General introduction and outline of the thesis

1. GENERAL INTRODUCTION

Prematurity is defined as childbirth that occurs before 37 completed weeks or 259 days of pregnancy. Worldwide, an estimated 15 million babies are born prematurely each year. That is more than 1 in 10 babies, affecting families all around the world.[1,2] In the Netherlands, about 7% of all live births were preterm (in 2015).[3]

Prematurity is the leading cause of newborn deaths (babies in the first 4 weeks of life) and death in children under the age of 5. Over 1 million children die each year due to complication of preterm birth.[2] However, improved neonatal care has dramatically increased the survival of premature babies. Furthermore, neonatal survival is extending to lower and lower extremes of gestational age. Survivors of prematurity, in turn, face specific health problems, many of which are unique to the preterm population. Because they are born before they are physically ready to face the world, these babies often require special care and are at risk for developing complications that result from anatomic and functional immaturity, like feeding difficulties, brain injury, severe infections and respiratory illnesses.[4,5]

The morbidity associated with preterm birth often extends beyond the neonatal period and throughout the life cycle. Premature babies are at greater risk for significant health problems later in life, such as neurodevelopmental disabilities and cognitive impairments, hypertension, metabolic syndrome and diabetes, respiratory abnormalities and exercise intolerance.[6]

Because they develop late in the embryo and are even far from mature at birth, the lungs appear to be most susceptible to damage in premature babies. Chronic respiratory morbidity is the most common serious adverse outcome affecting premature infants born prior to 32 weeks pregnancy, with up to 40% (23-73%) of preterm survivors having bronchopulmonary dysplasia (BPD). BPD, a severe chronic lung disease defined as supplemental oxygen requirement for at least 28 days,[7] is recognized a consequence of disrupted lung development, and is characterized by an arrest in vascular and alveolar growth,[7-9] as will be outlined in more detail in the next paragraphs.

1.1 Lung development

The lungs are the primary organs of the respiratory system. Already in the 4th week of gestation the development of the lungs starts with the appearance of a primitive lung bud from the ventral surface of the foregut. Lung development continues in five histological stages, namely embryonic (week 4-7), pseudoglandular (week 8-16), canalicular (week 17-25), saccular (week 26-38) and alveolar stages (week 38 – 3 year). During these stages, the respiratory tract develops by a branching process, which forms the bronchi, bronchioles, and ultimately the alveoli. The alveoli are thin walled small air sacs, located in the respiratory zone of the lungs and representing the smallest units in the respiratory tract, where air exchange occur. The number of alveoli in each lung increases from zero at 32 weeks' gestation to 50-150 million alveoli in term infants and 300-500 million in adults.[10-12]

At the same time and in the same spatial pattern, the pulmonary vasculature develops.[10,11,13,14] The target of bronchopulmonary development is the formation of an effective gas exchange organ where blood and air are in intimate contact of a large surface area. The major function of the lungs is to oxygenate blood and to clear carbon dioxide (CO_2) from the blood by gas exchange; the process of diffusion of oxygen from the inspired air into the blood and carbon dioxide out of the blood into the expired air. The blood gases in the pulmonary capillaries equilibrate with those in the alveolar air across the blood-air barrier, a very thin (≈ 2μm) diffusion membrane consisting of the walls of the alveoli and the endothelial cells of the pulmonary capillaries. Once oxygen is passively diffused to the blood, it binds to hemoglobin in red blood cells or dissolves in the plasma, is spread evenly throughout the body by the left ventricle, where it is used to sustain aerobic metabolism.

Premature infants are born in a critical stage of lung development (saccular or alveolar stage). At this stage, the immature lung has poorly developed airways with a much smaller surface area and relatively thick septa, insufficient for gas exchange. Furthermore, there is a surfactant deficiency, a decreased compliance, underdeveloped antioxidant mechanisms and inadequate fluid clearance. After birth, lungs of premature infants are exposed to several injurious stimuli, including hypoxia and/or hyperoxia, mechanical ventilation, infection and inflammation. Early injury to the developing lung can impair alveolarization, which result in simplification of the distal lung airspace and clinical manifestations of BPD.[14]

1.2 Pulmonary circulation

Pulmonary circulation refers to the movement of blood from the right ventricle, to the lungs via the pulmonary arteries and back to the left atrium via the pulmonary veins. Before birth, only 10% of the blood pumped out by the right ventricle enters the lungs, since the placenta, and not the lung, function as the organ of gas exchange.[15] This is due to a high pulmonary vascular resistance (PVR), driving the flow of blood away from the pulmonary circulation to the systemic and placental circulation, leading to a right-to-left shunt through the ductus arteriosus and foramen ovale. The high PVR in the fetus is maintained by compression of pulmonary vessels by the fluid-filled lungs, lack of rhythmic distention of the lungs (breathing) and hypoxic pulmonary vasoconstriction due to low alveolar oxygen tension. Humoral mediators such as endothelin-1 and lack of vasodilators such as nitric oxide (NO) also contribute to the high PVR.[16,17]

At birth, an impressive fall in PVR and an increase in systemic vascular resistance results in the transition from fetal to an adult circulation, including the closure of the ductus arteriosus and foramen ovale. Various mechanical factors and vasoactive agent signaling pathways contribute to this fall in PVR. Of these factors, pulmonary endothelial NO, acting via the cyclic guanosine monophosphate (cGMP) pathway, mediate pulmonary vasodilation and has a great importance in normal physiological pulmonary transition.[18,19]

As mentioned before, the development of the pulmonary vasculature is closely related to that of the airways. Vasculogenesis (de novo formation of blood vessels from angioblasts or endothelial progenitor cells) and angiogenesis (formation of blood vessels by direct extension of pre-existing vasculature) are the principal mechanisms governing the formation of the pulmonary vasculature.[20] Premature birth not only have deleterious impact on the development of the airways and alveoli, as mentioned before, but also cause early disruption of angiogenesis and vasculogenesis. Such disruption leads to a decreased vessel density, and thus to a reduction in the cross-sectional area of the pulmonary vasculature. Furthermore, hypoplasia of the pulmonary vasculature in combination with the underdeveloped airways results in hypoxic vasoconstriction. Chronic hypoxia in the lung tissues also alters vasoreactivity. Together, vascular simplification, hypoxic vasoconstriction and increased vasoreactivity lead to an increased PVR and causes structural remodeling with intimal hyperplasia and increased muscularization of small pulmonary arteries (pulmonary vascular disease; PVD).[21] Moreover, premature birth is associated with exposure to several injurious stimuli after birth. Intermittent hypoxia and hyperoxia, mechanical ventilation and infection/inflammation aggravate pulmonary vascular remodeling. If not prevented from progression, structural remodeling can result in pulmonary hypertension (mean pulmonary artery pressure ≥ 25mmHg), and ultimately right heart failure and death.[21,22]

The incompletely understood pathogenic cascade, as well as the absence of an effective treatment for neonatal pulmonary vascular disease (PVD) and PH renders neonatal PVD an urgent call for research. Additionally, with the increase in longevity of preterm infants with neonatal PVD and/or PH it is of critical significance to study long-term outcomes of this disease. Until now, most studies concerning long-term health outcomes have focused on respiratory outcomes. However, less is known about cardiovascular function in survivors of neonatal PVD.

1.3 Pulmonary vascular tone

Pulmonary hypertension, irrespective of the cause, is characterized by an increase in PVR. PVR is defined as mean pulmonary artery pressure minus mean pulmonary backpressure divided by cardiac output. The regulation of PVR occurs by changing the diameter of blood vessels, and the changes in vascular diameter are the sum of both passive (structural and mechanical) and active (smooth muscle tone) influences.

1.3.1 Passive influences

In the pulmonary circulation, there are two passive mechanisms at work being recruitment and distension of the small vessels.[23] Under normal conditions, when pulmonary artery pressure is low, perfusion pressures of pulmonary vessels vary between different lung segments. As pressure increases, vessels that were open but not conducting blood or were even closed are recruited simultaneously, thereby decreasing PVR. Moreover, the wall of the pulmonary

vessels is relatively thin, resulting in a large compliance that allows the pulmonary vessels to distend in response to increases in pulmonary pressure, leading to a further reduction of PVR. Although passive influences in the regulation of vascular diameter and resistance are important, this thesis focuses mainly on active regulation of pulmonary vascular tone.

1.3.2 Active regulation

Pulmonary vessels, like other blood vessels, have an inner lining of endothelial cells, which are surrounded by vascular smooth muscle cells (except the capillaries). Pulmonary vascular tone refers to the state of contraction of these vascular smooth muscle cells. It is the result of a complex interplay between a multitude of contracting (vasoconstrictor) and relaxing (vasodilator) factors that influence smooth muscle cell contraction or relaxation and thus the vascular diameter, thereby determining PVR. The factors that regulate pulmonary vascular tone can be divided in neurohumoral, mechanical, metabolic, endocrine, paracrine and endothelial influences. In addition, many other vasoactive factors have been shown to influence pulmonary vascular tone, including reactive oxygen species (ROS) and phosphodiesterases (PDE).[24,25]

In this thesis, we mainly focus on endothelial control of pulmonary vascular tone, and particularly the nitric oxide pathway (nitric oxide, PDE and ROS).

1.3.2.1 Nitric oxide

Nitric oxide was firstly described as an endothelial derived relaxing factor. It is synthetized in the endothelium from L-arginine by endothelial NO synthase (eNOS). eNOS is activated by mechanical forces (i.e. an increase in shear stress exerted by the blood flow on the endothelium) as well as by a host of chemical factors such as bradykinin, acetylcholine, substance P and noradrenaline acting on their respective receptors on the endothelium.[24] NO diffuses to the underlying smooth muscles, where it activates soluble gyanylyl cyclase (sGC), resulting in the production of cGMP. cGMP causes smooth muscle cell relaxation by activating protein kinase G (PKG), resulting in lowering intracellular Ca^{2+} and activation of myosin phosphatase, leading to a decrease in the sensitivity of the contractile apparatus to Ca^{2+}.[26]

NO has been implicated in normal pulmonary vascularization by stimulating endothelial proliferation through the VEGF-NO pathway. NO is an important downstream target for the proliferative effects of VEGF and for the differentiation of developing pulmonary artery endothelial cells. Furthermore, NO plays a critical role in the rapid fall in PVR during normal pulmonary perinatal transition. At birth, oxygenation and shear stress acutely increase NO production by increasing eNOS activity and by upregulating its expression. These mechanisms are likely to be involved in sustained reduction in PVR. Therefore, disruption of the NO pathway leads to impairment of pulmonary microvascular formation and has been implicated in the pathogenesis of (neonatal) PVD and PH.[10,21,27] However, the exact underlying pathophysiologic mechanisms remain incompletely understood.

Since there is increasing evidence that alterations in the NO-cGMP signaling pathway play an important role in the pathogenesis of neonatal PVD and PH, inhaled NO (iNO) was widely used in neonatal intensive care units as rescue therapy for preterm infants with respiratory disease undergoing ventilation. However, iNO treatment in premature infants (≤ 34 weeks) shows equivocal effects on pulmonary outcomes and survival and its use for preterm infants with respiratory failure is currently controversial.[28-30]

1.3.2.2 Phosphodiesterases

PDEs are enzymes responsible for the degradation of cyclic nucleotide second messengers cAMP and cGMP. Therefore, inhibition of PDEs in vascular smooth muscle has been recognized a powerful tool to reduce vascular tone by prolonging the half-life of cAMP and/or cGMP. To date, at least 11 different families of PDEs have been identified, all with different kinetic properties, localization and function.[31] The PDE isoform that are predominately present in vascular smooth muscle cells are PDE1, 3, 4, 5, 7 and 9.[32] Because the expression of PDE5 is 10 times more abundant in the pulmonary as compared to the systemic circulation, PDE5 inhibition preferentially dilates the pulmonary vasculature, with relatively little systemic vasodilation, and has been clinically validated as an effective treatment for PH.[33-35]

Oral sildenafil, a selective PDE5 inhibitor, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the treatment of PH in adults. Despite the safety and efficacy in pediatric patients had not been established, the drug has become a major component in the treatment of pediatric PH. However, there is a lack of licensing for its use in children below 1 year of age, meaning a significant number of patients are outside the approved remit including children with BPD-PH.[36] In 2013, the U.S. Food and Drug Administration (FDA) cautioned against the use of sildenafil in children with PH in light of the increases in mortality in children receiving high doses.[37] Despite such setbacks, sildenafil continues to be used off-license. Several recent studies, both in human and animals, are encouraging; sildenafil treatment in patient with BPD-PH was associated with improvement in clinical and hemodynamic parameters and a low mortality rate,[38,39] and sildenafil promoted adequate lung angiogenesis, decreased PVR, right ventricle hypertrophy and arterial medial wall thickness in newborn rats.[40]

1.3.2.3 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules with an unpaired electron.[41] Because of their highly reactive nature, ROS can react with various intracellular proteins and alter their structure and function. Small amounts of ROS are continuously produced in the human body, mainly during ATP production in the mitochondria, and have been shown to play a role in many signaling processes.[42-47] However, in order to prevent deleterious effects of ROS and to maintain proper cellular function, the amount of ROS needs to be carefully controlled. Under normal physiological conditions, most ROS

are scavenged by the anti-oxidant systems of the body (antioxidant vitamins and endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase).[42-48]

Because the pulmonary vasculature is by nature exposed to high levels of oxygen, production of ROS is likely to be more prominent in the pulmonary vasculature as compared to the systemic vasculature.[48] Modest variations in the balance between production and scavenging of ROS may contribute to regulation of normal function of the pulmonary vasculature (redox signaling).[49,50] The increase in ROS production results in pulmonary vasoconstriction and increase in PVR in vitro and in vivo.[51-54]

Neonates, and especially those who are born premature, are particularly vulnerable to oxygen toxicity, as their levels of antioxidant enzymes are inadequate and unable to protect the rapidly growing tissues, including the developing lung, from oxidative injury.[44,55-58] In the developing lung, oxidative stress lead to inactivation of surfactant, cellular dysfunction, and impaired cell survival, thereby playing a critical role in the pathogenesis and pathophysiology of neonatal PVD.[14,57-60]

1.4 Developmental Origins of Health and Disease

Nowadays, there is growing evidence that disruption of normal pulmonary vascular development in early life contributes to the development of PVD in adult life. In the late 1990s, it was already shown that a transient perinatal insult to the pulmonary circulation increases the risk of developing pulmonary hypertension.[61] It has also been shown that pulmonary artery pressure is elevated in offspring of mothers with pre-eclampsia, demonstrating that placental hypoxia causes pulmonary vascular dysfunction.[62] Underlying mechanisms of this so-called "fetal or perinatal programming" are currently unknown.

In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequences of perinatal pulmonary vascular events is imperative. Little is known about the cardiovascular function in this is relatively new patient population. More research to the long-term cardiovascular outcomes is necessary in order to improve the health of prematurely born survivors of neonatal PVD and to reduce the burden of adult cardiopulmonary morbidity and mortality.

2. AIMS AND OUTLINE OF THE THESIS

The general aim of this thesis is 1) to study peri- and neonatal (mal)adaptation, and 2) to investigate endothelial function in the adolescent pulmonary vasculature, both in an intact animal model of swine as well as in isolated small pulmonary arteries.

2.1 Peri- and neonatal (mal)adaptation

The main focus of this section is the effect of injurious stimuli in the peri-and neonatal period to the pulmonary vasculature. Both premature birth—with incomplete vascular growth, immature vascular function, and decreased host defenses—as well as exposure to injurious stimuli after birth, contribute to an abnormal development of the lung circulation.

Reactive oxygen species play a key role in the pathogenesis of neonatal PVD and can be caused by hyperoxia, mechanical ventilation, hypoxia, and inflammation. **Chapter 2** gives an overview of short- and long-term consequences of oxidative injury to the perinatal lung for the human cardiovascular system.

Failure of normal lung development will lead to neonatal PVD due to an altered function of the pulmonary vessels (with an increased vasomotor tone), as well as an altered structure of the pulmonary vasculature, i.e. vascular remodeling (including smooth muscle cell proliferation). PVD represents an underestimated and increasing clinical burden in the neonatal period, but also later in life. Despite decades of research, the exact mechanisms underlying PVD as well as to what extend PVD contributes to long-term cardiovascular morbidity and mortality are currently unknown. Consequently, we developed a new swine model for neonatal PVD allowing follow-up. In **Chapter 3** we demonstrate the surgical placement of catheters for long-term cardiovascular follow-up at rest and during exercise testing. **Chapter 4** describes the development and characteristics of the swine model of neonatal PVD, which is the first that allows exercise-testing and examination of long-term sequelae of a perinatal hypoxic insult, the course of the disease and the effect of therapy on long-term outcome.

It is well known that neonatal PVD is associated with multiple disruptions in the NO-cGMP signaling pathway, such as a decreased eNOS activity and reduced vasodilator response to NO.[63-68] However, little is known about disruptions more downstream in this pathway, including sGC- and cGMP-dependent mechanisms. Therefore, we investigated in **Chapter 5** the functionality of different parts of the NO-cGMP signaling pathway in the long-term, in vivo (at rest and during incremental exercise) and in vitro.

2.2 Endothelial function in the adolescent pulmonary vasculature

Endothelial function is a key factor in vascular development as well as in maintenance of vascular structure and function throughout life. Besides endothelial dysfunction is a crucial factor in neonatal PVD, it plays a crucial role in the pathogenesis of adult PVD, includ-

ing PH.[69-71] While the endothelial function of the systemic and coronary circulation is extensively investigated, studies into the endothelial function of the pulmonary vasculature received less attention. Therefore, in the second part of this thesis we present the results of studies concerning pulmonary endothelial function and vascular control.

Although the incidence of PH is higher in females, the severity and prognosis of PVD have been shown to be worse in male patients.[72,73] Until now, studies concerning sex differences in PH have mainly focused on the role of sex hormones. As it is unknown whether intrinsic sex-related differences in the NO-cGMP signaling pathway contributes to these difference between males and females, we investigated pulmonary vascular function in male and female swine in vivo and in vitro (**Chapter 6**).

By mimicking some aspects of endothelial dysfunction using hemoglobin-based oxygen carrier (HBOC)-201, an important pathogenic factor in PVD can be studied. HBOC-201 administration resulted in pulmonary (and systemic) vasoconstriction and thus elevated blood pressures. In **Chapter 7**, we determined the potential roles of NO, ROS and endothelin (ET) in mediating the observed vasoconstriction in resting and exercising swine.

As described earlier, PDE5 inhibition with sildenafil has been used as a therapeutic tool in treating patients with PH. ET receptor blockade has also been shown to induce pulmonary vasodilation and is also clinically used in patients with PH. However, little is known about whether the combination if those two treatments may have additional therapeutic effects. Therefore, in **Chapter 8**, we studied the effects of combined treatment of PDE5 inhibition and ET receptor blockade in the pulmonary circulation, as well as the mechanisms of interaction between the PDE5 and ET systems.

In the summary and general discussion (**Chapter 9**) the overall findings of this thesis, general considerations, recommendations and future perspectives will be addressed. Finally, a Dutch summary is provided in **Chapter 10**.

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