Summary and general discussion

1. SUMMARY

Annually, over 15 million babies are born prematurely (<37 weeks gestational age), accounting for more than 1 in 10 births worldwide.[1,2] In the Netherlands, about 7% of all live births were preterm (in 2015).[3] Because these babies are born before they are physically ready to face the world, they often are at risk for developing complications that result from anatomic and functional immaturity.[4,5] Because the lungs develop late in the embryo and are even far from mature at birth,[6,7] they appear to be most susceptible to damage in premature babies. Disruption of normal pulmonary vascular development plays a pivotal role in the pathogenesis of several neonatal pulmonary vascular diseases (PVD), including bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH). These chronic lung diseases are one of the most common adverse outcomes in these preterm neonates.[8]

Neonatal PVD complicates cardiopulmonary adaptations after birth, is associated with exercise intolerance later in life and predisposes to long-term cardiopulmonary disease.[9] It develops as a result of lung injury caused by several injurious stimuli. Oxygen tension is a key player in the pathogenesis of neonatal PVD.[10] Both antenatal and postnatal exposure to either hypoxia and/or hyperoxia contributes to disruption of the normal development of the pulmonary vascular bed, resulting in endothelial dysfunction, pulmonary vascular remodeling, and ultimately right ventricular dysfunction (**Chapter 2**).

PVD represents an increasing clinical burden, both in the neonatal period and later in life. Despite decades of research, the exact pathophysiologic mechanisms underlying PVD as well as to what extent PVD contributes to a decreased lung function, decreased exercise tolerance, increased vulnerability to cardiopulmonary disease throughout life, and cardiovascular mortality later in life remain incompletely understood. There is growing evidence that PVD is associated with multiple disruptions in the nitric oxide (NO)-cGMP-phosphodiesterase 5 (PDE5) signalling pathway.[11-17] However, no studies have been performed to investigate the long-term consequences of disruptions in this signalling cascade, and (large) animal models of neonatal PVD performing long term follow-up are lacking. Therefore, we developed a swine model for neonatal PVD showing clinical features resembling those found in patients in terms of pulmonary hemodynamics and abnormalities in the structure of the right ventricle and pulmonary vascular remodeling (**Chapter 3-4**). In this model for neonatal PVD we observed structural and functional changes in the pulmonary microvasculature that are still present several weeks after re-exposure to normoxia. Evidence from the literature as well as our study suggests that there is an impaired endothelium-dependent vasodilatation in piglets with hypoxia-induced PH in the first three weeks after re-exposure to normoxia that is due to a reduced responsiveness to NO, probably caused by altered sensitivity and/or activity of soluble guanylyl cyclase (sGC), resulting is an impaired cGMP production (**Chapter 5**). In our neonatal swine model for PVD we also showed more severe and more persistent PVD, limited exercise capacity, and more pronounced right ventricular remodeling during

follow-up in normoxia in male compared to female swine, consistent with neonatal clinical practice (**Chapter 4**).

Just as in infancy, the severity and prognosis of PVD have been shown to be worse in male adults, while on the other hand the incidence of PH is higher in females.[18,19] Consistent with these sex-dependent clinical observations, we demonstrated that endothelial NO synthase (eNOS) inhibition reduced bradykinin-induced vasorelaxation to a greater extent in male as compared to female pulmonary small arteries. Furthermore, we found an increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine, reflecting the sex-specific heterogeneity in treatment response (**Chapter 6**).

Prolonged endothelial dysfunction and persistent structural abnormalities in the pulmonary vasculature, as shown in this thesis, likely contribute to the exercise intolerance and increased vulnerability to cardiopulmonary disease throughout life. Thus, exposure of the developing lung to injurious stimuli in the perinatal period, may also contribute to the development of PVD later in life, trough so-called "fetal or perinatal programming". It is well known that endothelial dysfunction is not only a key factor in vascular development, but also in maintenance of vascular structure and function throughout life, and thus plays a crucial role in the pathogenesis of adult PVD.[20-22] While endothelial function of the systemic and coronary circulation has been extensively investigated, studies into the endothelial function of the pulmonary vasculature have received less attention. Therefore, in the second part of this thesis we present the results of studies concerning pulmonary endothelial function and vascular control, with a particular focus on the NO-cGMP-PDE5 pathway (**Chapter 6-8**).

Chapter 8 showed that PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of the endothelin (ET) pathway, thereby precluding an additional vasodilator effect of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition. After administration of hemoglobin-based oxygen carrier (HBOC)-201, mimicking endothelial dysfunction, ET_A/ET_B blockade prevented, and eNOS inhibition reduced the pulmonary vasoconstriction (**Chapter 7**).

Together, the research described in **this thesis** shows that we successfully created and characterized a large animal model of neonatal PVD. In this model, the abnormalities of the pulmonary vasculature and right ventricle persists despite normalization of pulmonary artery pressure (PAP), suggesting that the effects of disruption of normal lung development early in life could have consequences in adulthood, as recognized in the developmental origin of health and disease (DOHaD) concept.[23] Furthermore, we found sex differences in response to early postnatal injury, but also found sex differences in pulmonary vasomotor control later in life. These significant sex differences in the regulation of pulmonary vascular tone by the NO-cGMP-PDE5 pathway may contribute to understanding sex differences in incidence, treatment response and prognosis of PVD. **This thesis** contributes to the increasing insight in the mechanisms underlying PVD as well as the impact on cardiovascular health later

in life, and may lead to novel therapeutic and medical management strategies, including early interventions in the neonatal period, to decrease both the short- and long-term health problems in these patients.

2. GENERAL DISCUSSION

2.1 Peri- and neonatal (mal)adaptation

There is growing evidence that disruption of normal pulmonary vascular development in the perinatal period contributes to the development of PVD in adulthood. This so-called "fetal or perinatal programming", also known as the DOHaD concept,[23] has gained a great deal of attention in recent years. In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequence of perinatal pulmonary vascular events is imperative. Therefore, the main focus of the first section of this thesis was to study the short- and long-term effects of injurious stimuli in the peri- and neonatal period to the pulmonary vasculature.

2.1.1 Methodological considerations

To identify basic pathological mechanisms underlying neonatal PVD and to examine long-term sequelae of damage to the developing neonatal lung, we developed a neonatal swine model. Already, a number of animal models for neonatal PVD have been developed, relying on several injurious stimuli such as mechanical ventilation, oxygen toxicity and infection and sterile inflammation.[24-30] It is most commonly modeled in mice and rats, however these models are not without substantial drawbacks. Like patients with neonatal PVD, full-term mice and rats are born in the saccular stage of lung development. However, these newborn rodent pups are competent for proper gas exchange, which is in marked contrast to preterm human neonates. Furthermore, the small size leads to concerns in regard to parenteral administration of substances and difficulty of intubation and mechanical ventilation.[31] Other experimental animal models for neonatal PVD have proven to be useful. Both preterm rabbits and lambs appear to be translationally relevant in modeling neonatal PVD, and their larger size makes instrumentation and mechanical ventilation possible.[31] Piglets represent an alternative to other large animal models. Swine lungs share many anatomical, histological, biochemical, and physiological features with human lungs [32] and the relevance of the developing pulmonary circulation of neonatal piglets to human infants has been established already in the early '80s.[33,34] Although alveolar multiplication occurs faster in piglets (2-4 weeks compared to 3 years in human infants), the morphological development of pulmonary architecture in swine is comparable with that in humans.[32]

The use of oxygen as an injurious stimulus is likely to remain a key driver of pathology in experimental animal models of neonatal PVD. Both hyperoxia-based models,[35-38] as well

as models with hypoxia-induced PVD have been established.[39-42] In the clinical setting, however, premature infants experience episodes of intermittent hypoxia, normoxia (relative hyperoxia), and hyperoxia (due to high levels of supplemental oxygen). Therefore, we developed a neonatal swine model based on chronic exposure to a hypoxic environment, interspersed with daily re-exposure to normoxia for a short period and followed by hyperoxia during surgery and re-exposure to normoxia during follow-up. These alternations in oxygen tension in the neonatal period result in activation of various signal transduction pathways and transcription factors, especially hypoxia-inducible factors (HIF). Under hypoxic conditions, both HIF-1α and HIF-2α accumulate instantaneously, while HIF-1α, but not HIF-2α, protein disappears when hypoxia is sustained (>12h).[43] Re-oxygenation results in rapid degradation of both HIF-1α and HIF-2α.[44] Both HIF isomers have distinct roles in the pulmonary vascular response to hypoxia; HIF-1α promotes pulmonary vascular smooth muscle cell proliferation, whereas HIF-2α promotes pulmonary vascular endothelial cell proliferation.[45] Thus, in premature neonates as well as in this swine model, it is likely that both HIF-1α and HIF-2α contribute to the pulmonary vascular remodeling and PH.

To our knowledge, this new swine model for neonatal PVD is the first to comprehensively investigate long-term sequelae of damage to the developing lung. It confirms clinical observations that PH induced by chronic hypoxia is transient, although structural and functional changes in the right ventricle and the lung vasculature were still present after follow-up. Our model is an excellent model to examine the effect of exercise training and/ or pharmacotherapy on long-term outcome, as well as the molecular mechanisms underlying potential beneficial effects.

2.1.2 NO pathway

The NO-cGMP signalling pathway (figure 1) is important for the adjustments in the pulmonary vasculature that accompany the transition from pre- to postnatal life following birth. There is increasing evidence that alterations in the NO-cGMP signalling pathway play an important role in the pathogenesis of neonatal PVD.[11-13,41]. Perinatal hypoxia impacts the functionality of different parts of the NO-cGMP pathway.

First, hypoxia-induced PH is accompanied by impaired endothelium-dependent vasodilation in the pulmonary vasculature. Evidence from the literature as well as **Chapter 5** of this thesis suggests that the impaired endothelium-dependent pulmonary vasodilation is due to 1) a decreased eNOS activity and 2) a reduced responsiveness to NO.[11-15,17] Both Fike et al. and Berkenbosch et al. found an impaired production of NO through a reduced eNOS protein expression and/or activity [11,13] or dysfunction of eNOS [12,41,46]. In accordance with these findings, we showed a reduced vasodilator response to bradykinin in isolated pulmonary small arteries from hypoxia-exposed piglets that was abrogated after eNOS inhibition, also suggesting a reduced eNOS activity or eNOS dysfunction, resulting in an impaired NO production. In the pulmonary vascular endothelium, endogenous NO is produced by

Figure 1. Schematic representation of NO-cGMP-PDE5 signalling pathway, highlighting several therapeutic strategies.

eNOS from the metabolism of L-arginine to L-citrulline. In turn, the citrulline produced is recycled to arginine, providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine.[47] In addition to the evidence showing an impaired NO-production in hypoxia-induced PH, it has been shown that L-citrulline supplementation attenuates PH in oxygen-induced lung injury, both in rodents and swine.[48,49] Likewise, a recent case report showed that oral L-citrulline supplementation ameliorated chronic PH and reduced oxygen requirement in a premature infant born at 25 weeks of gestation with severe BPD.[50]

Besides the impaired production of NO, there is also evidence for a reduced responsiveness to NO. In agreement with findings of other studies,[11,46] chronic postnatal hypoxia was associated with a diminished vasodilator responsiveness to the exogenous NO-donor SNP in vivo in our study (**Chapter 5**). This reduced responsiveness of the pulmonary vasculature to NO could explain that no important clinical benefit was seen in preterm infant with respiratory failure treated with inhaled NO.[51-53]

Thus, secondly, the apparent reduction in responsiveness to NO suggests that there are disruptions in the NO-cGMP pathway more downstream to eNOS/NO production. sGC is the main enzyme activated by NO and catalyses the conversion of GTP into the second messenger cGMP in pulmonary vascular smooth muscle cells, causing vasorelaxation. A major prerequisite for the NO-induced activation is the presence of the reduced Fe^{2+} heme moiety. Oxidative stress, as for instance in hypoxia, causes removal or oxidation to Fe^{3+}, lead-

ing to the formation of an NO-insensitive form of the enzyme.[54,55] This implies that sGC also may be a fundamental mechanism that influences vascular structure and tone. Several in vivo and in vitro studies investigated the effect of sGC activators and stimulators in acute and chronic hypoxia. In acute hypoxia, it has been shown that sGC stimulation reversed the pulmonary vasoconstrictor response in pigs[56] and attenuate pulmonary hypoxic vasoconstriction in isolated perfused mouse lung.[57] In chronic hypoxia, sGC activation reduced PH, right ventricular hypertrophy and structural remodelling of the pulmonary vasculature in mice[57] and inhibit or reverse the development of chronic hypoxic PH in mice[58] and rats.[59,60] **Chapter 5** showed that the effect of PDE5 inhibition tended to be smaller at rest in the pulmonary vasculature of hypoxia-exposed as compared to normoxia-exposed piglets, suggesting an impaired cGMP production after chronic exposure to hypoxia. This implies that the reduced responsiveness to NO in vivo most likely is caused by altered sensitivity and/or activity of sGC. Interestingly, the effect of PDE5 inhibition during exercise was significantly larger in the pulmonary vasculature of hypoxia-exposed piglets. We hypothesize that the significant increase in PAP during incremental exercise causes secretion of natriuretic peptides by cardiomyocytes in response to cardiac stretch,[61] and thus particulate guanylyl cyclase activation, resulting in a normalisation of cGMP production. This hypothesis is supported by the highly correlated vasodilator effect of PDE5 and PAP. **Chapter 5** adds important information by showing that alterations in the NO-cGMP signalling pathway are still present several weeks after re-exposure to normoxia.

2.1.3 Sex differences

Throughout their lifespan, males generally have worse outcomes in PVD as compared to females. This gender disparity is particularly evident in preterm infants and is most marked in the respiratory morbidity of these preterm infants.[62,63] The prevalence of BPD is higher in male as compared to female premature infants,[64] and being male is also associated with more severe disease and thus a higher risk for the development neonatal PVD.[65] A meta-analysis by Liptzin et al. including data from over 500,000 preterm newborn infants highlighted a sex ratio ranging from 1.22 ($p < 0.05$) in favor of males for BPD compared to females.[66] The etiology of this disparity is mostly undetermined, but likely involves structural, genetic, physiologic and hormonal differences. Still, little is known about sex differences in the long-term outcome of neonatal PVD.

Consistent with clinical observations, significant sex differences were present in **Chapter 4** of this thesis. Male hypoxia-exposed piglets demonstrated more severe and more persistent disease that female hypoxia-exposed piglets, as evidenced by a higher PAP, that persisted for a longer period, and more pronounced right ventricular dilatation. Sartori et al. showed a greater altitude-induced increase in systolic PAP in young adults who had had transient PH.[44] Re-analyses of these data in **Chapter 4**, showed that males with perinatal transient PH displayed significantly higher systolic PAP at high altitude than both their controls and

females with perinatal transient PH, while baseline systolic PAP levels were not significantly different between all groups. These data suggest that a transient perinatal insult to the pulmonary circulation results in a higher pulmonary vasoreactivity in males, but not in females. Future studies are required to investigate the mechanisms underlying these sex-related differences in (neonatal) PVD.

2.1.4 DOHaD

The DOHaD hypothesis, formerly known as the "Barker" or "Fetal Origins of Adult Disease" hypothesis, postulates that exposure to certain environmental influences during crucial periods of development and growth may have significant consequences for an individual's short- and long-term health.[23,67] This concept has gained a great deal of attention in recent years, especially in pediatrics because of the dramatically increased survival of premature babies. Since approximately 10% of births are preterm, a growing cohort of prematurely born survivors reaches adolescence.[1,68] While the majority of research in this field has focused on the developmental origins of metabolic disease, it is increasingly recognized that disruption of normal pulmonary vascular development in the perinatal period contributes to the development of (pulmonary) vascular disease in adulthood. Already in the late 1990's, Sartori et al. showed a greater altitude-induced increase in systolic PAP in young adults who had had transient PH.[44] It has also been shown that PAP is elevated in offspring of mothers with preeclampsia, demonstrating that placental hypoxia causes pulmonary vascular dysfunction.[69] Lewandowski et al. showed with cardiac magnetic resonance (CMR) imaging that preterm birth is associated with global myocardial structural and functional differences even in adult life, with potentially clinically significant impairments in right ventricular systolic function.[70,71] In **Chapter 4** we demonstrated that exposure to chronic hypoxia in the neonatal period leads to a loss of pulmonary vasodilator capacity and a limited exercise capacity shortly after re-exposure to normoxia and, even more important, to structural and functional changes in the right ventricle and the lung vasculature that were still present after long-term follow-up. These data are consistent with the DOHaD hypothesis, in which a transient perinatal insult to the pulmonary circulation has persistent effects into adulthood. This possibly results in an increased risk for cardiovascular events later in life, like right ventricular failure, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.

2.2 Endothelial function in the adolescent pulmonary vasculature

Prolonged endothelial dysfunction and persistent structural abnormalities in the pulmonary vasculature, as shown in **Chapter 4**, likely contribute to the exercise intolerance and increased vulnerability to cardiopulmonary disease throughout life (DOHaD hypothesis). It is well known that endothelial dysfunction not only is a key factor in vascular development, but also maintenance of vascular structure and function throughout life, and thus plays a

crucial role in the pathogenesis of adult PVD.[20-22] While the endothelial function of the systemic and coronary circulation has been extensively investigated, the endothelial function of the pulmonary vasculature has received less attention. Therefore, the main focus of the second section of this thesis was to study the pulmonary endothelial function and vascular control in adolescence, with a particular focus on the NO-cGMP-PDE5 pathway.

2.2.1 NO-pathway

PH is associated with alterations in pulmonary vascular function and structure, resulting in an increased pulmonary vascular resistance (PVR) and thereby right ventricular afterload. The regulation of PVR occurs by changing the diameter of blood vessels by both passive (structural) and active (smooth muscle tone) influences. Pulmonary vascular tone is the result of a complex interplay between vasodilator and vasoconstrictor influences.[72] The vascular endothelium releases a variety of these vasoactive substances, including NO, prostanoids and ET, which play an important role in vasomotor control. Endothelial dysfunction, therefore, plays a crucial role in the pathogenesis of adult PVD.

HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction. HBOC-201-induced vasoconstriction has been ascribed to scavenging of NO.[73-76] Besides the disruption of the NO-mediated cascade, we found that the pressor effects of HBOC-201 results from an upregulation of ET production (**Chapter 7**). In accordance with these findings, several studies indicate that NO limits the influence of ET in the pulmonary vasculature. In swine pulmonary vasculature, combined ET_A and ET_B receptor blockade with tezosentan resulted in a larger decrease in pulmonary vascular resistance in the presence of NO synthase inhibition, as compared to the effect of combined ET_A and ET_B receptor blockade under control conditions.[77] Wiley et al. showed a direct modulatory effect of NO on the ET receptor binding,[78] whereas Kelly et al. showed that NO decreases ET secretion through the activation of sGC in pulmonary arterial endothelial cells.[79] Consistent with the findings of Kelly et al., we demonstrated that PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of ET, thereby precluding an additive vasodilator effect of combined blockade of these two vasoconstrictor pathways with PDE5 inhibition and ET receptor blockade.

Thus, NO induces pulmonary vasodilation not only through a direct effect on vascular smooth muscle cell via production of cGMP but also indirectly through inhibition of ET. Interactions between mechanisms involved in the regulation of pulmonary vascular tone must be considered in the development of new treatment strategies.

2.2.2 Sex differences

Just as there are sex difference in the prevalence and severity of neonatal PVD, there are also sex differences shown in PVD in adulthood. Although the incidence of PH is higher in females, the severity and prognosis of PVD, like in neonates, have been shown to be

worse in male subjects.[18,19] Until now, studies concerning sex differences in PH have mainly focused on the role of sex hormones, particularly female reproductive hormones. The effects of estrogens on the pulmonary vasculature are mediated trough both non-genomic (rapid) and genomic mechanisms. Via non-genomic mechanisms, it enhances the production of nitric oxide by upregulation of eNOS.[80-84] Estrogen receptor-dependent mechanisms, the genomic pathway, increases eNOS mRNA levels and eNOS activity in pulmonary endothelial cells.[82,84,85] Additionally, it is well known that estrogens downregulate gene expression of ET. However, the effects of estrogen on the pulmonary vasculature are complex and remain incompletely understood.[82,84,86]

Our study of sex differences in the regulation of pulmonary vascular tone also showed significant differences between male and female swine, both in vivo and in vitro (**Chapter 6**). NO synthase inhibition reduced bradykinin-induced vasorelaxation to a greater extent in male as compared to female in isolated pulmonary small arteries, which is consistent with observations that PVD is often more severe in men as compared to women. In vivo, however, we found comparable pulmonary vasoconstriction after administration of NO synthase inhibition. Possible explanations for this apparent discrepancy are 1) the absence of eNOS activation by circulating estrogens in vitro,[80-84] 2) the absence of contribution of nNOS in vitro,[87,88] 3) the different signaling pathways in receptor-mediated eNOS activation and shear stress-mediated eNOS activation[89,90] and 4) the presence of an unidentified alternative vasodilator pathway in NO signaling in females, in contrast to solely through cGMP-PKG-PDE5 in males.[91,92]

Furthermore, we demonstrated an increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine, which is consistent with recent post-hoc analyses of the PHIRST and SUPER trials showing that PDE5 inhibition leads to a greater improvement of 6 minute walking distance in male as compared to female patients with PH.[93,94] Finally, we found that concomitant NO synthase-inhibition enhanced the vasodilator responses to PDE5 inhibition at rest and during bradykinin-induced vasodilation, but only in females, suggesting that loss of endothelial function may not interfere with (males) or even enhance (females) the pulmonary vasodilator responses to PDE5 in patients with PH. Together, these results reflect the sex-specific heterogeneity in treatment response. Future studies are required to investigate the mechanisms underlying these sex-related differences in pulmonary vascular control mechanisms. A better understanding of the sex differences in pulmonary vascular control may allow for future therapeutic interventions in adult patients with PVD.

3. CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Several pathophysiologic insults in the prenatal and early postnatal period can disrupt normal pulmonary vascular development both in terms of the impaired alveolarization and dysmorphic vascular growth and, consequently, can lead to a variety of neonatal PVD. Preterm birth in itself already hampers the development of the lung, not only because preterm babies are born in a critical stage of lung development (saccular or alveolar stage), but also by exposing their immature lungs to several injurious stimuli, including hypoxia and/or hyperoxia, mechanical ventilation, infection, inflammation and oxidative stress.

According to the DOHaD concept,[23] disruption of normal pulmonary vascular development in the perinatal period not only contributes to morbidity and mortality in the neonatal period, but has also been shown to significantly increase the risk for a variety of health problems later in life. PVD can lead to endothelial dysfunction, vascular remodeling, and ultimately to cardiac dysfunction (right ventricular hypertrophy and failure). As improved neonatal care has dramatically improved the survival of premature babies, we may face a new epidemic of cardiovascular disease based on an as yet underestimated burden of PVD that originates from the time of birth.

Until now, most studies concerning long-term health outcomes of (extremely) premature infants have focused on respiratory outcomes. Indeed, it is well known that pulmonary function in childhood and adolescence is impaired in these patients, and this is even more pronounced in survivors of neonatal PVD like BPD.[95-100]

Less is known about cardiovascular function in survivors of neonatal PVD. The swine model of neonatal PVD we developed, shows clinical features resembling those found in patients with neonatal PVD (including PH), in terms of pulmonary hemodynamics, abnormalities in the structure of the right ventricle and disruptions in normal lung development (vascular remodeling). Consistent with clinical data, male swine develop more severe, and more persistent PVD, have a limited exercise capacity and exhibit more pronounced right ventricular remodeling during follow-up in normoxia. Therefore, our model is an excellent model to examine long-term sequelae of a perinatal hypoxic insult, the effect of therapy on long-term outcome and the course of the disease. It enables us to understand the mechanisms and potentially lead to new therapeutic strategies.

3.1 Long term sequelae of a perinatal hypoxic insult

It has been well-documented that exercise capacity is limited in long-term survivors of prematurity and/or neonatal PVD.[101-105] Exercise capacity is a resultant of pulmonary function and cardiovascular performance.[106,107] Until now, most studies concerning long-term health outcomes of (extremely) premature infants have focused on respiratory outcomes. Indeed, it is well known that pulmonary function in childhood and adolescence is impaired in these

patients. However, there is increasing evidence that the limited exercise capacity is not only due to the impaired pulmonary function, but is also due to cardiovascular dysfunction. Use of CMR imaging revealed distinct differences in mass, geometry and function of both the left and right ventricle.[70,71] Differences in right ventricular mass and function were proportionally greater than in the left ventricle. This observation corresponds well with our findings that, despite normalization of PAP, structural and functional changes in the right ventricle and the lung vasculature were still present after long-term follow-up. These alterations in cardiac function and structure may increase the risk for cardiovascular events later in life, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.

Nevertheless, the studies mentioned above were all performed under resting conditions. By subjecting the cardiopulmonary system to stress with exercise testing, subtle dynamic abnormalities that are not apparent during conventional static tests may be revealed. This may lead to early recognition of altered cardiac remodelling and heart failure, which is important for optimal management of these patients to improve long-term outcomes or even prevent future disease. Therefore, we are currently investigating cardiac function and structure during exercise in both controls and prematurely born adolescents, with and without neonatal PVD, by using CMR imaging.

3.2 Effect of therapy on long-term outcome and potential new therapeutic strategies

Several interventions in the neonatal period, including oral L-citrulline,[41,48] thromboxane inhibition,[108] angiotensin II type 1 receptor blockade[39] and ET-A receptor antagonists,[42] have been shown to ameliorate PH and/or pulmonary vascular remodeling in a similar model. However, the long-term outcome of these interventions remains to be established. Our porcine model is an excellent model to examine therapy on long-term outcome, as well as the molecular mechanisms underlying potential beneficial effects.

Evidence from several animal studies as well as evidence from our neonatal swine model suggests that the impaired endothelium-dependent vasodilatation found in PVD is due to a reduced responsiveness to NO, probably caused by altered sensitivity and/or activity of sGC, resulting is an impaired cGMP production. sGC stimulation and/or activation reduced or even completely reversed the pulmonary hypoxic pulmonary vasoconstriction, as well as reduce the structural remodelling of the pulmonary vasculature and right ventricular hypertrophy.[56,57,58,59,60] **Chapter 5** of this thesis adds important information to these previous studies by showing that alterations in the NO-pathway in a neonatal porcine model are still present several weeks after re-exposure to normoxia. Together, these data suggest that sGC stimulators/activators could be of benefit as a novel treatment strategy to stop or even reverse neonatal PVD, especially since the use of inhaled NO for preterm infants with respiratory failure is currently under debate.[51,109-111]

Besides pharmacological treatment, it would be very interesting to investigate the effect of exercise training on long-term outcome, as exercise training has been shown to be beneficial in adult patients with pulmonary arterial hypertension of any cause. Training is associated with improved pulmonary perfusion,[36] blood gas exchange[112] and right ventricular function.[113-115] Furthermore, exercise training has been shown to reduce smooth muscle cell proliferation.[116] Altogether, these beneficial effects improve exercise capacity and may reduce PAP, thereby improving quality of life.[116,117] Our swine model of neonatal PVD is the first that allows exercise-testing, but until now only the cardiovascular responses to acute exercise were assessed. Future studies should investigate the effect of exercise training on long-term outcome.

Beetroot juice provides another novel therapeutic target in PVD (figure 1). It has gained the attention of scientists because of its beneficial effects on cardiovascular health at both the macro-circulatory and the microcirculatory levels,[118-123] owing to the nitrate present in this food. Nitrate is reduced to nitrite in the oral cavity by commensal facultative anaerobic bacteria by the action of nitrate reductase enzymes, followed by the further reduction of nitrite to bioactive NO. This so-called nitrate-nitrite-NO pathway represents an important alternative source of NO to the classical L-arginine–NO-synthase pathway and is enhanced in hypoxia/ischaemia. Therefore, it might serve as a backup system to ensure NO bioactivity, particularly in situations when the endogenous NO-synthase dependent pathway is dysfunctional like in PVD.[124-126] We have recently performed some experiments to see if beetroot juice could mitigate PH and consequent right ventricular remodeling. However, the group was too small to detect significant changes, so it will be expanded in the future.

Finally, we aim to investigate if infusion of healthy neonatal endothelial colony forming cells (ECFCs) can be used as a therapy to prevent or reduce the development of neonatal PVD in our neonatal swine model of PVD. ECFCs are circulating bone-marrow-derived cells, which play two major roles in the cardiovascular system; endothelial healing and neoangiogenesis. Decreased numbers of these cells may directly hamper pulmonary vascular development and/or may contribute to the vulnerability of the developing pulmonary vasculature to injurious stimuli. Nowadays, there is increasing evidence that a decrease in number, but more importantly dysfunction of ECFCs in preterm infants may be a crucial step in the development of PVD in preterm infants.[127-129]

3.3 Course of disease

In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequence of perinatal pulmonary vascular events represents an emerging field. Since this is a relatively new patient population, there is a lack of consensus for the follow-up of high risk (formerly premature) patients. The American Heart Association and American Thoracic Society have made a guideline for diagnosis, evaluation and monitoring of pediatric patients with PH.[130] They recommend monitoring of children with

PH (or other neonatal PVD) in a multidisciplinary setting. It is a future challenge to evolve a follow-up program for (neonatal) PVD that facilitates an optimal transition of the patient from the pediatric to the adult setting and ensure early detection of health problems in these patients, thereby diminishing cardiovascular morbidity and mortality and improving quality of life. Furthermore, development of an exercise training program tailored for prematurely born adolescents, who may be at higher risk for early-onset adult diseases, should be considered. Establishing early, adequate levels of fitness and activity will have beneficial effects on overall health, thereby playing an important role in the prevention of diseases at long term. Thus, it should be a cornerstone in the follow-up of formerly premature adults.

In conclusion, **this thesis** contributes to the rapidly increasing insight in the process that can lead to neonatal PVD and its long-term consequences. The described research can lead to specific therapies that reduces or even reverses structural and functional changes of the pulmonary vasculature and right ventricle, thereby preventing cardiovascular diseases and improving the long-term outcome of prematurely born adults.

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