the reduction of their number has been implicated in the pathogenesis of BPD (Qi et al. 2013). Moreover, we observed that treatment with BRL37344 at both doses resulted in a significant increase in the number of CEPCs in the lungs of the hyperoxic pups. Interestingly, no substantial differences were observed in CEPC counts under normoxic conditions, irrespective of the treatment with BRL37344. The persistently low levels of CEPC in pups exposed to hyperoxia are not unexpected, as high oxygen levels are known to reduce circulating and lung endothelial progenitors in neonatal rodents and impair their homing ability (Balasubramaniam et al. 2007). Conversely, the hyperplasia of CEPCs observed in the lungs of the hyperoxia-exposed pups treated with the β3-AR agonist suggests that activating this receptor promotes the recruitment and homing of CEPCs to the lungs, helping to counteract the harmful effects of hyperoxia during the critical process of

vascularization. This hypothesis aligns with observations that β3-AR activation is essential for mobilizing hematopoietic stem cells from the bone marrow into the bloodstream in rodents (Méndez-Ferrer et al. 2010). Activation of this receptor could potentially enhance the regenerative capacity of the pulmonary vasculature, representing a promising avenue for therapeutic interventions in conditions exacerbated by hyperoxic environments (Filippi et al. 2025).

A final aspect that deserves attention is the significant mortality rate of the pups resulting from the treatment with BRL37344 at the highest dose (6 mg/kg). A similar outcome was observed in our study on a model of hyperoxia-induced gut alterations, where exposure to a lower 85% oxygen concentration was used (Filippi2023). The most likely explanation is that BRL37344, when administered at 6 mg/kg, exhibits a toxic effect. However, considering the elevated oxygen levels used in the current BPD model (95%), it is plausible that a synergistic effect —stemming from the combination of hyperoxia-induced damage and the sub-lethal toxic effects of 6 mg/kg BRL37344— could lead to lethal outcomes for the pups. To rule out the possibility of such harmful interactions, it would be necessary to evaluate this high dose of BRL37344 under normoxic conditions. Nevertheless, for ethical reasons, we decided against pursuing this option. Notably, treatment with BRL37344 at the effective dose of 3 mg/kg did not show any toxic effects or influence any of the evaluated parameters, thus suggesting a favorable therapeutic window at such a lower dose.

This study is subject to limitations. First, the pharmacological targeting of β3-AR was achieved using BRL37344, a compound that demonstrates greater selectivity for β3-AR than both β1-AR and β2-AR in rodents (Schena and Caplan 2019). Its suitability for subcutaneous administration makes it preferable for the neonatal rat model than other more selective second-generation β3-AR agonists, such as Mirabegron, which is administered via oral gavage and is less practical for newborn rodents. However, it is important to note that BRL37344 has been shown to act through β2-AR in some tissues, such as skeletal muscle (Ngala et al. 2009). Therefore, it is impossible to entirely rule out that some of the beneficial effects observed in our study may be attributed to the activity of BRL37344 as β2-AR agonist. Nevertheless, a recent in vivo study investigating the role of β3-AR in the pathogenesis of ROP revealed that the effects of BRL37344 were specifically mediated through β3-AR, as evidenced by its lack of efficacy when administered with SR59230A, a commonly used β3-AR

antagonist (Melecchi et al. 2024). Conversely, the selectivity of Mirabegron has also been recently questioned, as its induced detrusor relaxation appears to be mediated by either β2-AR agonism or α1-AR antagonism (Lim and Chess-Williams 2022). In this context, BRL37344 remains a validated candidate for activating β3-AR.

A second limitation of the study is the absence of specific lung functional assessments, since we only evaluated the efficacy of β3-AR pharmacological agonism by a mere analysis of the survival rate. As this represents the first investigation into the role of β3-AR in BPD, it serves primarily as a proof of concept. Therefore, additional morpho-functional evaluations are needed to further investigate the role of this receptor in lung maturation and the pathogenesis of BPD.

The last substantial limitation pertains to the adopted animal model of BPD-like disease. While neonatal exposure to hyperoxia is commonly used to reliably mimic the characteristics observed in preterm infants developing BPD, it must be pointed out that, in humans, this condition has a multifactorial etiology, with numerous factors contributing to its pathogenesis (O'Reilly and Thébaud 2014). Therefore, the lack of an ideal animal model that encompasses all the factors contributing to BPD remains a notable limitation, which can only be partially addressed through the refinement of study methodologies.

6. CONCLUSIONS

In conclusion, this study provides new insights into the role of β3-AR in lung development and the pathogenesis of BPD. By promoting alveologenesis, counteracting vascular impairments, and reducing fibrosis, β3-AR agonism plays a protective role against the onset and progression of BPD-like disease in newborn rats, ultimately leading to increased survival rates. While findings from animal studies must be approached with caution when applying them to humans, our research suggests that pharmacological targeting of β3-AR warrants exploration as a novel therapeutic strategy for BPD, capable of improving lung growth and differentiation in premature newborns. In this way, β3-AR agonism may reduce the need for long-term oxygen therapy, improve outcomes for premature infants, and minimize the long-term sequelae of BPD. Moreover, the potent antifibrotic effect of β3-AR agonism could also justify its therapeutic use against pulmonary disorders characterized by fibrosis, such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. Last, but not least, an easy bench-to-bedside translation of the notions from this study may be favored by the availability of effective and safe β3-AR agonists already registered as medications in the neonatal medicine repertoire. In this perspective, further research is needed to clarify the specific mechanisms through which β3-AR preserves lung maturation and to explore the long-term consequences of its agonism in chronic lung diseases.

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