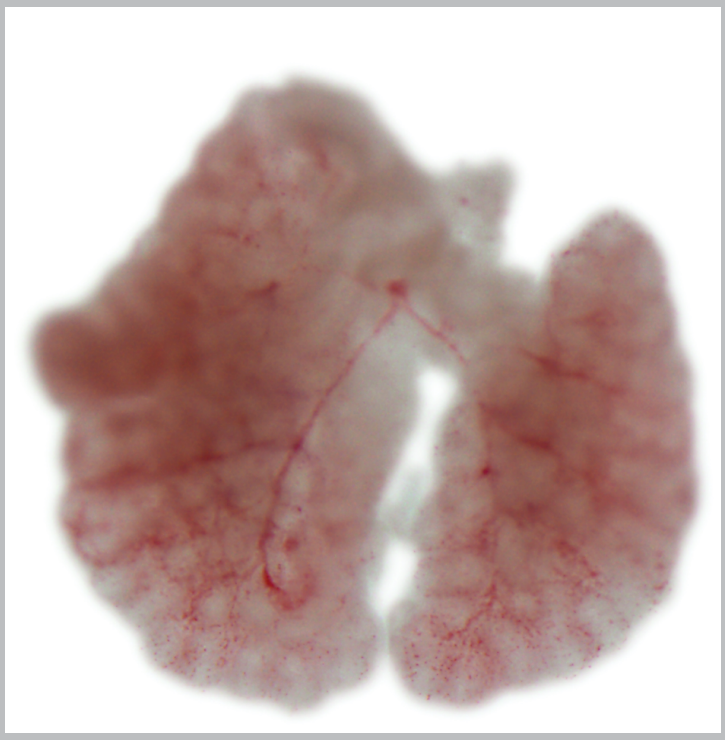
# Congenital Diaphragmatic Hernia: a vascular disease

Functional and structural studies of the pulmonary vasculature in Congenital Diaphragmatic Hernia



Ilona Sluiter

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Ilona Sluiter

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# Congenital Diaphragmatic Hernia: a vascular disease
# Functional and structural studies of the pulmonary vasculature in Congenital Diaphragmatic Hernia

## Congenitale hernia diafragmatica: een vasculaire ziekte
## Functionele en structurele studies van het pulmonale vaatstelsel in congenitale hernia diafragmatica

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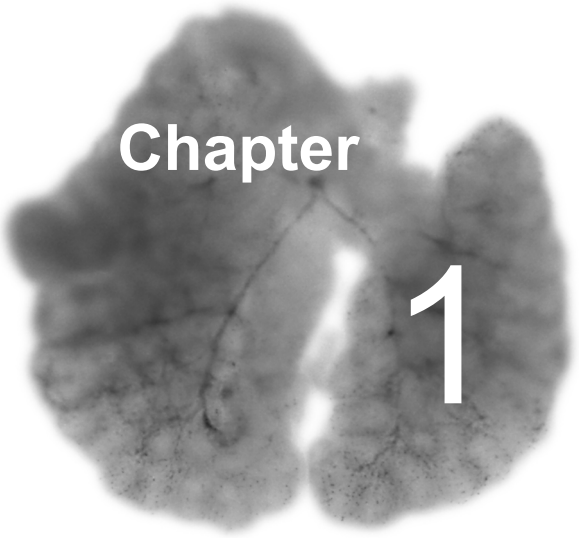

# Chapter 1

## Congenital Diaphragmatic Hernia: Still a moving target

I. Sluiter, C.P. van de Ven, R.M.H. Wijnen, D. Tibboel

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## Abstract

The primary therapeutic target of congenital diaphragmatic hernia (CDH) patients has shifted from emergency surgical repair towards a non-operative emergency of the newborn treated by interdisciplinary teams. The increased understanding of the epidemiological and pathophysiological aspects of CDH have lead to an improved knowledge and application of prenatal diagnosis, postnatal ventilation strategies, treatment of associated pulmonary hypertension and the role of extracorporeal membrane oxygenation therapy. In the surgical field, the perspectives have changed with delayed CDH repair, the introduction of minimally invasive surgery and use of prosthetic material for closure of large defects.

With decreased mortality, long-term multi-organ morbidity has increased in some survivors. In the near future, randomized controlled trials on different aspects of therapy will determine evidence-based optimal care.

## Introduction

Until the late 1980s, emergency surgery for patients with congenital diaphragmatic hernia (CDH) was considered the primary goal, based on the mechanical concepts of providing space for lung expansion and growth. Temporary improvement in the postoperative phase was known as the so-called 'honeymoon' period, preceding deterioration and death in many patients.
Until the late 1980s overall survival rates of 50% were published,[1-2] while individual centers published data on a wide range of survival from 20% to 70%.[3-5] The great disparities in survival were likely based on case selection. In the late 1980s, several centers switched from emergency surgery to delayed operative repair after patient stabilization. As a consequence, the treatment of patients with CDH evolved to a non-operative emergency of the newborn treated by interdisciplinary teams of neonatologists, pediatric intensivists and pediatric surgeons.
The institution of the CDH registry in 1995,[6] containing over 5000 patients with CDH today, has had a major impact on our knowledge of treatment strategies and risk stratification based on epidemiological approach. This development parallels international workshops dedicated to CDH (first organized in 2000) and the institution of the CDH-EURO Consortium in 2006. This initiative was primary aimed at international consensus on treatment modalities and the design of both pre- and postnatally evidence-based treatment strategies. In the mean time, operative procedures were developed in different species, including human,[7] resulting in increased understanding of the development of the diaphragmatic defect, as well as the pulmonary abnormalities in CDH.[8-11] The nitrofen model, a toxicological based rodent model of CDH has provided many answers in this respect.

Over the years a diverse spectrum of therapeutic approaches has developed:

1. Prenatal
    - Detailed evaluation of the fetus by ultrasound and/or fetal magnetic resonance imaging (MRI) to predict high risk fetuses and outcome.
    - Open and minimally invasive procedures on the fetus.
2. Postnatal (non-operative care)
    - Gentle ventilation to prevent pulmonary damage.
    - Application of inhaled nitric oxide (NO) to treat pulmonary hypertension.
    - Extracorporeal membrane oxygenation (ECMO) in selected cases.

3. Postnatal (surgical)
    - Early versus late closure of the defect.
    - Open versus minimal invasive repair.
    - The choice of material for closure of large defects.

Many of these strategies were originally described as 'magic bullets', mainly based on studies conducted in a single center without appropriate design and power to prove benefit of the newly applied therapeutic modality. Therefore, in considering CDH, it is extremely important to identify the players (medical specialists of different origin) and the subject they render important (Table 1). Today no standardized method of reporting case series of CDH is internationally agreed upon, nor the minimum demographic characteristics that should be available to properly evaluate a publication. Therefore, without detailed information with regards to population characteristics such as: (i) inborn versus outborn; (ii) prenatal versus postnatal diagnosis; (iii) standardized postnatal care versus local best practice; (iv) ECMO facility - yes or no; (v) open versus thoracoscopic repair; (vi) survival until discharge versus long term morbidity, it remains virtually impossible to perform benchmarking of treatment results in different centers.

**Table 1. Subjects considered important by medical specialists involved in the interdisciplinary treatment of congenital diaphragmatic hernia patients**

| Specialty | Confounder |
|---|---|
| Obstetrics | Quality of postnatal care |
| Neonatologists | Standard of care / ventilatory mode |
| Surgeons | Patient selection |
| ECMO specialists | Case selection |
| Developmental pediatrics | Selection-bias / mono specialism |
| Gastroentrologists | GER is the key issue |

ECMO, extracorporeal membrane oxygenation; GER, gastroesophageal reflux

A number of landmark studies can be identified, which changed the treatment algorithms for CDH significantly. For postnatal care the following publications should be mentioned:

- CDH – a tail of two cities: the Boston experiences[1] and the Toronto experience.[12]
- CDH: survival treated with very delayed surgery, spontaneous respiration and no chest tube.[13]
- UK collaborative randomized trial of neonatal ECMO.[14]
- Inhaled nitric oxide and hypoxic respiratory failure in infants with CDH.[15]
- Low dose nitric oxide therapy for persistent pulmonary hypertension of the newborn.[16]

- Current surgical management of CDH: a report of the Congenital Diaphragmatic Hernia Study group.[6]
- Late versus early surgical correction for CDH in newborn infants.[17]
- ECMO in infants with CDH: a systemic review of evidence.[18]

## Epidemiology of CDH

CDH has a worldwide incidence of 1 in 2500 – 3000 live births. In population-based studies the mortality remains high, while the CDH registry shows a timely decrease in mortality based on the data of over 50 centers worldwide. (current survival rate is 67% in the CDH registry).[19] Recently, a number of molecular genetic[9] as well as environmental[20] factors have been identified as playing a role in the etiology and pathogenesis of CDH. A specific CDH gene has not been identified yet, at least not in cases of isolated CDH. A number of candidate genes such as NR2F2, CHD2, and DISP-1 have been suggested.[21-22] Increasingly, disturbances in the retinoic acid pathways are suggested as a contributing factor in the etiology of CDH.[8-10]
As part of the debate of the maturational development of the pulmonary parenchyma, more and more investigators aim nowadays at understanding the pulmonary vascular abnormalities from a developmental point of view, integrating molecular genetics approaches and developmental pathways.[23-24]

***Pathophysiology of CDH***

The degree of pulmonary hypoplasia has long been considered as the main determinant for long term outcome. Following the landmark publication of Wung et al.[13] identifying the deleterious effects of 'aggressive' ventilation, treatment teams nowadays use the gentle ventilation approach as their standard philosophy. The cornerstones of this approach include low peak inspiratory pressures, acceptance of a relative hypercapnia, as well as right to left shunting in the absence of neuromuscular paralysis. The high incidence of bronchopulmonary dysplasia of 35% is another argument to reconsider the vulnerability of the hypoplastic lungs, in the absence of a proven primary surfactant deficiency.[25] In addition to pulmonary hypoplasia and preventing iatrogenic lung damage, the significance of pulmonary hypertension of the pathophysiology of CDH has emerged.[26] The decreased cross-sectional area and morphological vascular abnormalities, consisting of increased and extended muscularization of resistant vessels and medial hypertrophy, were described many years ago.[27] However, the consequences of the abnormal pulmonary vascular development for our understanding and therapeutic opportunities have not been investigated in a structured way.

***Prenatal diagnosis***

Pediatric surgeons, obstetricians and radiologists have put a major effort into our understanding of the three dimensional relationship between developing lungs, heart and abnormal position of the 'intra-abdominal' organs. A number of measures, such as the lung to head ratio (LHR),[28] nowadays observed/expected LHR (O/E LHR)[29] as well as the position of the liver and left- or right-sided CDH, are considered as predictors of survival, although debated by others.[30-31]

Apart form ultrasound, fetal MRI is suggested to describe the anomaly more accurately, including accurate measurements of lung volume.[32] These prenatal tests have also guided case selection for the application of fetal tracheal occlusion as a temporary measure to enhance lung growth. Today, fetoscopic tracheal occlusion is a subject of debate following the US experience[33] in which no difference was identified between patients treated with fetoscopic tracheal occlusion and patients treated postnatally using a standard protocol. By contrast, the ongoing fetoscopic endoluminal tracheal occlusion (FETO) experience in highly selected cases in Europe appears promising compared to historical controls.[34] Currently, three centers including Leuven, London and Barcelona are assessing the role of FETO in moderately severe CDH fetuses to improve mortality and long term morbidity in the tracheal occlusion to accelerate lung growth (TOTAL) trial (details available online). Case selection for fetoscopic tracheal occlusion has only been based on measures of pulmonary hypoplasia using sonographic LHR and liver position. Recently, the response of the fetal pulmonary circulation by Doppler flow measurement has been investigated by hyperoxygenation test (breathing 60% oxygen by the mother)[35] to predict postnatal pulmonary vascular response. Two major drawbacks regarding case selection for fetoscopic tracheal occlusion can be identified:

1. So far the predictive value O/E LHR is mainly based on data collected in different centers (low and high volume; different countries and level of expertise) without standardized postnatal care.
2. The fetal pulmonary vascular response takes place in a divergent circulatory stage from the human newborn and tremendous transitional changes occur in pulmonary vascular flow.

Properly designed randomized control trials (RCTs) are warranted to identify the relative contribution of these antenatal parameters. Moreover, risk stratification of individual patients and admittance to high volume centers with interdisciplinary treatment teams can be accomplished in this way.[36]

## Treatment

***Ventilation strategies***

As previously mentioned, the vulnerability of the hypoplastic lungs is increasingly recognized. Unfortunately, attempts to reach normocapnia have seriously damaged many newborns with CDH. Nowadays, the concept of gentle ventilation is widely used. Many centers restrict peak inspiratory pressures to 20 – 25 $cmH_2O$ and use high frequency oscillation (HFO) as a rescue therapy in case of $CO_2$ retention.[37] There are no data to show the superiority of either conventional ventilation (CV) or HFO as the primary ventilatory support. Since HFO is used as rescue therapy in selected patients, in which the inflammatory response of the lungs is already triggered, the outcome of patients with HFO is worse. These data are seriously flawed by selection bias. Recently the CDH-EURO Consortium started a RCT, the so-called VICI-trial (Dutch Trial Registry no 1310), on the use of CV or HFO as primary ventilatory mode from delivery. Primary endpoints of this study are the incidence of chronic lung disease and the number of ventilator-free days (evaluated at day 56).

Standardization of postnatal care is an essential step forward in the evolution of therapy in CDH and is recently accomplished through international collaboration.[38] This is prerequisite for comparison of treatment results and should be coupled to interdisciplinary long term pulmonary morbidity.

***Treatment of pulmonary hypertension***

The optimal treatment of CDH associated pulmonary hypertension is one of the major challenges in neonatal intensive care units. Support of the systemic circulation by variable dosages and/or combinations of vasopressor drugs and the acceptance of a variable degree of right-left shunting through the foramen ovale and ductus arteriosus are cornerstones of the therapeutic approach. Well-established therapies to lower the pulmonary vascular resistances, such as inhaled nitric oxide (NO) and/or phosphodiesterase (PDE) inhibitors, such as sildenafil, have not been properly investigated in CDH. A number of landmark studies on the use and effects of inhaled NO[15-16] were seriously underpowered for CDH and certainly not primarily aimed to evaluate the role of inhaled NO in the treatment of CDH. Approximately 30% of newborns with CDH respond to inhaled NO, and a debate exists on the definition of a positive response. With regards to PDE-inhibitors, both dosing as well as intervals and timing (early versus late) are subjects of investigation,[39] while the intravenous form of sildenafil is sparsely available. It is questionable whether the therapeutic target is to enhance vasodilation, as no data in human have proven a nitric oxide synthase deficiency. On the other hand, recent data have revealed increased endothelin plasma level as potential predictor of outcome.[26] Whether this is cause or effect is questionable.

In the near future, RCTs should be started to evaluate the different pharmacological approaches of treating pulmonary hypertension in CDH for single drug use, or in combination, taking into account to different metabolic pathways.[40]
Repeated echocardiograms are considered the best way to evaluate the degree of pulmonary hypertension and assess right heart function. Using serial echocardiograms, different therapies (inhaled NO; PDE inhibitors; endothelin receptor blockage; prostaglandin E1 to 'open' the ductus arteriosus) can be evaluated non-invasively and longitudinally in individual patients.
For many years the pre-postductal saturation difference was considered the best non-invasive way to evaluate right-left shunting, and may clinics aimed at decreasing the difference to <10% as a therapeutic target. Nowadays accepting that a high or at least increased pulmonary resistance is present in CDH patients has altered our practice. The therapeutic strategy should be to unload the right ventricle, for prolonged periods of time.

***Role of ECMO***

The role of ECMO in the treatment algorithms of CDH remains controversial, particularly since ECMO is not universally available. Solid guidelines for early transfer of high risk babies are prerequisite to identify to contribution of ECMO to individual survival rates.
The only trial examining the role of ECMO in neonatal respiratory insufficiency failed to show increased survival in CDH.[14] However, only 18 CDH patients were included, so the trial was underpowered to answer this specific question.
A recent report based on the CDH study group data showed that the duration of ECMO, nature of diaphragmatic repair and type of abdominal closure, either primary or with the use of a patch, and certain co-morbidities, such as severe cardiac anomalies, were independently associated with outcome.[19]
Over the years, the utilization of ECMO has decreased, but still ranges from 15% to 40%. The overall survival of CDH patients following ECMO is 51%.[41] Given the severe pulmonary hypertension in these patients, theoretically veno-venous-ECMO should be the primary mode, allowing oxygen-rich blood to enter the pulmonary circulation. However, in practice many patients need to be converted to veno-arterial-ECMO, such that veno-arterial ECMO has become the primary mode of ECMO in many centers. Whether the repair of the diaphragmatic defect should be performed on or off ECMO is institutionally dependent and no data conclusively support an optimal approach. A recent data analysis from the CDH Study Group revealed significantly more bleeding complications during repair on ECMO.[42] Bleeding complications may be reduced by using tranexamic acid or Amicar along with maintaining platelets >100 x $10^9$/L, reducing the activated clotting time and meticulous surgical technique. Some centers offer the highly debatable procedure of combining the ex-utero intrapartum treatment (EXIT) procedure with ECMO in the delivery room to prevent hypoxia, hemodynamic instability

barotrauma and acidosis.[43] Using this procedure, risk stratification of individual patients is critical.

## Surgery

Consensus exists that repair of the diaphragmatic defect should be done as a semi-elective procedure, but the definition of a stable patient remains a point of discussion. Important surgical aspects include the choice of either classical surgery versus a minimally invasive thoracoscopic approach. A recent meta-analysis revealed that thoracoscopic CDH repair had greater recurrence rates and operative times, but a similar survival rate and patch usage compared to open surgery.[44] For individual institutions, a learning curve has to be taken into account before benchmarking can take place. A minimal invasive repair will preclude the repair in the ICU as standard in many centers today to prevent transfer of vulnerable patients to the operating room.

***Timing of CDH repair***

With the evolution from an emergency operation shortly after birth to delayed surgery after an initial period of stabilization, the question remains: what is the right time for surgical repair? Two randomized trials showed no significant differences between early and late surgery, but risk stratification was difficult due to the small numbers.[45-46]

The CDH-EURO Consortium consensus states that surgical repair should be undertaken when following criteria are met:

- Mean arterial blood pressure that is normal for gestational age;
- Preductal saturation level between 85 and 95% with a fractional inspired oxygen <50%;
- Serum lactate <3 mmol/L;
- Urine production >2 mL/kg/h.

When ECMO is needed for stabilization, some centers advocate surgical repair on ECMO to allow lung expansion, ameliorate the mediastinal shift and alleviate post-operative risks due to surgical stress and increased abdominal pressure from reduction of viscera. A disadvantage of surgical repair on ECMO is the risk of hemorrhagic complications. The literature so far is equivocal about the advantage or disadvantage of surgical repair on ECMO.[42,47]

A standardized protocol to improve coagulation is important to reduce the risk of hemorrhagic complications; this is be done by decreasing the activated clotting time to 145 – 165 s, combined with the administration of either aminocaprioc acid or tranexamic

acid, for at least 24 h postoperatively and maintenance of platelets at around 100 x $10^9$/L. To reduce the thrombotic events in the ECMO circuit, the ECMO flow needs to be increased to 100 – 150 mL/kg/min. Our own data revealed that in 32 cases out of 129 patients received surgical repair on ECMO, surgical site bleeding was only 12.5%. The mortality rates and duration of mechanical ventilation were comparable between patients that underwent surgical repair on or off ECMO. Since there is selection bias favoring the group who were repaired off ECMO, this data may support repair on ECMO. New anti-coagulant products such as Tachosil® and Floseal® may further decrease intraoperative bleeding.

***Minimally Invasive Surgery for CDH***

Many institutions have reported successful minimally invasive surgery approaches to CDH. Initial laparoscopic attempts have been supplanted by the thoracoscopic approach which provides better visualization of the defect and increases working space after reduction of the intrathoracic content. Thoracic insufflation, with a $CO_2$ pressure of 4-5 mmHg, is helpful in reducing the viscera into the abdomen. Thoracoscopic repair was initially performed in less severe CDH patients with small defects, but more complicated cases have been successfully performed which require a patch closure. In our center, thoracoscopic repair is not offered in patients with liver herniation, during ECMO and in unstable patients. A recent meta-analysis showed an increased recurrence rate and a longer operative time in the thoracoscopic repair compared with open surgery.[44]

***Use of prosthetic material***

Keeping mind the importance of preserving the anatomical dome-shape of the diaphragm,[48-49] it is sometimes not possible to close the diaphragm defect primarily. In these cases, prosthetic material or muscle flaps can be used. Although polytetrafluorethylene (PTFE) is still widely used, newer bioprosthetic material such as porcine small intestinal submucosa-derived acellular biomatrix (Surgisis®) or porcine dermis-derived collagen matrix (Permacol®) have been developed and studied in this context. In retrospective case-series, the recurrence risk seems comparable between PFTE and Surgisis®.[50] Findings on the use of Permacol® were published only in one report; the results were promising but the smaller number precluded any statistical significance. Scarce reports in the use of muscle flaps (abdominal wall muscles or latissimus dorsi) are available, but fail to show better results than prosthetic material.

The development of autologous tissue using tissue engineering is very promising, but is still in an experimental stage requiring feasibility studies.[51] It is expected that the first clinical pilot studies will be started within five years. The goal of tissue engineering of the diaphragm is ingrowth of muscle cells into the scaffold, without the formation of scar

tissue. After the development of a muscular diaphragm, innervation of this regenerated organ can become the next step.

***Anti-reflux procedure***

Of the patients with CDH, 20 – 81% suffer from gastroesophageal reflux disease (GERD) after CDH repair.[52-53] Several causes have been suggested, such was weakened crurae of the diaphragm, an altered position and possible shortening of the esophagus or different relation between intra-abdominal and intrathoracic pressure. Also radial tension on the esophageal hiatus as result of repair of the diaphragm defect can lead to GERD.[54] Predictors for the development of GERD are liver herniation, need for patch repair and ECMO. The side of defect, an intrathoracic stomach and ventilation mode (CV versus HFO) have not been identified as significant predictors.[52,54]

A few reports mention a prophylactic fundoplication (PF) at the time of CDH repair. If the defect is repaired through a subcostal incision, a Nissen fundoplication or an incomplete wrap (Toupet or anterior hemiwrap) can be performed within the same procedure.

Performing a PF during CDH repair is not associated with major disadvantages, weight gain seems less impaired, and patients have fewer GERD-related symptoms.[52,55] One the other hand, only 30% of the patients without PF during initial surgery need surgical treatment for GERD in the first six months after CDH repair.[52,54] Not all patients with a PF are free of GERD symptoms: 17.6% are still symptomatic.[55] PF is not possible during a thoracoscopic procedure. In this era of minimal access approaches, an early anti-reflux procedure through laparoscopy in symptomatic GER is probably the treatment of choice. Because of the high incidence of GERD in CDH, early medical treatment is advised.[56] The contribution and timing of PF is not clear but should be based on clinical parameters.

## Long term outcomes and morbidity

With decreasing mortality in patients with CDH, short and long term morbidity of some survivors has increased and ushered in an interdisciplinary approach to follow-up. A mission statement by the American Academy of Pediatrics summarizes guidelines for post-discharge follow-up of these vulnerable patients.[56] Long term quality of life of both children and parents is highly dependent on the presence of dedicated CDH follow-up outpatient clinics involving pediatric gastroenterologists, pulmonologists, cardiologists, developmental pediatricians and physiotherapists. In this way, a tailor made intervention and surveillance program can be developed, maximizing the support of the individual patient. In this context, comparison between different centers becomes feasible by the

collection of prospective longitudinal data during the first decade of life as well as throughout adolescence and adulthood.[57-58]

It is desirable to have consensus about the timing and optimal timing of follow-up. These include repeated cardiac ultrasound and/or cardiac catheterization in individual patients, baby lung function testing,[59] ventilation-perfusion scans,[60] as well as standardized evaluation of GERD and developmental assessment.

The increased awareness of the presence of CDH by prenatal ultrasound may prevent hypoxic ischemic encephalopathy which occurred in many patients in the immediately postnatal period. On the other hand, the increased survival rates with our 'newer' therapeutic modalities results in a prolonged hospital stay of patients who previously would have died, and this increases the risk of developmental delay. Important financial incentives of institutions to create these dedicated teams may sometimes delay or 'prevent' this level of care and support.

## Conclusions

Patients with CDH remain a major challenge for pediatric surgeons and critical care specialists. Increased knowledge and improved outcomes are due to the emerging role of molecular genetics in our understanding of the etiology and pathogenesis, the progress in prenatal diagnosis, evolution of novel treatments options such as fetoscopic tracheal occlusion, the rich source of epidemiological data in the CDH registry, the use of standardized postnatal treatment protocols, and the institution of interdisciplinary follow-up teams. These advances will certainly contribute to better information for parents, proper risk stratification for benchmarking therapies and may lead to tailor made therapy for individual patients. International collaboration is essential to conduct RCTs on different diagnostic and therapeutic strategies to achieve evidence-based care and hopefully diminish both mortality and morbidity. CDH should still to be considered as a moving target.

**Practice points**

- CDH should be treated in high volume centers by experienced interdisciplinary teams.
- Fetal intervention for CDH should be only be practised at centers with significant experience in fetal therapy and only when included in randomized multicenter studies.
- CDH should be treated by standardized evidence-based postnatal treatment algorithms.
- CDH treatment results should be reported according to international guidelines of data publishing.
- CDH patients should receive long term follow-up by interdisciplinary teams.

**Research directions**

- To identify the primary defect and understand the differences in phenotype.
- To identify prenatal predictors of outcome and optimize patient selection for potential antenatal intervention.
- To design RCTs on non-surgical and surgical treatment modalities.
- To enhance our knowledge of the abnormal pulmonary vascular bed, using molecular biological and genetic approaches.
- To reach international consensus regarding an interdisciplinary follow-up plan.
- Developing preventive strategies based on knowledge of developmental pathways and epigenetic effects (e.g. the retinoic acid pathway).

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## Outline of this thesis

In this thesis we focus on pulmonary vascular disease (PVD) in the neonatal period. According to the new classification of Pediatric Pulmonary Hypertensive Vascular Disease;[1] neonatal pulmonary vascular disease can either be categorized as prenatal or developmental pulmonary hypertensive vascular disease, or as perinatal pulmonary vascular maladaptation. Despite this distinction considerable overlap exists between these two categories.
Clinically, neonatal PVD is characterized by severe respiratory insufficiency and hypoxemia as result of a persistence of the increased pulmonary vascular resistances. The changes within the pulmonary vasculature consist of vessels with a thickened wall, resulting in a smaller lumen and an abnormal vaso-reactive response. The early phases of pulmonary vascular disease seem to be largely reversible, because pharmacological treatment strategies in the postnatal period may induce recovery. However, in some cases this recovery fails and results in a fixed pulmonary hypertension, which is associated with right ventricular hypertrophy and failure and responsible for the high mortality and morbidity.

This thesis is divided in two major parts, describing studies related to the use of sildenafil in PVD, and studies related to the cellular mechanism leading to structural abnormalities of PVD.
The first aim was to elucidate the potential benefits of sildenafil as a therapeutic approach for PVD in neonates. We have analyzed the pharmacokinetics of sildenafil in neonates with PVD and subsequently studied the efficacy of sildenafil as a therapeutic strategy in a specific group of patients with neonatal PVD, congenital diaphragmatic hernia (CDH).
The second aim was to focus on the cellular basis of PVD, to obtain a better understanding of the normal and abnormal development of the pulmonary vasculature, especially the role of perivascular cells. Therefore, we analyzed the vascular smooth muscle cell and pericyte population to better understand the structural changes in neonatal PVD, with the special focus on PVD in infants with CDH.

***Chapter 1*** is an introduction to congenital diaphragmatic hernia (CDH), a complex congenital anomaly associated with pulmonary hypertension in which therapeutic options are scarcely investigated in a structured manner.

Part I is dedicated to the pharmacological strategies used to treat PVD. First, we review the current pharmacological treatment strategies used in neonatal PVD in ***chapter 2***. One of these therapeutic strategies is the inhibition of phosphodiesterase-5 by sildenafil.

We describe a pharmacokinetic study performed in our unit to elucidate the optimal dose regimen of oral sildenafil among newborns with PVD in ***chapter 3*** and analyze the effects of sildenafil among high risk infants with CDH in ***chapter 4***.

Part II focuses on the structural changes in PVD. We review the current knowledge about human neonatal PVD and the associated pulmonary vascular abnormalities in ***chapter 5***. Perivascular cells (ranging from vascular smooth muscle cells (VSMCs) in the proximal large and midsized arteries, until the pericytes in the most distal capillaries) are involved in the structural changes in the pulmonary vasculature. In ***chapter 6*** we describe the analysis of the VSMC population within the vascular wall in infants with CDH by using a tissue micro array approach, followed by the analysis of the pericyte population, the putative VSMC precursor, during normal and abnormal pulmonary vascular development by using the CDH rat model in ***chapter 7.*** Since PVD may be reversible early after birth, we have studied the hypoxia-induced PH rat model. Hypoxia is a trigger to induce vasoconstriction and chronic hypoxia leads to subsequent structural remodelling of the pulmonary vasculature resulting in pulmonary hypertension. The analysis of the VSMC population in pulmonary vascular remodelling and after recovery in normoxia in a hypoxia induced pulmonary hypertension rat model is described in ***chapter 8***.

In ***chapter 9***, the general discussion, we summarize the findings of the different studies and describe future perspectives within the field of PVD in newborns, especially in newborns with CDH.

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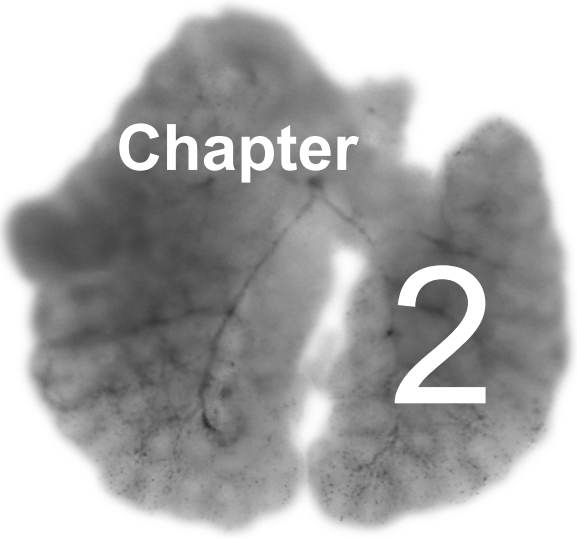
# Part I Pharmacological Strategies



# Chapter 2

# Pulmonary vascular disease in the newborn – From pathophysiology to therapeutic strategies

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## Abstract

Pulmonary vascular disease (PVD) summarizes all congenital or acquired pathologies that affect the pulmonary vasculature. One of these is pulmonary hypertension (PH), which is characterized by a mean pulmonary artery pressure of ≥ 25 mmHg in rest, or ≥ 30 mmHg during exercise. This definition applies for adults and children.[1]

Pulmonary hypertension is associated with a variety of diseases with different pathogenesis. A diagnostic classification of PH, based on the different causes, was made in 2003.[2]

There is some ambiguity about pulmonary hypertension in newborns (PHN). Both primary and secondary PHN are often collectively referred to as persistent pulmonary hypertension of the newborn (PPHN). In the last decades a variety of different terms describing PPHN: persistence of the fetal circulation,[3] persistent transitional circulation,[4] persistence of the fetal cardiopulmonary circulatory pathway,[5] progressive pulmonary hypertension[6] and persistent pulmonary vascular obstruction.[7] They all describe a failure or delay in the transition from fetal to neonatal circulation, exemplified by the failure of decreasing the pulmonary vascular resistance (PVR) after birth and abnormalities in the pulmonary vasculature. Clinically, each form of pulmonary hypertension in the newborn is referred to as PPHN.

In the WHO diagnostic classification of pulmonary hypertension, persistent pulmonary hypertension of the newborn is classified together with pulmonary arterial hypertension (Group I) and pulmonary hypertension that is associated with lung disease and hypoxemia is classified in group III. In this group there is a subgroup for abnormal development, for example in congenital diaphragmatic hernia (CDH).

In this review we will focus on pulmonary hypertension in newborns in relation to therapeutic strategies.

## Pulmonary hypertension of the newborn

Pulmonary hypertension in newborns (PHN) is a severe cardio-respiratory disorder. It is characterized by a persistence of a high pulmonary vascular resistance (PVR) after birth. This leads to a right-to-left shunt over the foramen ovale and ductus arteriosus, resulting in severe hypoxemia and eventually right ventricular failure. The incidence is 1.9 per 1000 live births, with variations of 0.43 to 6.8 per 1000 live births.[8]

PHN can be primary, or secondary, associated with a few neonatal disorders, such as congenital heart disease, lung disease, liver disease[9] and congenital diaphragmatic hernia.[10] Clinical syndromes that are associated with PHN are meconium aspiration, perinatal asphyxia, congenital sepsis and respiratory distress syndrome.[11-12] Primary PHN, without an obvious cause is considered as a separated disease entity with a characteristic pathology.

Initial signs of PNH are cyanosis and respiratory distress. The diagnosis can be made by echocardiography or by measuring the difference in pre and postductal arterial oxygen tension.[12] However, cardiac catheterization, which measures the pulmonary arterial pressure and PVR, gives an unambiguous diagnosis.[13] Recent studies have shown that biomarkers can be useful tools in the diagnostics and/or evaluation of pulmonary hypertension of the newborn. One of the biomarkers is the hormone B-type natriutric peptide (BNP), which is released by the ventricles as a pro-hormone in response to increased ventricular stress.[14-15]

Studies in infants and children have shown that plasma NT-proBNP levels, the amino terminal portion of the pro-hormone BNP, are increased in children with congenital heart disease or cardiomyopathy.[16] In infants with symptomatic patent ductus arteriosus[17] and PH[18] the NT-proBNP levels are also elevated. Recently it was shown that NT-proBNP could be an indicator for the severity of PH in CDH. Early elevated NT-pro-BNP in infants with PH in CDH is associated with a worse prognosis.[19]

## Pathophysiology

There are two specific characteristics of PHN: the failure of the physiological decrease in PVR, which is required for a normal transition after birth and the persistence and/or development of anatomical changes in the vasculature from heart and lungs, called vascular remodeling. These characteristics can contribute to a chronic pulmonary hypertension that is progressive and eventually irreversible.[20-21]

Endothelial dysfunction plays a prominent role in the failure of decreasing the PVR after birth. Normally, the interaction between endothelial and smooth muscle cells produces a balance between relaxation and constriction, which results in a slightly more relaxation

due to basal release of endothelium derived vasodilators, such as nitric oxide (NO) and prostacyclin ($PGI_2$).[22] The endothelial dysfunction in PH is responsible for an impaired vasodilator production and an increased expression of vasoconstrictors, such as endothelin (ET-1). This imbalance in vasodilators and vasoconstrictors is affecting the vascular tone and promotes pulmonary vascular remodeling.[21]

Vascular remodeling is a crucial process in the pathophysiology of pulmonary hypertension and is characterized as thickening of all the layers of the vascular wall (adventitia, media and intima). This thickening is caused by hypertrophy and/or hyperplasia of the predominant cell types within each layer (fibroblasts, smooth muscle cells and endothelial cells) and the increased deposition of extracellular matrix components (collagen, elastin and fibronectin).[23-24] This remodeling occurs in response to several physical and chemical stimuli (mechanical factors, hypoxia, mediators and tenascin-c). The consequences of vascular remodeling are increased vasoconstriction in response to contractile stimuli and a reduced vasodilator response due to narrowing of the vascular lumen and the reduced compliance of the vasculature.[23] The stimuli, responsible for the vascular remodeling, are activating a cascade of intracellular signaling pathways. This activation can lead to structural changes in the function of endothelial cells and smooth muscle cells.[24] This morphology is very different form the flow derived pulmonary hypertension which occurs in case of left-right shunts for prolonged periods of time as is known as plexiform arteriopathy.[25]

In primary PHN and PHN associated with CDH, a failure of normal evolution of the pulmonary vasculature is suggested to be the underlying etiological factor. Morphology remarks an identical structure of fetal vessels suggesting either a developmental delay of retarded switching on of genes responsible for maturational changes resulting in the possibility of muscular thinning and increased compliance of the resistance vessels is in the pulmonary vasculature.

## Pharmacological therapeutic strategies

The increasing knowledge of several signaling pathways involved in the regulation of vascular tone and the pathogenesis of pulmonary hypertension provides new therapeutic possibilities.[26] Pathways involving prostaglandin-cAMP, the NO-cGMP and endothelin are subject to intensive investigations and have led to the use of prostacyclin analogues, inhaled nitric oxide, sildenafil and endothelin receptor antagonists as therapeutic modalities. The latest interests and putative new therapeutic targets for the near future will be the platelet derived growth factor (PDGF) and Rho/Rho-kinase pathways.

***Prostaglandin-cAMP pathway***

Prostacyclin ($PGI_2$) is one of the endothelium-derived vasodilators that play a role in the transition at birth. The production of prostacyclin is decreased in pulmonary hypertension. There is also a reduced expression of prostacyclin receptors in human remodeled pulmonary artery smooth muscle.[27] Release of $PGI_2$ in late gestation and at birth is low for human newborn with PPHN.[28]

Introduction of prostacyclin therapy in the treatment of pulmonary arterial hypertension for human was a major breakthrough.[29] Intravenous epoprostenol, treprostinil (subcutaneous or intravenous), iloprost (aerosoled) and beraprost (oral) are all prostacyclin analogues, already used in adults and studies showed that they improve the symptoms of pulmonary arterial hypertension.[30-32]

Prostacyclin treatment for newborns was fairly recently described. A few case-reports are reporting about successful $PGI_2$ treatment in neonates with pulmonary hypertension.[33-35] Inhaled iloprost have shown to be effective in the treatment of persistent pulmonary hypertension of the newborn.[36] Prostacyclin treatment in newborn could be promising, but further trials are necessary. Studies in adults have shown that the treatment with prostacyclin is effective, but also has some disadvantages, like the adverse systemic effects, the short half-life and the high costs.[23]

***NO-cGMP pathway***

Nitric oxide (NO) is a selective pulmonary vasodilator and is one of the several vasoactive mediators that influence the tone of the pulmonary vascular wall. The release and response to NO in pulmonary arteries appears to be affected in neonatal pulmonary hypertension.[22]

In 1992 inhalation of NO (iNO) was introduced as a therapy to increase nitric oxide exogenously. It leads to a decrease in PVR and right ventricular (RV) afterload without a decrease in arterial pressure. There are no adverse systemic effects, because NO becomes inactive by binding to hemoglobin.[37] Most patients are responding on dose levels less then 20 ppm. Higher levels did not improve the response.[38] Overall, randomized trials with iNO showed improved oxygenation and a decreased need for extracorporeal membrane oxygenation (ECMO) treatment. A problem of iNO therapy is a rebound pulmonary hypertension with severe hypoxemia when NO is weaned. A decrease in endothelial nitric oxide synthase (eNOS) activity[39] and an increased endothelin level,[40] caused by exogenous nitric oxide, cause the rebound pulmonary hypertension. In spite of these iNO improvements the mortality wasn't improving.[38]

Although iNO is referred to as the standard therapy, there remains a group of about 30% who are not responding to iNO therapy.[41] This group includes infants with severe parenchymal lung disease, like in congenital diaphragmatic hernia, myocardial dysfunction or problems with the NO-cGMP signaling.[42-43]

Another therapeutic strategy that is interacting in the NO-cGMP signaling is inhibition of phosphodiesterase 5 (PDE 5). PDE 5 is an enzyme that converts cGMP to GMP, cGMP is activating protein kinase, and this will lead to a decrease of cytosolic calcium, and eventually vasodilatation. Studies have shown that the PDE-5 activity in fetal lungs is increased, maintaining the high PVR[44] and the activity is decreasing at birth. This is suggesting that decreased PDE-5 activity postnatal is important in maintaining the low PVR. In pulmonary hypertension there is an increased PDE-5 activity after birth.
Inhibition of PDE-5 activity, by sildenafil, results in an increased of cGMP levels inducing vascular smooth muscle cells to be hyperpolarized and relax.[45-46]
A study that compared sildenafil with iNO showed that sildenafil is more effective in decreasing the pulmonary artery pressure, but that both are equally effective and selective in reducing the pulmonary vascular resistance.[47] Sildenafil is augmenting the effects of iNO and is preventing pulmonary vasoconstriction and the rebound pulmonary hypertension during weaning of iNO.[8] The vasodilator effects of sildenafil are causing a reduction in the right ventricular afterload as well.[48]

***Endothelin pathway***

Endothelin-1 (ET-1) is a potent vasoconstrictor and mitogen, produced in the vascular epithelium. In the fetal pulmonary regulation of vasoconstriction and dilatation it is one of the important key players.[49]
Endothelin receptor antagonists are already used in the treatment of pulmonary hypertension in adults and children. Bosentan, a non-selective ET receptor antagonist, is evaluated in randomized controlled trails and have shown improvement in exercise capacity and hemodynamics.[50-51] It appears to be as effective as prostacyclin but better tolerated.[52] However, hepatoxicity is a significant adverse effect of bosentan.[51] Another aspect of bosentan is that it is non-selective, its blocks both ET-A and ET-B receptors.[53] Stimulation of ET-B receptors on endothelial cells will lead to a vasodilation.[54] It has been shown that in human neonatal lung ET-B receptors are overexpressed.[55-56]
Sitaxsentan and ambrisentan, specific ET-A receptor antagonists, will be more promising. A randomized controlled study in adults with pulmonary hypertension proved that sitaxsentan is effective.[57] Ambrisentan, only tested in a phase III study, shows also to be effective and is less hepatotoxic.[58]
The use of endothelin receptor antagonists, selective or non-selective, is not yet tested in neonates. There is a case report of a safe and effective use of bosentan in two neonates with pulmonary hypertension due to transposition of the great arteries.[59] Although the use of endothelin receptor antagonist has been shown to be effective in adults, further research in neonates is necessary.

***Platelet derived growth factor pathway***

Platelet derived growth factors (PDGFs) are potent mitogens. PDGF-B is mainly expressed in vascular endothelial cells and is forming PDGF-BB. This binds to the tyrosine kinase receptor (PDGFr-β) that is localized on vascular smooth muscle cells (VSMC). Activation of PDGF is leading to proliferation and migration of VSMC, engaged by activation of several signaling pathways involved in these cellular processes.[60-61]

Studies have shown that PDGF is upregulated in pulmonary hypertension.[61-62] Case reports about treatment with the PDGF inhibitor, Imatinib, in pulmonary arterial hypertension showed that it has a dose-dependent improvement on the right ventricle hypertrophy and reverse pulmonary vascular remodelling.[61,63] Recently, a case report described the using of a tyrosine kinase inhibitor in a neonate with pulmonary hypertension associated with congenital diaphragmatic hernia leading to a gradual decrease in the pulmonary artery pressure and an improved the clinical condition.[64] Further research is necessary to study the effects of inhibition of PDGF in pulmonary hypertension.

***Rho/Rho-kinase pathway***

RhoA and Rho-kinase, which are highly expressed in vascular tissues,[65] are forming a key pathway in regulating the pulmonary vascular tone.

Contraction of smooth muscle cells is regulated by the phosphorylation of the myosin light chain (MLC). Phosphorylated MLC initiates stimulation of myosin ATPase activity by actin, leading to a contractile state. Dephosphorylation of MLC by myosin light chain phosphatase (MLCP) initiates vasodilatation.[66] The Rho/Rho-kinase pathway enhances the phosphorylated state of MLC, by inhibition of MLCP,[67] causing vasoconstriction despite the decreasing cytosolic calcium level. This phenomenon is called calcium sensitiziation.[68]

Fasudil, a Rho-kinase inhibitor improves pulmonary hypertension, right ventricular hypertrophy and also reverses the endothelial dysfunction in monocrotaline-induced pulmonary hypertension in rats.[69] In neonatal rats with induced pulmonary hypertension, either by hypoxia or bleomycin, which were unresponsive to nitric oxide, fasudil was generating a vasodilatory effect.[70] Therefore, fasudil may be a promising therapy, although further research is necessary.

## Conclusion and future perspectives

The increasing knowledge of the pathophysiological mechanisms of pulmonary hypertension and the signaling pathways involved in the regulation of the vascular tone have lead to more therapeutic targets. Some of these therapeutic targets are already

introduced in clinical settings, at least in adults, and tested in randomized control studies with pulmonary arterial hypertension.

For pulmonary hypertension of the newborn only iNO is tested in randomized controlled studies. Therapies with prostacyclin analogues, sildenafil and bosentan are already used quite often in clinical settings and case-reports have been published. They all describe the positive effects of these therapies in pulmonary hypertension of the newborn. Randomized controlled studies are necessary to confirm the positive effects of these therapies in pulmonary hypertension of the newborn.

Aside from the single use of the therapeutic agents, combinations of different treatments are being employed. Infants with severe parenchymal lung disease, like in congenital diaphragmatic hernia, myocardial dysfunction or problems with the NO-cGMP signaling need a more combined therapy focused on vasodilatation and anti-proliferation. Advantages of these combination therapies are the additive effects and the prevention of rebound pulmonary hypertension.

More research is necessary to unravel the basic pathophysiological mechanisms behind the vascular development, the neonatal transition and exploring the different signaling pathways that are involved in these mechanisms. This may lead to possible new therapeutic targets.

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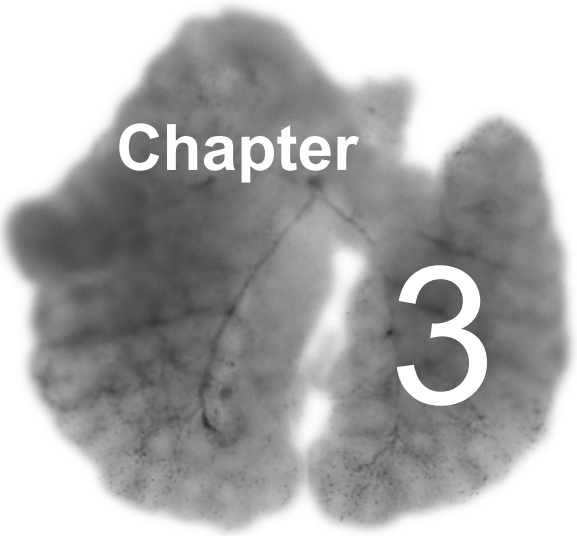

# Chapter 3

## Sildenafil exposure in neonates with pulmonary hypertension after administration via a nasogastric tube

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## Abstract

**Objective** This study was set up to describe the pharmacokinetics and exposure of oral sildenafil in neonates (2-5 kg) with pulmonary hypertension.

**Design** We included 11 neonates (body weight 2.05 kg, postnatal age 2 – 121 days) who received sildenafil and extracorporeal membrane oxygenation (ECMO) treatment for pulmonary hypertension. Sildenafil capsules were given via a nasogastric tube. Blood samples were collected via a pre-existing arterial line to quantify sildenafil and metabolite plasma levels (219 samples). Nonlinear mixed effects modeling was used to describe sildenafil (SIL) and desmethylsildenafil (DMS) pharmacokinetics.

**Results** A one-compartment model was suitable for both SIL and DMS. Inter- and intra-patients variability for clearance at 100% bioavailability were 87% and 27% (SIL) and 62% and 26% (DMS). Patient weight, postnatal age and post-ECMO time did not explain variability. Concomitant fluconazole use was associated with a 47% reduction in sildenafil clearance. The exposure expressed as average plasma concentration area under the curve over 24 h ($AUC_{24\ (SIL+DMS)}$) ranged from 625 to 13579 ng/h/ml. An oral dose of 4.2 mg/kg/24h would lead to a median $AUC_{24\ (SIL+DMS)}$ of 2650 mg/h/ml equivalent to 20 mg three times a day in adults. Inter-patient variability was large, wit a simulated $AUC_{24\ (SIL+DMS)}$ range ($10^{th}$ and $90^{th}$ percentiles) of 1000–8000 ng/h/ml.

**Conclusion** Sildenafil pharmacokinetics are highly variable in post-ECMO neonates and infants. In a median patient, the current dose regimen of 0.5-2.0 mg/kg four times a day leads to an exposure comparable to the recommended adult dose of 20 mg three times a day. Careful dose titration based on efficacy and the occurrences of hypotension, remains necessary. Follow-up research should include appropriate pharmacodynamic endpoints with a population pharmacokinetic/pharmacodynamic analysis to assign a suitable exposure window or target concentration.

## Introduction

Pulmonary hypertension (PH) is an important cause of cardio-respiratory failure in the newborn.[1] Treatment options include, amongst others and in addition to nitric oxide inhalation (iNO) and extracorporeal membrane oxygenation (ECMO), the potent phosphodiesterase (PDE-5) inhibitor sildenafil. The latter has been licensed under the trade name Revatio® for the treatment of PH in adults and is used off-label in neonates. A dose of 0.5-2.0 mg/kg four times daily is considered appropriate to treat PH in newborns,[2] but supporting evidence is limited to case reports and small pilot studies with varying dose regimens.[3-5]

Recently, Mukherjee et al. published the first population pharmacokinetic study in which they characterized the pharmacokinetics of intravenous sildenafil via continuous infusion in term neonates up to 7 days of age, postulating rapid maturation of the CYP3A-mediated clearance of sildenafil.[6] The CYP3A-mediated metabolism also makes sildenafil prone to drug-drug interactions with commonly used co-medication such as bosentan and fluconazole.[7] Considering this maturation and the remaining substantial inter-patient variability of clearance and volume of distribution, an adequate sildenafil dose would vary substantially from one patient to another and within a patient over the course of a couple of days. Since the intravenous formulation is not available in The Netherlands, we treat our patients orally by administering the contents of extemporaneously prepared capsules via a nasogastric tube. In patients of 7 days and older, sildenafil clearance and exposure have not been studied in detail, especially in the post-ECMO period.

Goals of this study were to characterize the pharmacokinetics of sildenafil and its metabolite desmethylsildenafil in post-ECMO neonates after nasogastric administration, to explain variability by age, weight and other covariates and to simulate the effects of different dose regimens on sildenafil exposure.

## Methods

### *Patients*

This study was approved by our institutional ethics review board as part of a larger study into pharmacokinetics during ECMO at the intensive care unit (ICU) of the Erasmus-MC Sophia Children's Hospital. After verification via repeated cardiac ultrasound scans, patients were initially treated with iNO according to the standard departmental protocol, but eventually had to be placed on venoarterial ECMO to maintain sufficient oxygenation. Criteria for ECMO in our institution have been published in prior publications describing different aspects of pharmacotherapy during ECMO.[8-10] After written parental consent,

we included 11 patients who had undergone ECMO with PH who had an arterial line during their stay in the ICU. Sildenafil therapy was given on ECMO, based on persistent PH, either via an echocardiogram or the inability to wean from ECMO, and was continued after decannulation. Sildenafil capsules (1, 2, 5 and 10 mg) were prepared extemporaneously by the hospital pharmacy form commercial Viagra® tablets, with pharmaceutical grade lactose as filler and only excipient. Intermediate doses were given by combining multiple capsules. Production facilities and procedures were in accordance with the Good Manufacturing Practice guidelines for hospital pharmacy production as issued by The Netherlands Association of Hospital Pharmacists (NVZA). Content uniformity was assessed in each individual batch as described in the European Pharmacopoeia.[11] Sildenafil content was within 90% to 110% of the specified amount for each batch of capsules. Capsules were opened and their contents dispersed in a syringe filled with water before administration via the nasogastric tube. The syringe was flushed with water and afterwards the tube was flushed with a final aliquot of water. Initially, 0.5 mg/kg of sildenafil three or four times daily was given via a nasogastric tube. The dose was titrated up to a maximum of 10 mg/kg/24h to reach adequate pre- and postductal saturation, while maintaining adequate systemic blood pressure. Concomitant drug therapy consisted of inotropics, diuretics, sedatives, analgesics, bosentan, fluconazole and antibiotics as required, adjusted to the individual needs of each patient. Sildenafil and concomitant medication were recorded, as well as patient characteristics, clinical parameters, laboratory results and ECMO and ventilation settings.

***Blood sampling and analysis***

Arterial EDTA-decoagulated blood samples (100-200 μl) were taken from an existing line. Sampling took place between disconnection from ECMO and discharge from the ICU for as long as a line was present; the first sample was taken once the parents had given written consent. On the first day of sampling, a pharmacokinetic (PK) curve of five points was taken at 0, 1, 2, 4 and 6 hours after a sildenafil dose. Over the following days, a maximum of three blood samples were taken per day at varying sampling times for as long as an arterial line was present, with a median of 13 samples per patients (range 7 – 55). For newborns cannulated within maximum 3 days after birth, the last sample was taken maximum 4 weeks after decannulation. On average, patients were followed for 228 h (range 54 – 528h). Plasma was separated via centrifugation and stored at -80°C until analysis. Sildenafil (SIL) and desmethylsildenafil (DMS) were quantified in 50 μl of plasma with a validated liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method after liquid-liquid extraction.[12] Accuracy, intra- and interday precision were within 13% for both compounds. The lower limits of quantification were 1 ng/ml. Matrix effects were present, but inter-plasma batch variability in recovery was under 12%.

***Pharmacokinetic analysis***

In total, 219 SIL and 219 DMS concentration time points were analyzed simultaneously using nonlinear mixed-effects modeling software (NONMEM VI 2.0, Globomax LLC, Ellicott City, MD, USA), with the first-order conditional estimation (FOCE) method, which allows interaction between structural and residual variance components. Models were parameterized in terms of volume of distribution and clearance, as if the absolute bioavailability (F) and conversion ratio of DMS/SIL ($F_c$) equaled unity. Using NONMEM, we can estimate average pharmacokinetic parameters for the population, as well as inter- and intra-individual variability and a residual error. Model fit was assessed using plots of population and individual predicted concentrations versus observed concentrations and weighted residuals over time. Models were compared using the minimum value of objective function (MVOF) via the log likelihood ratio test for nested models. An MVOF-drop of 3.84 ($p<0.05$) was considered statistically significant, following a $\chi^2$ distribution with 1 degree of freedom. Inter-individual variability was modeled as a separate variance terms for periods of 2 days in a row, allowing clearances and volume of distribution to vary overtime for each individual. Residual error was estimated with a proportional error model. The influence of demographics, clinical parameters and co-administered drugs on clearance and volume of distribution were analyzed via univariate analysis of each covariate. Statistically significant covariate effects ($p<0.05$) were included in an intermediate multivariate model. Subsequently, covariates were deleted stepwise and removed from the model if the MVOF did not significantly increase. The resulting model was considered the final model. The tested covariates included the use of all co-administered drugs (including bosentan, inotropics and fluconazole), postnatal age, body weight, gender, blood pressure, saturation, liver function, time after ECMO decannulation, heart rate and sildenafil dose in mg/kg/24 h.

Using NONMEM, the average plasma concentration area under the curve over 24 h ($AUC_{24\ (SIL+DMS)}$) was calculated as a measure of drug exposure. Since DMS possesses activity itself (50% as potent as SIL[13]) 50% of $AUC_{24\ (DMS)}$ was added to $AUC_{24\ (SIL)}$. To correct for differences in dose frequency, AUCs were calculated over 24 h periods. The model was validated with the bootstrap resampling method, in which the model is repeatedly fitted on a reduced dataset. All, 1250 resampled datasets were used to calculate the median parameter estimates and 95% confidence interval. The model was considered valid if the original parameter estimates fell within this interval. Plasma concentration time profiles of SIL and DMS were simulated with NONMEM using the final parameter estimates in 800 patients, replicated from the original dataset, with dose regimens of 2, 3, 5 and 7 mg/kg/24h.

## Results

Table 1 contains demographic data of the study population and the calculated median $AUC_{24\ (SIL+DMS)}$ for each patient. The high pharmacokinetic variability was visible in both the calculated $AUC_{24\ (SIL+DMS)}$ and the original plasma concentrations; see figure 1 for the

**Table 1. Patient characteristic and calculated SIL and DMS exposure**

| Patient | Sex | PNA (days)[a] | Time after ECMO (days)[a] | Body weight (kg)[a] | Primary diagnosis[b] | Sildenafil Dose (mg'/kg)[c] | CYP3A Comedi-cation[d] | Average $AUC_{24\ (SIL+DMS)}$ (ng/h/ml)[e] | survived |
|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 121 | >100 | 4.8 | VSD | 2.0 (t.i.d.) | - | 1598 | N |
| 2 | F | 22 | 0.2 | 4.0 | PA | 2.5 (t.i.d.) | BOS | 8284 | Y |
| 3 | M | 2 | 0.1 | 2.3 | CDH | 2.0 (q.i.d.) | - | 19514 | Y |
| 4 | M | 19 | 2,5 | 4.0 | TGA | 2.5 (q.i.d.) | BOS, FLU | 13579 | Y |
| 5 | F | 17 | 1.4 | 2.0 | CDH | 1.5 (t.i.d.) | FLU | 625 | Y |
| 6 | F | 22 | 10 | 4.1 | ASD, VSD | 1.5 (t.i.d.) | - | 8297 | Y |
| 7 | F | 11 | 6.5 | 4.3 | PPHN | 0.5 (q.i.d.) | - | 939 | N |
| 8 | F | 11 | 0.5 | 2.7 | PPHN | 2.25 (t.i.d.) | - | 3935 | Y |
| 9 | F | 20 | 4.5 | 3.0 | CDH | 0.33 (q.i.d.) | BOS, FLU | 1618 | Y |
| 10 | F | 22 | 8.4 | 3.0 | CDH | 2.5 (t.i.d.) | - | 4232 | Y |
| 11 | F | 101 | 93 | 5.1 | CDH | 3.0 (t.i.d.) | - | 2404 | Y |

[a], at inclusion; [b], ASD, atrial septal defect; CDH, congenital diaphragmatic hernia; PA, pulmonary atresia; PPHN, persistent pulmonary hypertension of the newborn; TGA, transposition of the great arteries; VSD, ventricular septal defect; [c], average dose after titration, given three (t.i.d.) or four (q.i.d.) times daily; [d], bosentan (BOS) or fluconazole (FLU) that was used in conjunction with SIL for any length of time during the study period ; [e], calculated over a period of 24 h, as a combination of SIL and DMS assuming a DMS potency of 50%

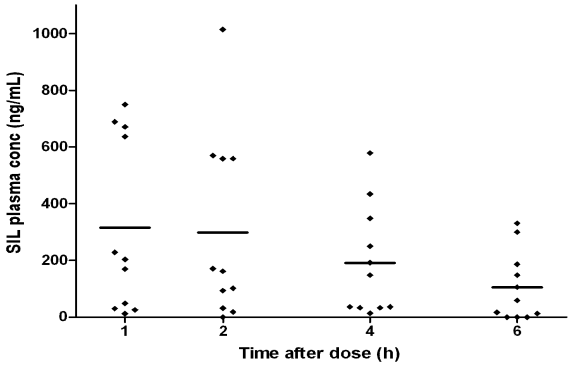


**Figure 1. Sildenafil plasma concentrations for each individual at sampling times of a full PK curve (1, 2, 4 and 6 hours post-dose).** Lines indicate the mean concentration at each time point.

measured sildenafil concentrations at 1, 2 4 and 6 h post dose. None of the patients required renal replacement therapies during the observation period. Median ECMO-duration (range) was 148 h (42 – 292h). Pharmacokinetics (PK) could be described adequately with a sequential one-compartment model for SIL and DMS with first-order absorption (fig. 2).

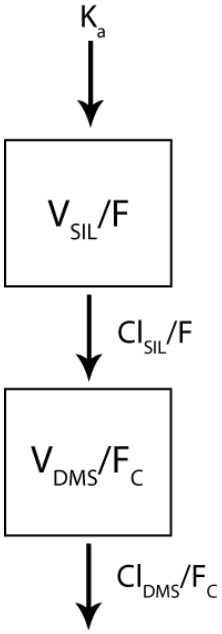


**Figure 2. Sequential one-compartment model describing sildenafil (SIL) and desmethylsildenafil (DMS) pharmacokinetics.** $K_a$ = absorption rate constant, $V_{SIL}/F$ = apparent sildenafil volume of distribution, $CL_{SIL}/F$ = apparent sildenafil clearance, $V_{DMS}/F_C$ = apparent desmethylsildenafil volume of distribution, $CL_{DMS}/F_c$ = apparent desmethylsildenafil clearance. True pharmacokinetic parameters require correction for bioavailability (F) and the DMS/SIL conversion ratio ($F_c$), respectively.

Pharmacokinetic parameter estimates, together with their standard error estimates generated by NONMEM's covariance option, are given in table 2. Inclusion of inter- and intra-patient variability improved the model, as can been seen in the plots of population prediction versus observed concentration (figure 3). The residuals versus time plot revealed no structural deviation form zero. Residual variability was modelled with a proportional error model for each analyte. Shrinkage of variance terms, which has the potential to distort covariate effects and induce model misspecification with sparse sampling study designs,[14-16] was acceptable at 5.7% (ε) and max 7.0% (η's). In a forward-inclusion procedure, postnatal age, heart rate, bosentan use and fluconazole use were identified as potential covariates ($p<0.05$), but only fluconazole remained in the

final model after the stricter backward covariate exclusion ($p<0.001$). Concomitant fluconazole use led to a 47% drop in clearance (see figure 4).

**Table 2. Pharmacokinetic parameter estimates**

| | Sildenafil | | Desmethylsildenafil | |
|---|---|---|---|---|
| | Model estimate (CV %) | Bootstrap median ($5^{th}$-$95^{th}$ percentile) | Model estimate (CV %) | Bootstrap median ($5^{th}$-$95^{th}$ percentile) |
| **Population Average** | | | | |
| $K_a$ ($h^{-1}$) | 2.4 (4.2) | 2.4 (2.0-4.5) | - | - |
| V/F (l)* | 34 (5.0) | 35 (25-56) | 14 (35) | 14 (9.5) |
| CL/F (l/h)* | 7.3 (17) | 7.3 (5.1-12) | 9.7 (19) | 9.7 (7.1-14) |
| CL-reduction with Fluconazole use (%) | 47 (28) | 47 (23-69) | - | - |
| **Intrapatient variability** | | | | |
| CL (%) | | | | |
| **Interpatient variability** | 27 (56) | 24 (10-35) | 26 (29) | 25 (17-31) |
| V (%) | | | | |
| CL (%) | | | | |
| **Residual variability** | 94 (54) | 86 (44-127) | - | - |
| Proportional error (%) | 87 (42) | 83 (49-111) | 62 (46) | 59 (36-80) |
| | 38 (5.3) | 37 (32-41) | 30 (17) | 30 (24-39) |

* Apparent values, divided by F to correct for bioavailability (sildenafil) and $F_c$ to correct for the metabolic conversion ratio of SIL to DMS (desmethylsildenafil); F absolute bioavailabilityl; $F_c$ conversion of ratio DMS/SIL; $K_a$ absorption rate constant; V volume of distribution

Inter-individual variability on SIL clearance ($CL_{SIL}$), DMS clearance ($CL_{DMS}$) and SIL volume of distribution ($V_{SIL}$) significantly improved fit. Intra-patient variability explained 4.5% (SIL) and 6.2% (DMS) of variability in clearance. Bootstrap validation results are shown in table 2. All estimates were within the $5^{th}$ and $95^{th}$ percentile range. The median $AUC_{24\ (SIL+DMS)}$ was 3935 ng/h/ml (Table 1: range 625 – 13579 ng/h/ml). The AUC ratio of DMS versus SIL ($AUC_{DMS}/AUC_{SIL}$) was on average 0.94 (range 0.14 – 2.16). Of the 11 patients, one patient died of sepsis (28 mg SIL/day, $AUC_{24\ (SIL+DMS)}$ = 939 ng/h/ml) and one died of progressive cardiac failure caused by therapy-resistant PH (8 mg SIL/day, $AUC_{24\ (SIL+DMS)}$ = 1598 ng/h/ml). The correlation coefficient between weight-normalized sildenafil dose and $AUC_{24\ (SIL+DMS)}$ was only 0.36 due to large remaining inter-patient variability in SIL and DMS clearance (87% and 62%, respectively). The correlation between $AUC_{24\ (SIL+DMS)}$ and simulated dose regimens of 2, 3, 5 and 7 mg/kg/24h is displayed in figure 5. A dose of 4.2 mg/kg/24h corresponds to an $AUC_{24\ (SIL+DMS)}$ of 2650 ng/h/ml, with large variability, as indicated by the $10^{th}$ and $90^{th}$ percentiles (1000 and 8000 ng/h/ml, respectively).

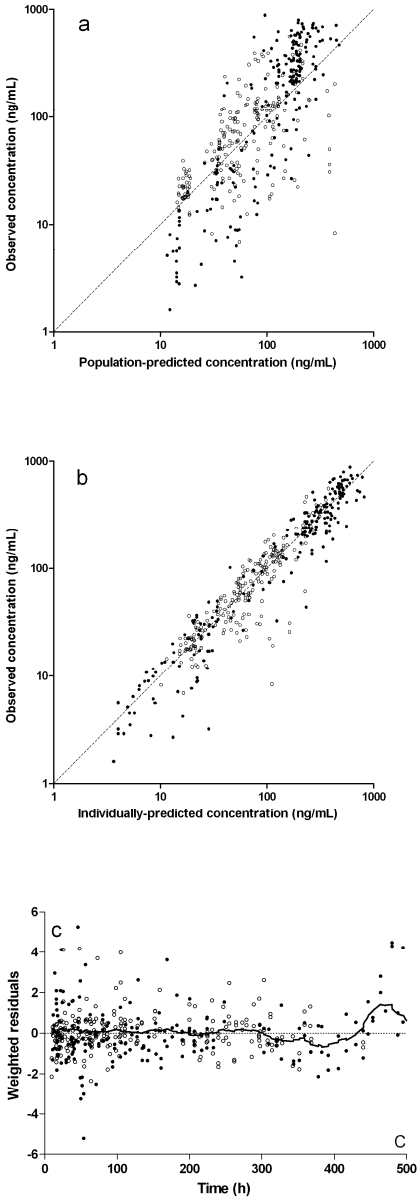


**Figure 3 Goodness-of-fit plots of sildenafil (closed) and desmethylsildenafil (open) for the final model.** A. Population predicted concentrations vs. observed concentrations, B. Individual predicted concentrations vs. observed concentrations. C. Weighted residuals vs. time with trend line.

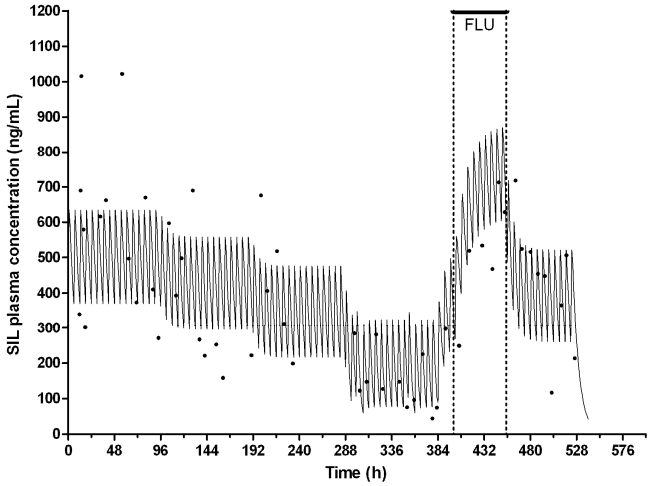


**Figure 4. Plasma concentration vs. time plot for a patient who received three doses of fluconazole (6 mg/kg) during sildenafil therapy (10 mg/kg/24h).** The dots are measured sildenafil concentrations, the solid line is the individual .Bayesian prediction of sildenafil concentrations based on the final model. Sildenafil concentrations are higher during fluconazole treatment (between 400 and 455 h). After the fluconazole treatment has ended, sildenafil pharmacokinetics return to normal.

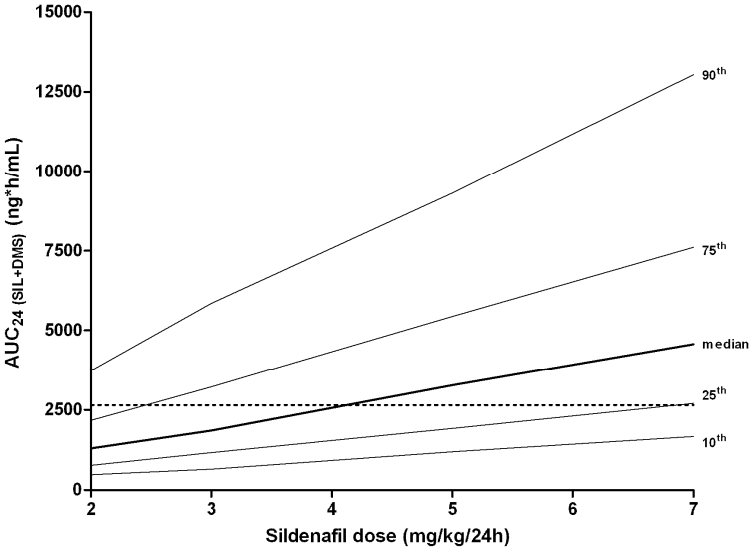


**Figure 5. Simulated area-under-the-curve ($AUC_{24\ (SIL+DMS)}$) for sildenafil and desmethylsildenafil with dose regimens of 2, 3, 5 and 7 mg/kg/day.** Curves represent the $10^{th}$, $25^{th}$, $50^{th}$ (median), $75^{th}$ and $90^{th}$ percentiles of AUC distribution.

## Discussion

The calculated exposure and the measured plasma concentrations of SIL and DMS were both highly variable between one patient and the next, which might led to inadvertent under- or overdosing upon administration of a standard dose. The underlying variation in PK parameters could not be explained by age or body weight, time after ECMO decannulation or other covariates, which might have been caused by the relatively narrow age and weight range: most patients were between 10 and 22 days old.

Variable gut absorption might in part be responsible, which has thus far been described for enteral feeding only.[17] Another explanation could be flow-limited hepatic clearance in combination with hemodynamic changes; even through SIL is considered to have and intermediate extraction rat in healthy adults.[18] Unfortunately, we did not have reliable markers for hepatic blood flow for these patients. There as no evidence of hepatic dysfunction; median (range) alkaline amino transferase (ALAT) and aspartate amino transferase (ASAT) blood levels were 14 (10-16) and 29 (15-40) IU/l. The vasopressor score, as a composite marker of overall hemodynamic instability (via quantification of the required amount of inotropic support),[19-20] was not correlated with SIL or metabolite clearance.

These patients had an average $CL_{SIL}/F$ of 7.3 l/h, equivalent to an adult of 62 l/h/70 kg after allometric scaling with a ¾ power exponent,[21] which is higher than the clearance found in adults tested for PH after oral administration (30.7 l/h).[22] The high clearance is offset by an increased volume of distribution: this could be in part be a remnant of the preceding ECMO treatment with its associated higher distribution volumes, an effect that has been described for a multitude of drugs.[23] After intravenous administration, Mukherjee et al. found an increased sildenafil clearance from a postnatal age of 1 day (0.84 l/h or 8.1 l/h/70 kg up to 7 days (2.6 l/h, or 25 l/h/70 kg) and attributed this to maturing CYP3A metabolism. The apparent trend was probably captured in our patients by modelling inter-patient variability. Co-administration of fluconazole was associated with a decreased $CL_{SIL}$ (-47%), but data from more individuals are needed to conclusively determine covariate effects.

When we compare our $CL_{SIL}/F$ of 62 l/h/70 kg to a clearance of 25 l/h/70 kg after intravenous administration (which is the value Mukherjee found in 7-day-old patients), we can calculated a biological availability of 40%. This could imply that the bioavailability of our sildenafil capsules is similar to that of the commercial tablets in adults.[22] Enteral feeding might have an effect on bioavailability, but this would have been reflected in statistically significant differences between CL or V for patients of different indications. However, the oral administration could provide an extra source of variability compared to intravenous administration, related to variable absorption capacity which might be linked to specific diseases states, diarrhea or vomiting. Unfortunately, these aspects are difficult

to model quantitatively and were not present in our dataset. A direct comparison between oral and intravenous sildenafil could be used to find out whether variability in absorption is an explanation for the high inter-patient variability we have seen in our study. Mukherjee estimated inter-patient variability on $CL_{SIL}$ and $V_{SIL}$ to be 55% and 43%, respectively. This is much smaller than the variability we found in our dataset (87% and 94%), which would indicate less variability with the intravenous formulation. This could in part be caused by our smaller number of patients, which makes it difficult to accurately estimate inter-patient variability and statistically significant covariate effects. In general however, it would seem that the intravenous formulation might lead to a more predictable clearance and volume of distribution, making it desirable over the oral capsules. In the absence of accepted efficacy parameters in this population, it is difficult to assign a suitable dose regimen. As a surrogate efficacy parameter, we used a corresponding efficacious exposure in adults after oral administration. In adults with PH, a dose of 20 mg three times a day is considered effective with acceptable side effects.[24] Assuming a ratio of $AUC_{DMS}/AUC_{SIL}$ of 0.74 and $CL_{SIL}/F$ of 30.7 l/h in adults with PH,[7] the corresponding $AUC_{24\ (SIL+DMS)}$ for a dose of 20 mg three times a day is 2650 ng/h/ml (equation 1).

$$AUC_{24\ (SIL+DMS)} = (Dose_{24} / ( CL_{SIL}/F))*(1 + 0.5*0.74) \qquad \text{(equation 1)}$$

All of the patients with a $AUC_{24\ (SIL+DMS)}$ >2650 ng/h/ml survived. Of our four patients with AUCs <2650 ng/h/l, one died of cardiac failure, which may indicated undertreatment. Unfortunately, we do don not know why her dose was not titrated upwards; she received 0.67 mg/kg three times a day from the onset of treatment. She experiences hypotension, but it started abruptly and only after having received sildenafil for three consecutive days, which makes sildenafil an unlikely cause. Several patients reached higher AUCs (up to 13579 ng/h/ml, equivalent to an adult dose of 100 mg three times a day). We could not detect an increased change of hypotensive episode in patients with high drug exposure. To reach a median $AUC_{24\ (SIL+DMS)}$ of 2650 ng/h/ml in our patients, sildenafil should be dosed orally at 4.2 mg/kg/24 h. This is equivalent to a dose of 1.0 mg/kg given fours times a day, but even a dose as high as 7 mg/kg/24 h a quarter of patients will have an average $AUC_{24\ (SIL+DMS)}$ under 2650 ng/h/ml (figure 4). This high variability in exposure implies that careful dose titration is necessary.

There is an ongoing debate on the best marker of PH severity in neonates, comparable with the 6 min walking test in adults.[24-26] In the absence of a generally accepted endpoint, current dose titration is based on a subjective perception of efficacy which includes the difference between pre-and postductal saturation, predetermined vascular parameters obtained by echocardiogram, a decreased tension following sildenafil administration and/or the ability to wean from ECMO, in addition to acute occurrence of

hypotension as a dose-limiting side effect. Unfortunately, all of these parameters are affected by concomitant medication (e.g. inotropes) and underlying illness (e.g. congenital cardiac deformities), which confound assessment of efficacy. To confirm a target concentration or exposure window in these specific patients, we need valid, generally accepted pharmacodynamic endpoints and a large study to define their correlation with exposure. Recently, the National Heart, Lung and Blood Institute (NHLBI) working group on Pediatric Respiratory Diseases Research published a detailed overview of the areas in pediatric pulmonary requiring additional research.[27] This strategic plan explicitly states the need for research into, among others, appropriate diagnostic markers and pharmacodynamic endpoints, differences in drug efficacy between adults and children, age-dependent patterns of pharmacokinetics and dose optimization for the individual patients. We concur, and would like to stress the important contribution that population pharmacokinetic modelling can make to the optimization of pharmacotherapy: by quantifying inter-patient variability, by evaluating the effects of age and other covariates on pharmacokinetics and by making maximum use of sparsely sampled plasma concentrations, thereby facilitating studies in populations of vulnerable patients.

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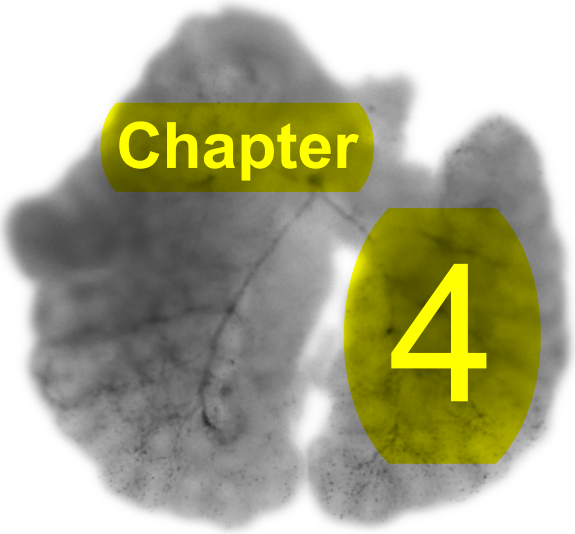


# Sildenafil treatment in Congenital Diaphragmatic Hernia: End of another Saga?

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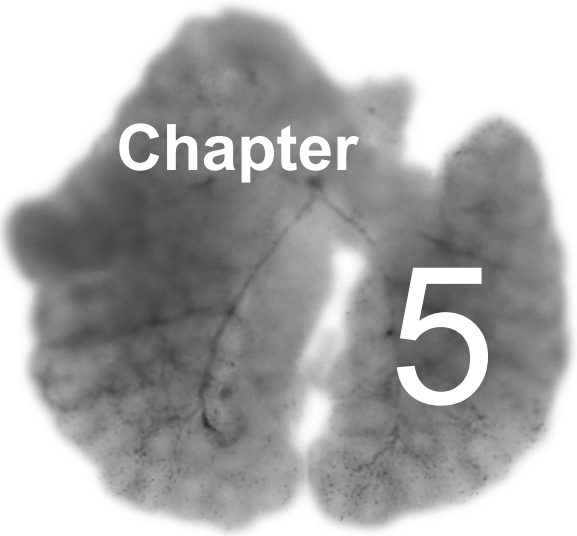
*Submitted Pediatr Pulmonol*

# Part II Structural Vascular Abnormalities



# Chapter 5

## Vascular abnormalities in human newborns with pulmonary hypertension

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## Abstract

Pulmonary vascular disease (PVD) embodies all congenital or acquired pathologies that affect the pulmonary vasculature. One of them is pulmonary hypertension of the newborn (PHN), which is clinically characterized by a persistent high pulmonary vascular resistance postnatally and an abnormal vascular response. Morphologically, the vascular walls of the small pulmonary arteries become thickened, leading to increased resistance of these vessels and thus a worsening of gas exchange. PHN occurs as a primary disease or in association with abnormal lung development, for example as in congenital diaphragmatic hernia, and is a critical determinant of morbidity and mortality.
Here we review the current knowledge about vascular abnormalities in PHN and discuss the vascular abnormalities in different conditions associated with pulmonary hypertension in human newborns in relation to recent findings from molecular biology.

## Background/Definition

Pulmonary hypertension of the newborn (PHN) is a severe cardio-respiratory disorder with an incidence of approximately 5 per 1000 live births.[1-2] Clinically it is characterized by a persistence of a high pulmonary vascular resistance (PVR) and abnormal vascular responsiveness. This leads to a right-to-left shunt over the foramen ovale and ductus arteriosus, resulting in severe hypoxemia and eventually right ventricular failure. Cardiac ultrasound is the cornerstone in diagnosis and management of PHN. Measurement of tricuspid regurgitation, right ventricular and pulmonary arterial dimensions, position of the intra ventricular septum, right to left shunting and forward flow across the pulmonary valve predicts the existence of increased right ventricular or pulmonary arterial pressures.

PHN can occur as a primary disease or in association with neonatal disorders, such as congenital heart disease (transposition of the great arteries), lung disease (respiratory distress syndrome, pneumonia), liver disease,[3] congenital diaphragmatic hernia (CDH)[4] and Down syndrome.[5] Furthermore, clinical syndromes that are associated with PHN are meconium aspiration (MAS), perinatal asphyxia and neonatal sepsis.[1,6]

Persistent pulmonary hypertension of the newborn (PPHN) is defined as a syndrome with severe hypoxemia and persistent pulmonary hypertension caused by the fact that the PVR failures to decrease at birth.[7-9] According to the updated clinical classification of pulmonary hypertension[10] persistent pulmonary hypertension of the newborn (PPHN) identifies a subgroup of group 1 (pulmonary arterial hypertension). However, pulmonary hypertension owing to lung diseases and/or hypoxia (group 3) contains a subgroup for developmental abnormalities. Since PPHN does not distinguish between these two groups, we will refer to PHN throughout this review to mark the cases with pulmonary hypertension as result of developmental abnormalities.

PHN is associated with a significant mortality and morbidity rate, which may vary according to the experience of the neonatal treatment team and/or the availability of different treatment modulation such as inhaled nitric oxide (iNO) and Extracorporeal membrane oxygenation (ECMO). Considerable neurosensory loss was observed in about a quarter of the 1 to 2-year-old infants that suffered moderate to severe PHN.[11-13]

Already in 1984 Geggel classified PHN histologically in three groups; 1) abnormal reactivity of the pulmonary vasculature (maladaptation), 2) remodeled pulmonary vasculature without parenchymal disease (maldevelopment) and 3) hypoplastic pulmonary vasculature (underdevelopment).[9]

Both functional and structural changes in the pulmonary vasculature are observed in most clinical conditions associated with PHN. The structural changes affect the functional properties as well.[14]

Morphologically, a well identified thickening of the vascular wall obstructs these vessels

and reduces the gas exchange. This vascular remodeling process is caused by abnormal neo-muscularization of the small pulmonary arteries, which normally are partially or non-muscularized.

## Normal vascular development

Understanding the process of normal vascular development is a prerequisite to comprehend the progression of PVD, and specifically PHN. The pulmonary vasculature is formed in two distinct, but overlapping phases of 1) the foundation of the primitive vasculature and 2) by the maturation of these vessels by stabilization and remodeling, eventually leading to a functional pulmonary circulation.

The lung develops from the foregut and reciprocal interactions between the lung endoderm and the surrounding mesenchyme lead to the formation of the bronchial tree. This is followed by patterning of distinct cell types along the proximal/distal axis of the lung.[15] Proper epithelial and mesenchymal interaction is essential to ensure a well-developed pulmonary vasculature.[16-17]

Studies using primarily histological material described the pulmonary trunk, the most proximal part of the pulmonary circulation, to be derived from the truncus arteriosus,[18] which later connected to the pulmonary arch arteries, which themselves originated from the sixth branchial arch.[19] Intrapulmonary arteries and veins will develop within the splanchnic mesoderm that surrounds the endoderm foregut.[20]

Both angiogenesis and vasculogenesis may contribute to the development of the pulmonary vasculature, but how these two processes add to the vasculature is beginning to be unraveled. Initially, studies were limited to histology and these concluded that the main pulmonary arteries form by angiogenetic sprouting of the dorsal aorta or aortic sac and would subsequently connect to the capillaries formed in the distal lung mesenchyme by vasculogenesis. By the end of the pseudoglandular phase the pulmonary circulation is formed.[21-23] However, later it was shown that already early in development the vessels in the lung are connected to the embryonic circulation to form vascular networks that expand with the lung endoderm growth, through a process called distal angiogenesis.[24] The finding that the circulation in the lung is already present from the earliest onset of lung development was confirmed with more molecular data.[25]

While angiogenesis is the major process of pulmonary vascular development during lung formation, vasculogenesis may be involved as well. Both vasculogenesis and angiogenesis generate primitive endothelial tubes. These primitive, endothelial tubes are stabilized by surrounding cells – perivascular cells, or mural cells - that closely associate with the endothelial cells upon signals emanating from the endothelium. Endothelial cells form the innermost cell layer of the vasculature, which functions as a barrier to control

the exchange of materials and cells from the bloodstream, to control of angiogenesis and to modulate of blood pressure.

The primitive vessels are remodeled through subsequent interactions between the endothelial cells and the mural cells. This results in the inhibition of endothelial cell proliferation, increased proliferation of mural cells and deposition of extracellular matrix (ECM) proteins.[20]

ECM proteins form a basement membrane that separates different layers within the vascular wall and functions as a scaffold to maintain the histological vessel wall structure.[26] In addition, ECM proteins regulate bioavailability of several signaling molecules, that are involved in cell proliferation, survival, migration and differentiation.[27]

To maintain homeostasis, the basement membrane remodels continuously by degradation and reassembly of the ECM proteins in response to transmitted signals from the ECM receptor or through the influence of ECM modifying proteins, called matrix metalloproteinases (MMPs). The control in degradation of the basement membrane is regulated by the ratio of MMPs and tissue inhibitors of metalloproteinases (TIMPs).[28]

Mural cells are defined as those cells that after differentiation can be identified as either a pericyte or a SMC, depending on the anatomical position of the vessels in the lung. Thus, mural cells are not well defined and may even be a heterogeneous population of cells of different origin. Lineage tracing experiments in mice showed that approximately thirty percent of the SMCs derive from the mesothelium and thirty percent from endothelium-mesenchyme transitional cells.[29-30] The remaining percentage is of unknown source(s). However, perivascular cells recruited from the mesenchyme, may be a potential source.[31]

The mature pulmonary vasculature ranges morphologically from simple capillaries in the septa of the distal alveolar spaces to the thicker elastic arteries at the proximal end of the lung. Vessel morphology gradually changes from elastic arteries as the most proximal vessels, partial muscular arteries and non-muscular arteries at the most distal intra-acinar level and muscular arteries in between.[32-33] The media of the elastic arteries and muscular arteries consist of more than one continuous layer of SMCs. The partial muscular arteries have a spiral smooth muscle coat of one layer of intermediate cells, phenotypically between SMCs and pericytes. The non-muscular arteries have one layer of pericytes, which share the basal membrane with the endothelial cells.

Once vessels are stabilized and gradually mature along the proximodistal axis in the lung, the media being formed consists of a heterogeneous SMC population. This population can either have a contractile phenotype or a synthetic phenotype, based on the presence or absence of contractile and cytoskeleton proteins.[34-35] The contractile cells control the vascular tone, whereas the synthetic cells are involved in injury repair. The unique aspect of SMCs is the switch between the two phenotypes in response to environmental factors.[35]

***Molecular aspects of vascular development***

Above we have seen that proper epithelial and mesenchymal interactions are essential for a well-developed pulmonary vasculature.[16-17] One of the earliest signaling cascades in the primary lung is the Fgf10-Fgfr2 pathway. Fgf10 is secreted from mesodermal cells located at the distal tips of the branching airways and induces budding by binding to its receptor, Fgfr2, which is expressed on the epithelial tip cells. This signal triggers the epithelial cells to express several factors, like Spry2, SHH and Bmp4, which subsequently induces yet another cascade of events. This subject is outside the scope of this review, and we would like to refer to some excellent recent reviews.[17,36] How the pulmonary vasculature develops in relation to the airways is still being researched, but initial reports suggest that primitive vessels guide the branching airways.[37-39] Vascular development is primarily studied in relation to the systemic circulation and cancer, and data about the development of the pulmonary vasculature is still very limited. Whereas early research in pulmonary vascular development was based on histological studies, more recent studies using animal models start to provide information about the molecular aspects of vascular development. One of the most potent mitogen for vascular development and angiogenesis is vascular endothelial growth factor (VEGF). VEGF interacts with its receptors Flt-1 and Flk-1 that are expressed in the endothelium. Interaction of VEGF with Flk-1 induces the proliferation of endothelial cells,[40-41] whereas VEGF/Flt-1 interactions are important for the vascular organization.[42] VEGF activity is critical throughout vascular development.[43-44] In early stages VEGF is expressed in mesenchyme and epithelium, in later stages VEGF is only expressed in epithelium,[45] where it is essential for the epithelial branching morphogenesis.[46] VEGF expression is regulated by oxygen levels through the activity of the hypoxia inducible factor-1 (HIF-1), which is a heterodimer of a constitutive subunit (HIF-1β) and an oxygen sensitive alpha subunit. Under normoxic conditions, prolyl hydroxylases will hydroxylate specific proline residues in the HIF-α subunits, which subsequently are targeted for proteosomal degradation by the von Hippel-Lindau protein (pVHL).[47] These prolyl hydroxylases are inactivated under hypoxic conditions, and then this process will not take place.[48-49]

Stabilization of the developing immature vasculature is achieved, as we have seen above, by the recruitment of mural cells and the generation of ECM. Several signaling pathways are involved in this process, such as platelet-derived growth factor B (PDGF-B), sphinosine-1-phosphatase (S1P), Angiopoietin-1 (Ang-1) and TGF-β with PDGFR-B, endothelial differentiation sphingolipid G protein coupled receptor (EDG1), TIE-2 and activin-receptor like kinase (ALK) as their receptors, respectively.[20] These pathways are involved in the recruitment of mural cells, the proliferation and differentiations of these cells towards smooth muscle cells and pericytes.[50-51] With the recruitment of mural cells and the formation of a basement membrane, cell-cell and cell-matrix interactions are

formed. These interactions are necessary for the further maturation of the vasculature.[52-55]

***Transition at birth***

Pulmonary vascular transition at birth is a complex process which is characterized by the clearance of lung fluid, decrease in PVR and rapid increase in pulmonary blood flow to adapt to extra uterine life. Before birth, fetal gas exchange occurs through the placenta and the PVR is high caused by the constriction of pulmonary vessels.[56] The lumen of the small pulmonary arteries is narrow and the media appears compressed with cuboidal-shaped SMCs. Moreover, the anatomy of the fetal heart (foramen ovale) and major arteries (ductus arteriosus) shunts the circulating blood directly into the systemic circulation, so that approximately ten percent of the cardiac output is circulated through the pulmonary vasculature.

Directly after birth, increased oxygenation, shear stress and breathing cause the pulmonary vessels to dilate. In addition, intra-acinar blood vessels are recruited to accommodate an estimated 10-fold increase in pulmonary blood flow. As a result the PVR will decrease.[57] The increased blood flow coincides with a decrease in the relative thickness of the vessel wall.[56] The smaller arteries and arterioles of the lung undergo normal physiological remodeling within the first one to two months, whereas the large elastic arteries achieve this over a period of months, which results in a gradual decrease of the PVR. The endothelial cells flatten and the SMCs obtain the classical spindle shape. The vessel lumen is widened by reorganization of the cytoskeleton in the SMCs and endothelial cells[58] leading to morphological changes in both cells. This process may be facilitated by the paucity of fixed connective tissue in the vessel wall at birth.

***Balance vasoconstrictors/dilators / functional aspects of vascular tone***

Besides the morphological adaptations after transition at birth, the function of the pulmonary vasculature is also changing as can be monitored by several functional aspects, like the regulation of the vascular tone. This regulation is the result of the balance between endothelium derived vasoconstrictive and vasodilatative mediators. Although several pathways are involved, we will focus on three pathways that are targets of current therapeutic strategies in pulmonary arterial hypertension; the NO pathway, the $PGI_2$ pathway and the ET-1 pathway, for review see.[59]

Nitric oxide (NO) is produced by endothelial Nitric Oxide Synthase (eNOS) from arginine. NO activation of a cGMP dependent protein kinase will relax the contractile apparatus in SMCs. This kinase subsequently reduces the intracellular calcium concentration and decreases the myosin contraction. eNOS is activated by several stimuli, such as shear stress, estrogens, and the rise of intercellular calcium under influence of substances, such as bradykinin, histamine and serotonin. The calcium is bound by calmodulin, which

binds to eNOS and leads to activation.[60] $PGI_2$ also has a vasodilatory effect by activating adenyl cyclase through a receptor G protein-coupled mechanism. The ET-1 pathway can have both a vasoconstrictive and a vasodilatory effect on SMCs. Activation of either the ET-A or ET-B receptor on the SMCs leads to vasoconstriction; activation of ET-B receptors on endothelial cells (ECs) leads to vasodilatation.[61]

Expression of eNOS in human lung increases until 31 weeks of gestation, followed by a marked decline.[62] eNOS may also play a role in lung maturation, because the antenatal peak of eNOS expression is concomitant with the onset of alveolarization.[63] In sheep, eNOS increases during the late fetal and early postnatal periods.[64-65] In pigs, NO-mediated vasodilatation is increased directly after birth and declines after the second week postnatally. This suggests a role for NO in optimizing the pulmonary vasodilatation at time of transition and immediately after birth.[66-68]

In fetal lambs, ET-1 is involved in the regulation of the pulmonary vascular tone.[69] However, a lowering in the level of ET-1 does not affect the PVR, suggesting that other factors or mechanisms are also influence in the antenatal vascular tone. In human lungs, the expression of ET-1 and its ET-A receptor are stable throughout gestation, whereas the ET-B receptor expression increases from mid-gestation until birth.[62]

Thus, both human and animal studies suggest that the production of NO and $PGI_2$, as well as the expression of ET-B receptor increase during gestation. These three mechanisms are associated with induction of vasodilatation of the pulmonary vasculature.[66]

However, in pulmonary hypertension the production of some of the vasoreactive mediators deviate to some extent. For example, prolonged hypoxia triggers the development of pulmonary hypertension of the newborn, which is associated with a decreased NO production.[70] In addition, in newborn calves suffering from hypoxia induced pulmonary hypertension the production of $PGI_2$ was attenuated.[71]

## Therapeutic aspects

The therapeutic approach of PHN is based on inducing vasodilatation of the vascular bed to reduce the PVR.[72-73] Vasodilatation can stimulated by intervening with the NO or $PGI_2$ pathway or by reducing the activity of the ET-1 pathway, which inhibits vasoconstriction. Most of the knowledge of the use of these different therapies is based on their response in pulmonary arterial hypertension in adults,[59] or animal PAH models, such as the prenatal ductus ligation lamb model[74-75] or the monocrotaline induced rat model.[76] Inhaled NO is the most studied therapeutic strategy in newborns and it has been shown to improve oxygenation.[77] If oxygenation is not improving despite treatment with iNO, ECMO could be considered to optimize the respiratory and cardiac

function.[73,78] NO induces SMC relaxation by stimulating a molecular cascade starting with cGMP production. cGMP, however is degraded by phosphodiesterase type 5 (PDE-5). Sildenafil inhibits PDE-5 and thus improves oxygenation by inhibiting PDE-5, but the clinical evidence is still limited[2,79] and optimal dosing remains problematic.[80] Prostacyclin therapy in newborns is only described in case-reports[81-83] and properly designed clinical trials are warranted, since one major disadvantage is the non-selective effect on the systemic circulation.[81-83] The use of endothelin receptor blockers is not tested in newborns yet, but only described in a case-report.[84] A number of recent reviews are available describing the different therapeutic strategies in pulmonary hypertension in the newborn.[73,85]

## Vascular abnormalities

PHN is morphologically characterized by the anatomical change in the cardio-pulmonary vasculature, called vascular remodeling, evidenced by a combination of hypertrophy and/or hyperplasia of the cells within each layer of the vessel wall (fibroblasts, smooth muscle cells and endothelial cells) and increased extracellular matrix deposition (collagen, elastin and fibronectin).[86-87] Moreover, an expansion of smooth muscle cells is observed in partially and non-muscularized peripheral arteries, called neo-muscularization. Neo-muscularization is thought to be the result of aberrant differentiation of precursor cells, pericytes and “intermediate” cells, into SMCs.[88] In addition, experimental data obtained from mainly hypoxia induced PAH models, representing pulmonary hypertension in adults; have suggested that other cells may also contribute to remodeling of the media. These are: 1) recruited circulating progenitor cells that differentiate into smooth muscle (like) cells;[89-92] 2) resident vascular progenitor cells that become active and differentiate;[93-94] 3) activated adventitial fibroblasts that migrate into the media and/or intima;[95-96] and 4) endothelial cells that transdifferentiate into mesenchymal cells.[97] Vascular remodeling is also associated with other processes that play a role in the pathogenesis of PHN, like production of hypoxia- or inflammation-induced growth-factors, angiogenic factors, inflammatory mediators and vasoconstrictors.

Vascular remodeling and neo-muscularization progressively narrow the vascular lumen, resulting in increased vasoconstrictive response and a decreased vasodilatory response.[86] These structural changes are partly mediated by dysfunction of the endothelial cells. An altered production of endothelial vasoactive mediators (nitric oxide, prostacyclin, endothelin-1, serotonin and thromboxane) affects the growth of the SMCs, facilitating the development of pulmonary hypertrophy and structural remodeling.[98]

Most of the aspects about the vascular remodeling process and the development of the structural changes are described for PAH in adults. However, intrauterine events may contribute to the development of neonatal pulmonary hypertension. Adverse intrauterine stimuli in late gestation, such as decreased blood flow, changes in substrate or hormone delivery to the lung, chronic hypoxia, chronic hypertension or inflammation, have the potential to change the functional and structural aspects of the pulmonary vasculature, thereby contributing to abnormalities as seen in pulmonary hypertension.[99]
In persistent pulmonary hypertension of the newborn it was hypothesized that the transition of fetal vascular smooth muscle cells to an adult phenotype fails. Muscularization of the immature pulmonary vasculature leads to migration of adventitial fibroblasts and medial smooth muscle cell, and increased production of extracellular matrix proteins fixes the vessel wall in an incompletely dilated state.[14]

## Pulmonary vascular diseases in the newborn

Pulmonary hypertension is associated with a variety of disorders or syndromes in infants, but they all share similar pulmonary vascular abnormalities. This review focuses on pulmonary vascular abnormalities in human newborns with “idiopathic” persistent pulmonary hypertension of the newborn (PPHN), pulmonary hypertension in lung hypoplasia either caused by congenital diaphragmatic hernia (CDH) or by renal disorders or early premature rupture of membranes (PROM), and pulmonary hypertension caused by meconium aspiration syndrome (MAS).

***‘idiopathic’ persistent pulmonary hypertension of the newborn (PPHN)***
Idiopathic PPHN is the second most common etiology of pulmonary hypertension in the newborn.[78] In idiopathic PPHN, associated causes of the pulmonary hypertension could not be diagnosed. However, in some cases of idiopathic PPHN, infants had a constriction of the fetal ductus arteriosus in response to exposure to non-steroidal anti-inflammatory drugs (NSAIDs) in the third trimester[100] Furthermore, the relation between the use of selective serotonin reuptake inhibitors (SSRIs) in pregnant women and the occurrence of pulmonary hypertension is described.[101-102]
The pulmonary vasculature is significantly remodeled with increased vessel wall thickness and smooth muscle cell hyperplasia. The intra-acinar arteries are muscularized,[103-104] but the preacinar arteries have a normal muscular coat.[9] These abnormalities prevent a normal transition after birth.
Recently, it was suggested that disruptions of NO, prostacyclin and endothelin pathway are responsible for the structural abnormalities seen in idiopathic PPHN.[78] Reduced expression and activity of eNOS have been noted[105-106] as well increased ET levels.[107-

[108] Lassus et al. reported an increased expression VEGF in the vascular endothelium, but a decreased VEGF plasma level compared to term infants with normal lungs.[109] This may reflect an overall disturbance in vascular development.
In conclusion, idiopathic PPHN is a complex pathological process with direct or indirect involvement of multiple signaling pathways regulating vascular tone and structural remodeling of the pulmonary vasculature that already occurs in utero.[78]

***Lung hypoplasia in congenital diaphragmatic hernia (CDH)***
CDH is one of the developmental defects that are thought to be due to genetic predisposition[110] or susceptibility to environmental factors.[111] Pulmonary vascular disease in CDH is characterized by abnormal pulmonary vascular development, abnormal vasoreactivity and a disorganization of postnatal vascular remodeling.[112] The abnormal vascular development is characterized by fewer capillaries, which are unevenly distributed and embedded in a thicker mesenchyme.[113]
In CDH both the media and the adventitia show structural abnormalities of the vessels. Both were reported to be much thicker in the pulmonary arteries of newborns and stillborns with CDH. This suggests that the intrapulmonary arteries in CDH may already become excessively muscularized during fetal life.[114] Shehata et al. and Yamataka and Puri both showed that the adventitial layer in CDH infants was significantly thicker than in controls.[115-116] The adventitial layer normally decreases after birth as a part of the structural remodeling, but it seems that in CDH this structural remodeling is impaired.[115] Treatment with extra corporal membrane oxygenation (ECMO) showed a reduction in the adventitial thickness, but persistent medial hyperplasia explaining their unresponsiveness to vasodilatation therapy.[117]
The deposition and turnover of extracellular matrix proteins is also involved in vascular remodeling. The balance between deposition and turnover are matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). In CDH lungs, MMP-1 expression was increased in capillaries and fibroblasts, whereas TIMP-2 expression was decreased. In the arterioles of CDH lungs, the positive area of MMP-2 and 9 and TIMP2 expression was increased.[28] This suggests a shift in the balance between the MMPs and TIMPs. This may play a role in the abnormal remodeling of interstitium and pulmonary arteries in CDH.[28]
Sonic Hedgehog (SHH) signaling is involved in the development of respiratory bronchioles and thinning of the interstitium. SHH expression normally peaks at 16 – 18 weeks gestation and completely disappeared at 35 weeks gestation.[118] In CDH however, decreased SHH expression was already detected in early development and maximal mRNA expression was observed in the late canalicular/saccular period (21 – 32 weeks gestation), which continued until term. Overall, the SHH expression seems to be delayed

in CDH compared to control, which suggests that changes in SHH expression may contribute to pulmonary abnormalities in CDH.[118]

Since the vasculature of CDH patients is clearly affected, various groups have investigated the expression and influence of a diverse set of angiogenic factors. One of the most potent angiogenetic factors is VEGF, which is strongly activated under hypoxic conditions by the activity of HIF-1, a dimer of an oxygen sensitive α-subunit and a stable β-subunit. VEGF is a signaling factor with pleiotropic functions, including the stimulation of NO production in endothelial cells.[47] In control lungs, HIF-1α was expressed in the endothelium and medial layer of the pulmonary arteries, whereas in CDH it was only expressed in the media.[119] In the same study, however, the expression patterns of several angiogenetic downstream targets, such as VEGF, Flk-1 and eNOS did not significantly differ between CDH infants and controls,[119] although many other target genes have not been investigated in detail. Another finding in this study was that the expression of pVHL, the protein responsible for targeting HIF-1α for degradation, was increased in the medial layer in CDH lungs compared to control lungs.[119] Boucherat et al hypothesized that changes in Ang-1/TIE2, a ligand/receptor required for correct organization and maturation of newly formed vessels, may be responsible for the structural vascular abnormalities.[113] However, the Ang-1 concentration in human lung samples did not significantly change during pregnancy and no differences were observed between controls and samples with lung hypoplasia. Likewise, the expression of TIE-2 did not differ. On the other hand, the Ang-2 concentration in lung hypoplasia samples was increased throughout pregnancy in compare to controls. Ang-2 competes with Ang-1 in binding to TIE-2, and blocks the function of Ang-1, the stabilization of vessels. In contrast to the study of de Rooij,[119] these authors found elevated levels of VEGF-A in fetuses with lung hypoplasia. The biological effects of VEGF-A and Angiopoietins are mediated by eNOS. eNOS levels were even more decreased in CDH patients compared to patients with lung hypoplasia of other causes then CDH, and both were lower than in control (25% and 45% of control, respectively).[113]

Two studies found high concentrations of ET-1 in the plasma of infants with CDH;[120-121] another study found increased expression of ET-A and ET-B receptors in the pulmonary arteries.[122]

Altogether, several studies have indicated changes in levels of important regulatory proteins. However, until now a causative relation between these individual changes and the occurrence of PHN has not yet been demonstrated.

***Lung hypoplasia in renal disorders and early premature rupture of membranes (PROM)***

The underlying mechanism to explain the lung hypoplasia in renal disorders, other than the supposition that the pulmonary hypoplasia is secondary to oligohydramnios and thoracic compression in utero.[123-126]

Experimental research showed that antenatal drainage of lung fluid leads to a decrease in lung volume and an abnormal maturation of the alveolar wall.[127] The lung normally produces one-third of the amniotic fluid,[128] but the lung drainage is more excessive in the absence of amniotic fluid. The kidney is also responsible for the production of amniotic fluid; however this is relatively late in gestation. The decreased number of airways in lung hypoplasia due to renal disorders implies interferences of the lung growth before 16 weeks gestation. Thus oligohydramnion can not be the only explanation of the lung hypoplasia in these cases. Another explanation for the lung hypoplasia is the role of proline, an amino acid, since the kidney is an important source of proline during fetal development. Injection of nephrotoxins into chick embryos and followed the production and metabolism of labeled proline. The morphology of the lung showed hypoplasia with sparse mesenchyme.[129]

Early premature rupture of membranes (PROM) before the 26$^{th}$ week of gestation is associated with lunghypoplasia.[130-131] The pathophysiological mechanism suggested the is leakage of amniotic fluid, which comprises the normal fetal breathing movements. These fetal respiratory movements are required to mechanically stimulate lung growth, and absence or reduction is a poor prognostic sign.[132]

The hypoplastic lungs showed a delayed maturation with poor saccular branching and retarded epithelial maturation at the periphery of the acinus, which associated with poor development of the interstitial tissue and delayed vascularization of the saccules.[133] Infants with bilateral renal agenesis and dysplasia had reduced number of arteries, probably associated with the reduced number of airways.[134] The pulmonary arteries were normal in size in most of the lungs, whereas in some lungs the arteries were reduced in size, although these arteries still appeared large compared to the lung volume. The vessel wall thickness was reduced in almost all cases of renal agenesis, but was normal for the gestational age in the cases of renal dysplasia cases. In some of the infants with renal agenesis or dysplasia the smaller and more peripheral vessels had an extended muscularization, whereas in two cases there was less muscularization compared to normal.[134]

As reported by Boucherat et al, the eNOS level in lung hypoplasia not caused by CDH was lower compared to controls, but higher than in CDH.[113] This low eNOS level could lead to a disruption in the molecular and cellular aspects of the interaction between epithelium and mesenchyme in the coordination of airway branching and vascular development, although data analyzing this aspect in depth are scarce.

***Meconium aspiration syndrome (MAS)***

Meconium aspiration syndrome (MAS) is defined as respiratory distress in infants born with meconium stained amniotic fluid, in which the respiratory symptoms could not be explained otherwise. It is a life-threatening disorder that is complicated by respiratory failure, pulmonary airway leakage and persistent pulmonary hypertension in 15% to 20% of cases.[135] In 8% to 25% of all the deliveries the amniotic fluid is contaminated with meconium, as a result of fetal hypoxia stress. Aspiration or diffusion of meconium into the fetal airways will lead to MAS in 5%.[136-137] Aspirated meconium obstructs the small airways. The chemical pneumonitis inhibits the surfactant function and causes inflammation of the lung which plays an important role in the pathogenesis.

The number of intra-acinar arteries is normal in MAS, but there is an extensive muscularization, extending into the arteries in the alveolar wall. This extensive muscularization of the distal pulmonary arterioles was already observed in infants dying after MAS, suggest that other factors than only aspiration of meconium contributes to the pulmonary symptoms. The structural abnormalities, as seen in MAS, suggests that these vascular abnormalities already developed in utero.[138]

## Expert commentary

Pulmonary hypertension in newborns is a challenge for neonatologists because of the associated morbidity and mortality. So far, treatment of PHN is largely based on trials in adult PAH, and scarce data on evidence based treatment with iNO in newborns with pulmonary hypertension.

Although the etiologies differ for the various causes of pulmonary hypertension, the vascular abnormalities consist of increased vessel wall thickness and/or extensive muscularization of the small arterioles (resistance vessels). These facts were mainly discovered by analyzing (immuno)-histological studies of limited number of human post mortem lungs. Our knowledge about the vascular abnormalities in pulmonary hypertension in newborns is mainly based on research in animal models and nonstructural analysis of patients who died under hypoxic conditions. These data are also of relevance for our understanding of pulmonary hypertension in case of CDH. The animal models provided more insights in the normal pulmonary development and the pathophysiological mechanisms involved in development of pulmonary hypertension due to CDH. The development of the pulmonary vasculature is very complex and it is still largely unknown how the many factors involved interact in the development of the vasculature. Other fields of research such as angiogenesis and vasculogenesis during embryonic development and tumor biology in which vascularization plays a central role may also contribute, given the similarities in vascular growth.

## Five years view

PHN still results in a significant mortality and morbidity in part because of the lack of proper evaluated treatment modalities. Moreover, as this review clearly indicates, translational PHN research is largely descriptive and still lacks the knowledge about (epi)genetic factors that are implicated in PHN development, or the factor(s) and pathways involved causing predisposition for the development of vascular abnormalities. We like to highlight key aspects of future attention, arbitrarily separated in clinical and translational aspects.

Recently a strategic plan for lung vascular research is published, outlining recommendations for future research to get a better understanding of PAH in adults.[139] Some of these recommendations can be extrapolated to PHN as well.

***Clinical aspects***

Adult pulmonary arterial hypertension (PAH) is explicitly defined as a pulmonary arterial pressure of at least 25 mm Hg at rest and 30 mm Hg during activity,[140] based on cardiac catheterization as the "golden" standard. However, PHN lacks an unambiguous diagnosis, and therefore, it is necessary to reach an indisputable definition for PHN. So far, in the newborn and pediatric age groups, cardiac ultrasound is an important tool to aid in the diagnosis of PHN, although large patient data sets are currently unavailable.

Aside from the improvement of diagnosis, a clear distinction between primary or secondary PHN must be made, as well as separation of associated abnormalities which may influence clinical outcome. This will benefit the patient, as treatment modalities will become individualized, based on the patients' genetic background (see translational research for details).

Based on research in animal models and first trials in PAH, possible therapeutic strategies for the near future are the inhibition of tyrosine kinases and Rho-kinases. These strategies are based on the discovery of the involvement of these signaling pathways in the remodeling of the vasculature in pulmonary hypertension. Also a combination of different therapeutic strategies needs to be examined in multicentre trials.

To improve the long term follow up of survivors, a globally accepted standardized follow-up program must be implemented. It is already known that the morbidity of pulmonary hypertension, characterized by neurodevelopmental impairment and chronic lung disease, is partly caused by the clinical management. This situation requires attention of all physicians involved in the patients' care, and careful multimodal documentation (cardiac ultrasound, biomarkers, lung biopsy and genetic evaluation) of the treatment results, to be able to improve the current approaches. Within this context recently Haworth and Hislop published follow up data of the UK pulmonary hypertension service for Children.[141]

***Translational aspects***

In order to understand the pathogenesis of PHN, we need to unravel the biology of vascular development and repair mechanisms. A wealth of data is available on the development of the systemic vasculature, but it is clear that the pulmonary circulation deviates from the systemic vessels at important points. Although mechanisms of cellular interactions are initially comparable, it is required to extrapolate the current systemic data to the pulmonary research field, and extend the research in the areas that are different from the systemic data. Especially unraveling the molecular and cellular adaptations at the transition point after birth is a prerequisite for understanding the (in)ability of the pulmonary vasculature to reduce the PVR observed in PHN.

The unpredictable outcome of current treatment is potentially based on individual genetic background. Therefore, it is of critical importance to collect patient material to categorize the PHN cases in a better and more efficient manner. This material can be used to perform whole genome sequencing, which will be fundamental in the identification of putative mutations that are in common between PHN patients. Furthermore, the obtained data will be used to pinpoint loci that may result in a genetic predisposition to develop PHN. In order to realize these goals, it is necessary to setup patient biobanks containing pathology samples and patient cell lines to obtain DNA for karyotyping and genome sequencing. The search to identify biomarkers to determinate early the severity of pulmonary hypertension will help us to start multimodal therapeutic strategies earlier.

Current research using animal models to identify and optimize putative therapeutic approaches are required to extrapolate the possibility to use specific drugs regimens. The drugs can be based on PAH experiences, but may also be based on knowledge obtained through the basic understanding of molecular and cellular processes involved in the transitional stages of the pulmonary vasculature. The identification of specific factors involved in this process may contribute to the development of specific drugs targeted to single molecules.

**Key-issues**

- Randomized control studies are needed to improve and develop (new) evidence based therapeutic strategies.
- Genetic and protocollized diagnostic evaluation of each newborn with pulmonary hypertension using standardized, innovative methods will accelerate and contribute to an optimized therapeutic approach.
- Close collaboration between clinicians and scientists involved in PHN is a prerequisite for the success of a useful biobank. Patient material needs to be collected, categorized and stored in a proper and standard way to ensure high quality.
- The -omics era (genomics, proteomics, metabolomics) will enhance the knowledge of pulmonary vascular development, and in turn contribute to a better understanding of the pathophysiology of vascular abnormalities in PHN.
- Individual factors of several metabolic and signaling pathways are described to influence vascular remodeling and vasoreactivity of the pulmonary vasculature in PHN, but the interaction between of these factors is still largely unknown.

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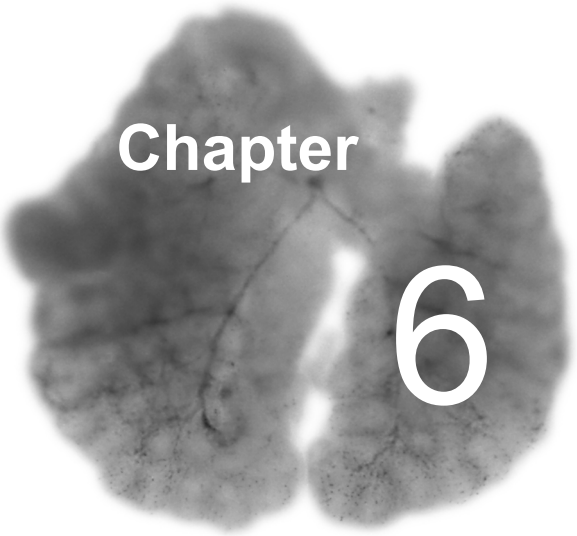

# Chapter 6

## Premature differentiation of vascular smooth muscle cells in human congenital diaphragmatic hernia

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*Submitted*

## Abstract

**Background** Pulmonary hypertension is a major determinant of mortality and morbidity in congenital diaphragmatic hernia (CDH), which is associated with characteristic thickening of the media and adventitia of the vascular wall. The media consists of a heterogeneous population of smooth muscle cells (SMC), ranging form synthetic to contractile cells, which is associated with their regulatory (vascular tone) and structural functions. These populations are regulated by developmental and environmental cues and thus may play a role in the development of the structural changes.

**Methodology** We first analyzed the protein expression of specific markers associated with either synthetic or contractile SMC phenotypes in human lungs at different developmental stages and secondly, we compared immature / premature and term infants with congenital diaphragmatic hernia or lung hypoplasia due to renal agenesis or PROM with age-matched control infants

**Results** Synthetic and contractile SMCs are distributed in a temporal and spatial specific pattern, associated with the proximodistal axis of the lung. Infants with CDH have contractile SMCs which are more widely distributed and already more distally located compared to controls. This different distribution is already observed form 19 weeks gestation onwards.

**Conclusion** We show for the first time that the more extensive distribution of contractile SMCs is associated with an early maturation of the vasculature, contrasting to the general hold concept that the lung shows delayed maturation in CDH.

## Introduction

Pulmonary hypertension is a severe cardio-respiratory disorder that is associated with a variety of diseases.[1] An increased pulmonary vascular resistance caused by structural and functional changes in the pulmonary vasculature results in a restricted pulmonary flow and ultimately in right ventricle failure leading to death.[2]

In congenital diaphragmatic hernia (CDH) pulmonary hypertension, in combination with lung hypoplasia, is the major determinant of the significant mortality and morbidity in these patients.[3-4] Morphologically, the structural abnormalities in the pulmonary vasculature of CDH patients are well described and characterized by a reduced vascular bed, increased thickness of arterial media and adventitia and an excessive muscularization of arterioles.[5-7] Modern treatments aim to reach a diminished pulmonary vascular tone and restrict iatrogenic damage of the epithelial components of the lung by gentle ventilation.[8-9] However, even high technological approaches such as ECMO can not improve the outcome due to our lack of knowledge of the underlying pathology as we recently reviewed.[9]

The vascular wall of muscular vessels consists of three layers, the intima, media and adventitia and each layer contains specific cells. The endothelial cells of the intima are surrounded by medial vascular smooth muscle cells (VSMCs) in arteries and veins, which are involved in the (pulmonary) vascular function (regulation of vascular tone) and structure (remodelling). The VSMCs are heterogeneous and their phenotypes associate with their function, ranging from synthetic (immature) to contractile (mature). The different phenotypes can be distinguished based on morphology, expression of cytoskeleton and contractile markers (figure 1) and responsiveness to several growth factors.[10-11] The VSMC phenotype is developmentally and environmentally regulated and may play a role in vascular remodelling as seen in pulmonary hypertension.[12] A case-report of two patients with primary pulmonary hypertension showed that VSMCs with an immature phenotype were present in the intima, whereas the media only had mature, contractile VSMCs.[13]

Morphological analysis of the lung hypoplasia in CDH revealed an early arrest in branching of both airway and vasculature,[14-15] with a histological immature lung,[16] suggesting that lung hypoplasia in CDH results from a delay in development. However, in human the maturation of alveolar type II cells and surfactant production was not delayed.[17]

Therefore, we analyzed the expression of specific markers associated with distinct VSMC phenotypes to investigate the pathogenesis of pulmonary hypertension in infants with CDH. Since VSMC play an important role in abnormal vascular remodelling and the structural abnormalities in CDH already develop in utero,[18-19] we hypothesized that the VSMC population in CDH is already intrinsically different early in development.

We show clear differences in the expression of VSMC markers in CDH compared to age-matched controls, which suggests that VSMCs in CDH infants do not have a delayed development, but rather display an early differentiation into a mature, contractile phenotype.

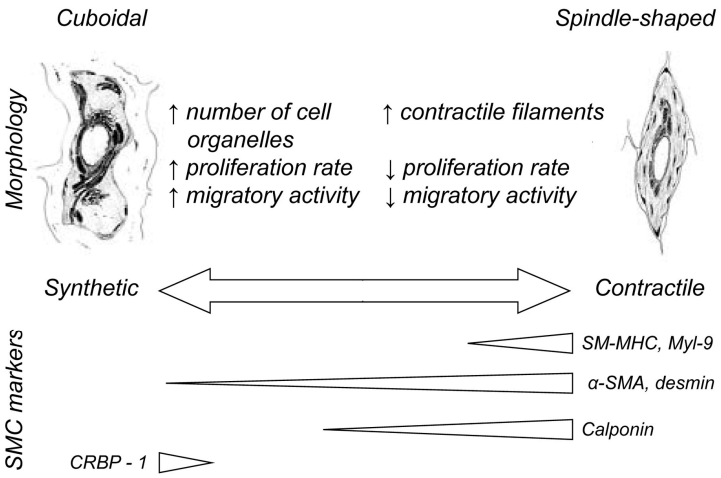



**Figure 1. Characteristics of smooth muscle cell phenotypes.** Smooth muscle cells display different phenotypes ranging from synthetic to contractile, which can be characterized by cell morphology (cuboidal versus spindle shaped), function (proliferation, migration or regulation vascular tone) and the expression of specific SMC markers. (Figure adapted form S.S.M. Rensen et al. Neth Heart J 2007;15:100-108)

## Material and Methods

### *Human lung samples*

Human lung samples were retrieved from the archives of the Department of Pathology and the Erasmus MC, Rotterdam, following approval by the Erasmus MC Medical Ethical Committee. Fifty control lungs were used for the tissue microarray for normal lung development, subdivided in pseudoglandular, canalicular, saccular and alveolar stage (table 1).

For the CDH versus control experiments, 20 CDH patients were selected (mean lung/body weight of term CDH patients 0.0032, normal ratio 0.012), which did no show severe hemorrhage and/or necrosis. The control group consisted of age-matched patients (15 – 40 weeks) that either died in utero or the duration of postnatal survival and spontaneous breathing and/or ventilation was comparable with the CDH patients (table 2).

**Table 1. Characteristics of control infants selected for normal developmental tissue microarray**

| Developmental stage | N | Gestational age in weeks | Postnatal age | Birth weight in grams |
|---|---|---|---|---|
| Pseudoglandular | 8 | 15.5 (10 – 17) | - | 79 (36 – 117) |
| Canalicular | 15 | 22 (18 – 25) | - | 350 (138 – 695) |
| Saccular | 11 | 31 (26 – 37) | 1 hr (0 – 24 hrs) | 1750 (758 – 2790) |
| Alveolar | 16 | 40 (32 – 41) | 5 wks (3 hrs – 2 yrs) | 2910 (1300 – 4025) |

Median and ranges (in brackets) of gestational age, postnatal age and birth weight of controls divided in pseudoglandular, canalicular, saccular and alveolar.

**Table 2. Characteristics of CDH patients and controls selected for tissue microarray**

| | Developmental stage | N | Gestational age in weeks | Postnatal age | Birth weight in grams |
|---|---|---|---|---|---|
| Control | Immature / Premature | 10 | 31.5 (15 – 36) | 1 hr (0 – 24 hrs) | 2000 (57 – 2700) |
| | Term | 10 | 39.5 (38 – 41) | 36 hrs (1 hr – 1 wk) | 3220 (2490 – 3950) |
| CDH | Immature / Premature | 10 | 31.5 (15 – 36) | 1 hr (0 – 48 hrs) | 1032 (30 – 2515) |
| | Term | 10 | 39 (37 – 40) | 7 hrs (1 hr – 3 days) | 2835 (2000 – 3000) |

Median and ranges (in brackets) of gestational age, postnatal age and birth weight of CDH patients and controls divided in immature / premature and term subgroups

We divided the CDH patients and age-matched controls in two groups; immature / premature (gestational age < 37 weeks) and term (gestational age > 37 weeks). Five patients with lung hypoplasia due to renal agenesis and 5 patients with lung hypoplasia due to premature rupture of membranes (PROM) were selected to determine if possible differences in expression between CDH and control were caused by CDH or by lung hypoplasia in general (table 3).

***Tissue micro array***

Tissue micro arrays were constructed as described by Kononen et al.[20] For each sample, three tissue core biopsies of 1.5 mm in diameter and 3.2 mm in depth were taken from preselected regions to ensure adequate representation of all lung structures. These biopsies were placed in linear arrays into empty recipient paraffin blocks, two for the

normal development stages, one for CDH, and one for CDH control. Tissue cores of adult multi-slides were used as controls.

**Table 3. Characteristics of patients with lung hypoplasia not due to CDH**

| Lung hypoplasia (no CDH) | N | Gestational age in weeks | Postnatal age | Birth weight in grams |
|---|---|---|---|---|
| Renal agenesis | 5 | 34 (22 – 36) | 1 hr (0 – 48 hrs) | 1550 (616 – 1670) |
| Premature rupture of membranes | 5 | 29 (29 – 33) | 2 hrs (1 – 19 hrs) | 1305 (1078 – 2050) |

Median and ranges (in brackets) of gestational age, postnatal age and birth weight of patient with lung hypoplasia due to either renal agenesis or premature rupture of membranes.

***Immunohistochemistry***

Immunohistochemistry was performed on 4 μm sections of TMAs according to standard protocols, using the EnVision™ detection system (DakoCytomation, Glostrup, Denmark).[21] Primary antibodies used were SMMS-1 against SM-MHC (1:50, Dako, The Netherlands), α-SMA (1:300, Biogenex, San Ramos CA, USA), Myl-9 against myosin light chain (1:500, Santa Cruz, Heidelberg, Germany), calponin (1:100, Dako), desmin (1:200, Monosan, The Netherlands) and CRBP-1 (1:25, Abcam, Cambridge, UK). Antigen retrieval with Tris-EDTA buffer (pH 9.0) was used for SMMS-1, α-SMA and desmin; the remaining antibodies did not require antigen retrieval. Negative controls were performed by omission of the primary antibodies.

The expression of the various VSMC markers in the vascular wall was scored as either present or absent. Since the human lungs were not fixed at a pressure of 20 $cmH_2O$, but by immersion, distinction based on quantification of the vessel diameter was difficult. Therefore the distinction was made between small, medium-sized and large vessels, based on the morphology of the vessels stained with α-SMA. Small vessels are defined to have a small lumen and consisted of one layer with VSMCs, medium-sized vessels had a larger lumen compared with the small vessels and had of one or two layers of α-SMA positive cells, and the large vessels had several layers of α-SMA positive cells in combination with a large lumen.

## Results

***Expression pattern of vascular smooth muscle cells during normal development***

First, we analyzed the distribution of SMCs using the general, non-SMC specific marker α-SMA. At the earliest gestational period tested, the pseudoglandular stage (10 – 17 weeks), the peribronchial cells and the medial layer of pulmonary arteries, except for the

most distal precapillary areas, already expressed α-SMA. This expression was maintained throughout development and also undefined interstitial cells showed positive staining for α-SMA (figure 2A-D). Secondly, we analyzed the expression of additional, more specific SMC markers, which showed a clear developmental expression pattern. Two typical markers for contractile SMCs, SM-MHC and Myl-9, were already expressed from the pseudoglandular stage onwards (figure 2E and 2L). SM-MHC showed a prominent staining of VSMCs in large and medium-sized vessels, as well as in peribronchial cells. Myl-9 showed a typical vascular pattern with positive staining of VSMCs and sometimes the endothelium in the large vessels. Calponin started to be expressed in the VSMCs of large vessels in the saccular stage (data not shown). The expression of the synthetic SMC marker, CRBP-1 is only observed in some lungs in the large and medium-sized vessels in the alveolar stage (compare figure 2M-O with 2P). The same phenomenon is observed for the expression of desmin (data not shown). The peribronchial SMC express desmin and Calponin already in the pseudoglandular stage (data not shown).

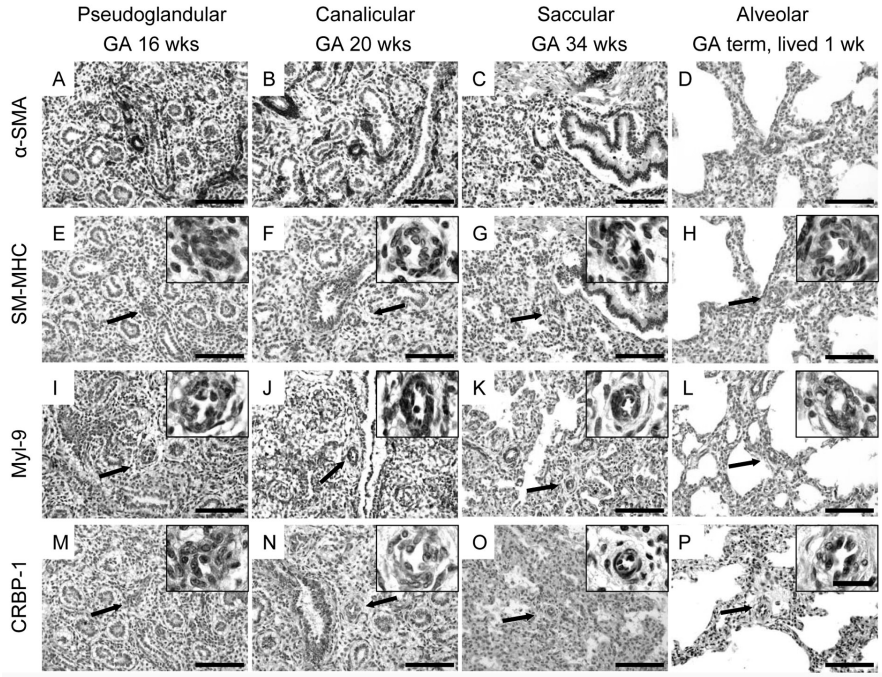



**Figure 2. Expression pattern of SMC markers during normal development.** Immunohistological staining of α-SMA (A-D), SM-MHC (E-H), Myl-9 (J-L), and CRBP-1 (M-P) during different developmental stages; pseudoglandular 16 weeks (A, E, I, M), canalicular 22 weeks (B, F, J, N), saccular 37 weeks (C, G, K, O), alveolar, > 40 weeks (D, H, L, P). Scale bars 100 μm. The insert is a higher magnification of the vessel that is indicated by the arrow. Scale bar 20 μm.

***Expression pattern of vascular smooth muscle cells in CDH patients versus control***

Expression of α-SMA was detected throughout the vasculature in unaffected as well as CDH immature and premature infants, whereas Calponin was only restricted to the large vessels (figure 3A, E and data not shown). SM-MHC is more widely expressed in the large and medium-sized vessels in both the immature and premature CDH infants compared to control (figure 3B, F), and is even expressed in lungs as early as 19 weeks of gestation (figure 3B). Myl-9 expression showed an expression throughout the whole vasculature. However, more CDH infants showed a positive staining for Myl-9 compared to control infants (figure 3C, G). The expression of CRBP-1 was only observed in the large and medium-sized vessels of CDH infants and was not found in unaffected infants (figure 3D, H).

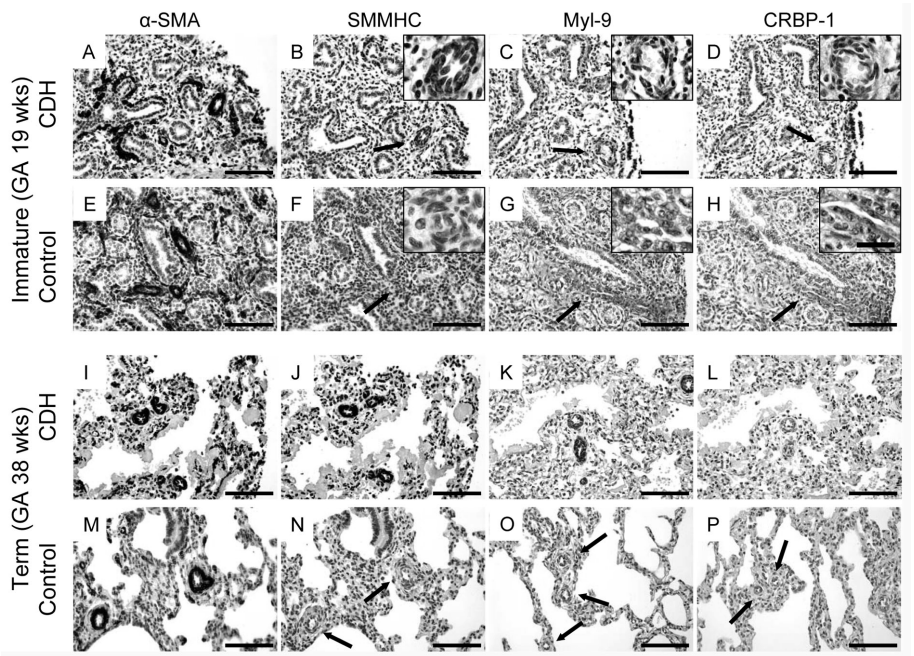



**Figure 3. Expression pattern of SMC markers in immature / premature and term CDH and age-matched controls.** Immunohistological staining of α-SMA, SM-MHC, Myl-9 and CRBP-1 for both immature (19 weeks) foetus (A-D) and term CDH (38 weeks) infant (I-L) in comparision with age-matched controls (E-H and M-P, respectively). Scale bars 100 μm. The insert is a higher magnification of the vessel that is indicated by the arrow. Scale bar 20 μm.

In term CDH infants, α-SMA was comparable to term controls (figure 3I, M), but SM-MHC was more broadly distributed in the large and medium sized vessels compared to term control infants (figure 3J, N). In addition, and in contrast to control infants, CRBP-1

was only expressed in the large and medium-sized vessels of term CDH infants (figure 3L, P). The expression showed a similar pattern in both immature / preterm and term CDH infants, indicating that structural changes are already present early in development. Since we detected significant differences in expression patterns, we wondered whether our findings were specific for CDH lungs. Therefore, we analyzed the expression of SM-MHC and CRBP-1 in infants with lung hypoplasia due to renal agenesis and lung hypoplasia due to early PROM. SM-MHC expression was observed in the large and medium-sized vessels, comparable to the pattern observed in CDH (figure 4A, B). CRBP-1 on the other hand was not expressed in infants with lung hypoplasia unrelated to CDH (figure 4C, D). This indicates that the expression of SM-MHC is not CDH-specific and associated with lung hypoplasia whereas the expression of CRBP-1 is CDH specific.

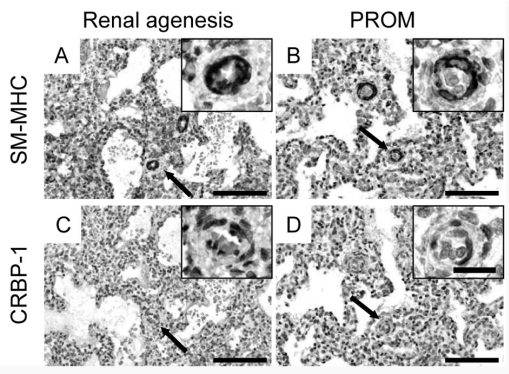
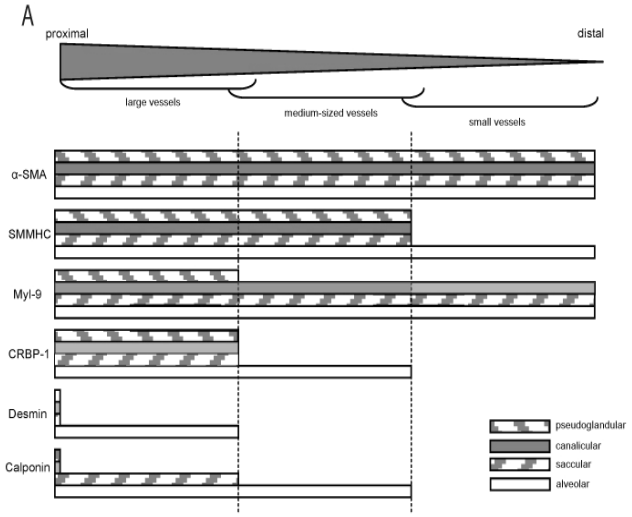



**Figure 4. Expression of SMC markers SMMHC and CRBP-1 in lung hypoplasia unrelated to CDH.** Immunohistological staining of SM-MHC and CRBP-1 in hypoplastic lungs due to renal agenesis (A, C) or early PROM (B, D). Scale bar 100 µm. The insert is a higher magnification of the vessel that is indicated by the arrow. Scale bar 20 µm.

In summary, a temporal and spatial gradient of contractile makers is established during embryonic and fetal lung growth (figure 5A). The expression domains of the different SMC markers are already observed along the proximodistal axis early in development, most prominently in the large and medium-sized vessels and for some SMC markers even in the smallest vessels (figure 5A). The expression domains of SM-MHC and CRBP-1 along the proximal-distal axis are expanded more distally in CDH infants compared to controls and these differences are already present during early development, from 19 weeks gestation onwards (Figure 5B versus 5C).




| | | Pseudo-glandular | Canalicular | Saccular | Alveolar |
|---|---|---|---|---|---|
| α-SMA | Large | 100% | 100% | 100% | 100% |
| | Medium-sized | 100% | 100% | 100% | 100% |
| | Small | 100% | 100% | 100% | 100% |
| SM-MHC | Large | 37.5% | 78.6% | 83.3% | 93.8% |
| | Medium-sized | 37.5% | 57.1% | 50% | 81.3% |
| | Small | - | - | - | 8.3% |
| Calponin | Large | - | - | 37.5% | 93.8% |
| | Medium-sized | - | - | - | 25% |
| | Small | - | - | - | - |
| Myl-9 | Large | 62.5% | 100% | 100% | 100% |
| | Medium-sized | - | 57.1% | 87.5% | 100% |
| | Small | - | 14.3% | 50% | 87.5% |
| Desmin | Large | - | - | - | 18.2% |
| | Medium-sized | - | - | - | 18.2% |
| | Small | - | - | - | - |
| CRBP-1 | Large | 12.5% | 7.14% | 12.5% | 43.8% |
| | Medium-sized | - | - | - | 25% |
| | Small | - | - | - | - |

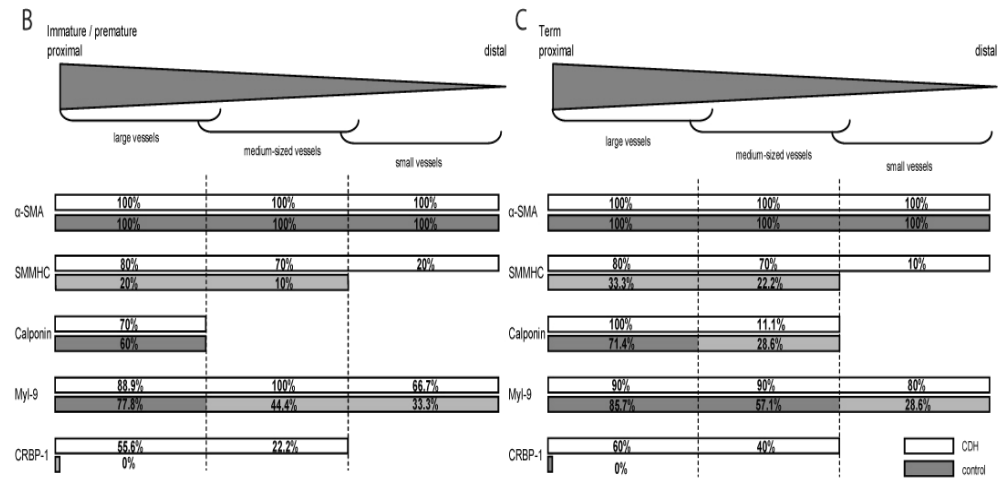



**Figure 5. Schematic overview of the expression pattern of SMC markers during normal development (A) and in immature / preterm CDH (B) and term CDH (C) with age-matched controls.** A) Developmental expression pattern for the different SMC markers used in this study, subdivided large, medium-sized and small vessels. Columns represent the expression of the indicated markers during the different lung development periods, the pseudoglandular, the canalicular, the saccular and alveolar stages, respectively. The table shows the percentages of infants that expressed the particular SMC marker. B and C) Schematic representation indicating the expression for the different SMC markers in immature / premature CDH with age-matched control infants in B and for term CDH with age-matched control infants in C, subdivided for large, medium-sized and small vessels. For each infant, individually, the expression was scored, as either present or absent. The number of infants with a present expression is calculated in percent

## Discussion

Infants with CDH display a variety of clinical problems, of which pulmonary hypertension is increasingly recognized to cause most of the mortality and morbidity. The associated structural changes in the pulmonary vasculature are prominent in the medial layer, in which VSMCs form a heterogeneous cell population and are involved in the functional and structural aspects of the pulmonary vasculature.[22] Vascular remodelling as seen in pulmonary hypertension may be associated with an altered VSMC phenotype.[12] Therefore, we studied the differentiation and maturation pattern of VSMCs in human CDH infants and controls.

Our results show that both SM-MHC and CRBP-1 are more widely distributed in the pulmonary vasculature of infants with CDH, indicating a changed heterogeneity of the VSMC population within the vascular wall in CDH. The heterogeneity of SMC subpopulations can be displayed by the different phenotypes, that vary from quiescent, contractile cells to proliferative, synthetic cells.[23] Contractile cells are typically flattened, quiescent cells with a functional contractile apparatus, whereas synthetic cells proliferate and secrete extracellular matrix proteins. The contractile and synthetic cells can be clearly distinguished based on their morphology[24-25] and on the expression of different SMC markers. Studies performed in human and animal models revealed that contractile cells express proteins such as SM-MHC, Myl-9 and Calponin, whereas synthetic cells can be distinguished by the expression of CRBP-1.[11,26-27]

In this study we evaluated the expression of the different markers from proximal to distal by analyzing large, medium-sized and small vessels in human lungs. We found, in agreement with earlier findings, a clear expression pattern for the different markers along the proximodistal axis, with VSMC differentiation ranging from immature, distally located VSMCs to differentiated, mature proximally located VSMCs.[28] However, VSMCs are not terminally differentiated and adapt their phenotype in response to changes in the local environment.[23,29]

In CDH both SM-MHC and CRBP-1 are more widely expressed. SM-MHC appears in two isoforms, which are developmentally regulated.[30-31] SM-1 is expressed in fetal arteries already early in gestation, whereas SM2 starts to be expressed during late fetal and postnatal development.[30] The specificity of our antibody does not discriminate between these two isoforms. The differences in expression of SM-MHC and CRBP-1 between CDH and age-matched controls were already observed in our evaluation of immature / premature infants. This suggests that early in development the fate of VSMCs is changed.

To find out if these differences in expression of SM-MHC and CRBP-1 were specific for CDH, we also analyzed the expression of these markers in the pulmonary vasculature of infants born with lung hypoplasia either due to renal agenesis or early PROM (before the

26th week). SM-MHC was expressed in the large and medium-sized vessels in cases of both lung hypoplasia due to renal agenesis and early PROM. This pattern was comparable to infants with CDH. This suggests that the different expression of SM-MHC is not CDH-specific, but is related to lung hypoplasia.

However, there are major differences related to different causes of lung hypoplasia. Morphological analysis of lung hypoplasia in CDH showed an early arrest (around 10 to 14 weeks), whereas it is thought that lung hypoplasia in renal agenesis and early PROM occurs later in gestation. In renal agenesis lung hypoplasia is associated with a retarded epithelial maturation in the distal acinus,[32] whereas in CDH the alveolar type II cells are not delayed in maturation.[17] In renal agenesis the lungs had a reduced number of arteries,[33] similar as seen in CDH. However, the structural changes in the pulmonary vasculature in renal agenesis only compromise extensive muscularization of the peripheral vessels,[33] whereas in CDH the media is thickened as well.[5-7]

CRBP-1 was specifically expressed in the arterial media in lungs of CDH infants, but could not be detected in the vasculature of cases with lung hypoplasia other than CDH. CRBP-1 is involved in the retinoic acid (RA) metabolism and SMC that express CRBP-1 are thought to have an altered RA metabolism.[34] RA is involved in physiological developmental and pathological processes.[35-36] Recently we documented the involvement of the RA pathway in the pathogenesis of human CDH.[37]

The expression of both SM-MHC and CRBP-1 in CDH showed an altered distribution already early in gestation, which corresponds to the morphological analysis of the vasculature in patients that died after ECMO, who still had medial hyperplasia.[7] Another study revealed that the increased vessel wall thickness occurred in utero in stillborns with CDH,[19] which suggests that the intrinsic differences already occur early in development. Our study implies a different heterogeneity of VSMC subpopulation in CDH compared to controls already early in development. Moreover, the expression and localization of SM-MHC in the pulmonary vasculature of CDH shows an aberrant development, in stead of a delayed development. This is in contrast with the general concept of overall delayed development of the lung in CDH, based on findings of an arrest in airway and vascular branching in combination with an immature histological aspect of the lung.[14-16] Further support for a delayed maturation was based on studies using animal models, the nitrofen induced CDH rat model or the surgical model of CDH in rabbits or lambs[38-41] but was never documented in human studies so far.

Overall, the pulmonary vascular development in CDH contains VSMC displaying an early differentiation into a mature, contractile phenotype. The disappointing results of vasodilatory therapy such as the low response to inhaled nitric oxide,[42] ECMO with a positive response of only 30 percent,[43] and other drugs, suggested to modulate the pulmonary vascular tone, such as sildenafil (chapter 4) might well be caused by this phenomenon.

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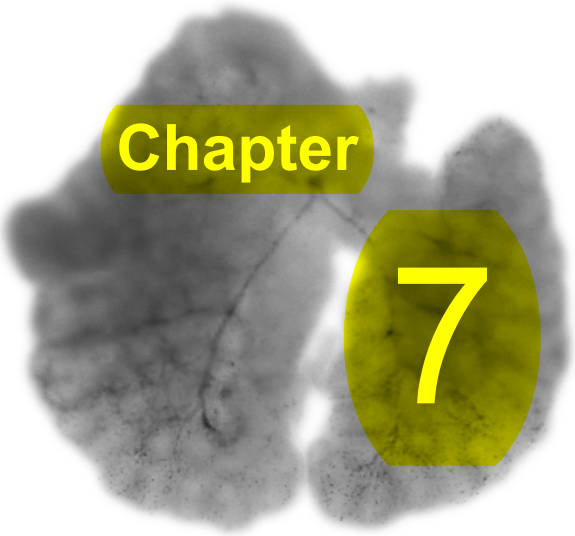

# Chapter 7

# The central role of pericytes in pulmonary hypertension in congenital diaphragmatic hernia

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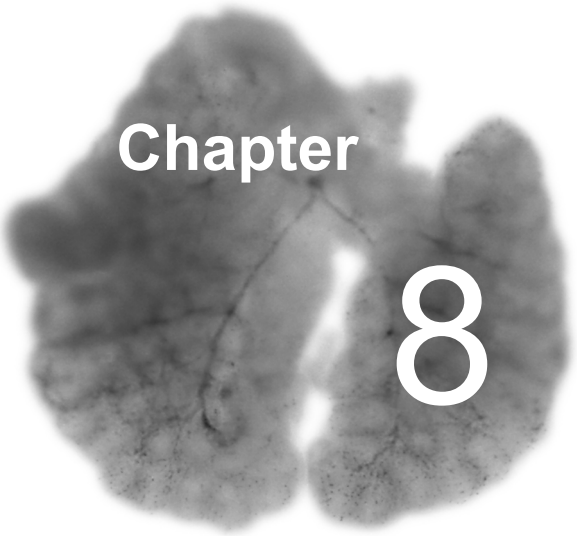
*Manuscript in preparation*



# Chapter 8

## Reversal of pulmonary vascular changes after recovery from chronic hypoxic pulmonary hypertension in rats

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## Abstract

Pulmonary hypertension is responsible for significant mortality and morbidity among newborns and infants, the pathology is characterized by pulmonary vascular remodelling with medial hypertrophy and adventitial thickening. Since it is unknown whether these abnormalities are permanent, we studied the reversibility of pulmonary hypertension induced changes in the hypobaric hypoxia induced rat model. Exposure of rats to hypoxia for 4 weeks induced signs of pulmonary hypertension, such as increased right ventricular systolic pressure (RVSP), increased right ventricular (RV) weight and considerable pulmonary vascular remodelling. Vascular changes were associated with the expression of SMemb in the pre-acinar vessels and increased expression of α-SMA, SM-MHC 2 and Calponin in the intra-acinar vessels. The RVSP and RV weight gradually decreased after 1, 2, 4 and 6 weeks of recovery in normoxia, although this reversal of the hypoxia induced changes did not reach baseline levels. However, the vessel wall thickness did return to the normal range and the expression of the SMC markers was comparable to the normal control group. Thus, the morphological pulmonary vascular changes are completely reversible after 6 weeks recovery.

## Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a severe cardio-respiratory disorder and is responsible for significant mortality and morbidity among newborns.[1-3] Pulmonary hypertension can be related with several different diseases.[4] Pulmonary hypertension in newborns can be primary, the so-called idiopathic PPHN, but is usually associated with respiratory or cardiac disorders such as meconium aspiration syndrome, pneumonia, congenital diaphragmatic hernia, transposition of the great arteries or total abnormal pulmonary venous return.[5-8]

Persistent pulmonary hypertension of the newborn is characterized by an increased pulmonary vascular resistance (PVR). In the early phase of exposure to hypobaric hypoxia, the increase of PVR is mainly caused by vasoconstriction, but sustained exposure to hypoxia results in structural changes in the pulmonary vasculature. These structural changes, also called vascular remodelling, consist of an increasing thickness of the medial and adventitial layer of pulmonary arteries, and of neo-muscularization of the smaller partial and non-muscular arterioles.[9-10] In clinical practice respiratory insufficiency is treated by using ventilation with high inspiratory oxygen concentrations. Rodent models have shown that hyperoxia disturbs several cellular processes, which potentially hampers vascular differentiation and have a negative effect on the growth of the pulmonary vasculature.[11]

The pulmonary vasculature develops as primitive sprouts emerge from an expanding network of capillaries surrounding the growing lung buds.[12] These primitive endothelial tubes are stabilized by the direct interaction with perivascular cells derived from the surrounding mesenchyme to form an immature vessel. Perivascular cells can either be vascular smooth muscle cells (VSMCs) or pericytes based on morphology and expression of specific markers. In general, smooth muscle cells form the medial layer in the large and midsized vessels and are separated from the endothelial cells, whereas pericytes are sharing the basement membrane with the endothelial cells.[13-14]

VSMCs can be subdivided into contractile and synthetic cells, depending on their morphology and the expression of molecular markers. Contractile VSMCs are characterized by the expression of markers such as Smooth Muscle Myosin Heavy Chain (SM-MHC), Calponin and Caldesmon and they appear as the typical elongated cells that are mainly involved in the regulation of the vascular tone.[15] On the other hand, synthetic VSMCs lack these contractile markers, but rather express non-muscle Myosin Heavy Chain-B, also known as SMemb, and are cuboidal-shaped.[16] Synthetic VSMCs are involved in production of extracellular matrix proteins and proliferation of VSMCs in case of injury.

The media of the large pulmonary arteries contain a heterogeneous population of VSMCs[17-19] and these different populations have a unique response to pathologic stimuli,

like hypoxia.[20-23] Hypoxia induced vascular remodelling, consisting of the increased medial and adventitial thickness, is the result of increased proliferation of residing cells, such as the VSMCs in the media and fibroblasts and myofibroblasts in the adventitia combined with the deposition of extracellular matrix proteins (elastin, collagen, fibronectin and tenascin).[24] Recently, resident vascular progenitor cells were identified at the border of media and adventitia that have the potential to differentiate into mesenchymal cells and contribute to vascular remodelling.[25-26] Moreover, the sustained exposure to hypoxia results in an inflammatory micro-environment that leads to the accumulation of monocytic cells (leukocytes, monocytes and lymphocytes) and circulating mesenchymal progenitor cells in the vascular wall.[27] The monocytic cell population is associated with the increased thickening of the adventitia[27] whereas the circulating mesenchymal progenitor cells could contribute to vascular remodelling of the media and adventitia.[27-28] Exposure to chronic hypoxia leads to changes in endothelial cells, which have a significant effect on the phenotype of VSMCs.[29-30]

Animal models revealed that vascular remodelling of the pulmonary proximal vasculature is responsible for significant pulmonary hypertension, which contributes directly to the increased right ventricle afterload and the development of right ventricular hypertrophy.[31-33] The distal vasculature also remodels as a result of changes in flow dynamics.[34]

The pulmonary vascular wall remodels during the development of pulmonary hypertension and concomitantly the VSMC phenotype. However, it is not clear whether these structural changes are reversible once the hypoxic stimulus has disappeared and what the role of the different VSMCs is in this process. Therefore, we used a hypoxia-induced rat model for pulmonary hypertension to study the recovery of the animals by assessing hemodynamic factors, pulmonary vascular morphology and the VSMC characteristics after the hypoxic stimulus was ended.

We showed that chronic hypoxia results in functional and structural adaptation of the cardio-pulmonary system. The pulmonary vascular remodelling showed nearly complete reversal after recovery in normoxia.

## Material and Methods

### *Animals*

Sixty adult male Wistar rats (aged 8 wks, mean weight 325 ± 34 g at the start of the experiment) obtained from laboratory stock were studied. They were fed a standard pelleted diet (Sniff Spezialdiäten, Germany) and water *ad libitum*. All rats had a photoperiod of 12 h light, alternating with 12 h darkness. Ten of them (control rats) were selected randomly to be kept in room air at normal pressure (760 mmHg ($FiO_2$ 0.21)). In

the same room, fifty rats (hypoxic rats) were maintained in a hypobaric hypoxic chamber (410 mmHg ($FiO_2$ 0.11)) for 4 weeks. These rats were removed from the hypobaric chamber twice a week for half an hour to provide fresh food and water and to clean the cages. After 4 weeks of hypobaric hypoxia they returned to normoxic conditions. Control rats (control) and ten hypoxic rats (recovery 0) were then euthanized. The others were euthanized in groups of ten animals after a recovery period in normoxia of 1 (recovery 1), 2 (recovery 2), 4 (recovery 4) and 6 (recovery 6) weeks, respectively. The local ethical review committee for animal experiments approved all animal procedures.

***Hematocrit***

All rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) and intubated by tracheotomy with a polyethylene tube (14 G). They were artificially ventilated with room air *(60/min, PIP 12 and PEEP 2)*. Since hypoxia induces an increase in hematocrit, to enhance oxygen transport, in blood samples drawn by puncture of the right orbita, the hematocrit was determined.[35]

***Hemodynamic measurements***

An incision was made in the neck, 0.5 cm to the right of the midclavicular line. The right external jugular vein was isolated. An ultra miniature catheter (model SPR-320, Millar Instruments, Inc., Houston, Texas), attached to a pressure transducer, was introduced through the right external jugular vein into the right ventricle by pressure tracing. Once it was in its right position the right ventricular systolic pressure was measured (MIDAC software, Radboud University Nijmegen, Nijmegen, The Netherlands). The pulmonary artery pressure could be assessed, since the right ventricular systolic pressure and the systolic pressure in the pulmonary artery are identical, provided there is no valvular stenosis.[36-38]

After the hemodynamic measurements the rat was heparinised (200 IU/kg iv.) to prevent clot formation in the pulmonary arterial blood vessels.

***Measurement of right ventricular weight***

The atria were separated from the ventricles at the atrio-ventricular ring. After dissection of the right ventricular wall (RV) from the left ventricle and septum (LV + S), both were weighed separately. Right ventricular weight was calculated as the ratio of RV to LV + S weight.[39-40]

***Tissue preparations***

After the physiological measurements, the thoracic cavity and abdominal wall were opened and the abdominal aorta was punctured to exsanguinate the rats. The lungs and heart were removed *en bloc*. To exclude effects of vasoconstriction and/or vasodilatation

on the thickness of the vascular wall, pulmonary arteries were fixed under standard conditions as described by Rabinovitch.[37,41] To allow maximal perfusion of the lung vessels, the left atrium was cut open. A cannula (24 G) was introduced into the pulmonary artery through the right ventricle. Thereafter the lungs were irrigated with phosphate buffered saline (PBS, pH 7.2-7.4) through the pulmonary arteries until the effluent from the left atrium was clear.[42] This was followed by injection with a hot (60 °C) mixture of barium sulphate (6 %) and gelatine (2.5 %) at 100 cm $H_2O$ pressure for 7 minutes.[37] This fills the pulmonary arterial blood vessels up to the pre-capillary level. The lungs were then inflated with 4 % formalin at 36 cm $H_2O$ pressure. Both, pulmonary artery and trachea were occluded by ligation to maintain fixation pressure. Thereafter the lungs were fixed with 4 % formalin for at least 1 week.[43-44] Finally the lungs were paraffin embedded.

***Histology and morphometry***

Serial sections of 5 μm thickness of paraffin embedded lungs were mounted on poly-L-lysine-coated glass slides and stained with Masson trichrome staining, which stains elastic fibres dark blue/black, collagen fibres green and smooth muscle red.[45]

Masson trichrome stained sections of the left lung were analysed at 400x magnification. Pulmonary arteries were identified based on their location and structure. They mainly run along the terminal and respiratory bronchioles and the alveolar duct level and are characterized by a distinct inner and outer elastic lamina. Only arteries fulfilling these criteria and that were approximately cross-sectioned, so that the maximal external diameter exceeded the minimal external diameter by less than 50 %, were analysed using a computerised measurement program (GrabStore W2K, version 1.01, Nijmegen, The Netherlands).[45]

Three dimensions, were measured along the shortest arterial diameter and expressed in microns: the luminal diameter (LD) between the internal elastic laminae, since the thickness of the intima was minimal in all cases, the external diameter (ED) between the external elastic laminae and the arterial diameter (AD) (including the adventitia). From these variables medial and adventitial thickness (MT and AT) were calculated. The distance between the internal and external elastic laminae represents the media. The distance between the external elastic laminae and the outer border of the vessel represents the adventitia. MT and AT were calculated as followed: MT = [ED – LD] / 2 and AT = [AD – ED] / 2. The thickness of the arterial media and adventitia was expressed as percentage of the external diameter, (MT% = [2 x 100 x MT] / 2 and AT% = [2 x 100 x AT] / 2), a parameter for medial hypertrophy and adventitial thickening, respectively.[31,40,46-48]

Pulmonary arteries were measured in two groups; the intra-acinar arteries with an external diameter ≤ 50 µm and pre-acinar arteries with an external diameter between 50 – 150 µm.[49] For morphological analysis we only used lung samples in which we could at least measure 10 pre-acinar and 10 intra-acinar pulmonary arteries to calculate the medial and adventitial thickness. In each of the 6 groups, the lungs of at least 8 rats were analyzed. The average number of calculated pulmonary arteries in each sample was 23 in total, a mean of 11 intra-acinar arteries and 12 pre-acinar arteries respectively.

Immunohistochemical staining was performed according to standard protocols, using antigen retrieval by boiling sections for 15 minutes in Tris-EDTA buffer (pH 9.0), except for Myl-9. The signal was enhanced with the Envision™ detection system (DakoCytomatic, Glostrup, Denmark).[50] Negative controls were performed by omission of the primary antibody. The used antibodies and their dilutions are listed in table 1.

The slides were examined by two individuals independently and the expression of SMC markers in the vascular wall were scored as either clear or weak staining (1) or no staining at all (0).

**Table 1. Antibodies used with the experimental dilutions**

| antibody | dilution | Compony |
|---|---|---|
| α-SMA | 1:300 | Biogenex, San Ramos, CA, USA |
| SM-MHC 1 | 1: 75 | Abcam, Cambridge, UK |
| SM-MHC 2 | 1:800 | Abcam, Cambridge, UK |
| Calponin | 1:100 | DAKO, The Netherlands |
| SMemb | 1:4000 | Abcam, Cambridge, UK |
| Myl-9 | 1:500 | Santa Cruz, Heidelberg, Germany |
| MLC1a | 1:5000 | Abnova, Heidelberg, Germany |

### *Analysis and statistics*

Values of the hematocrit, right ventricular systolic pressure, right ventricular weight expressed as the ratio of RV to LV + S as percentage of body weight, and the thickness of the arterial media and adventitia, expressed as percentage of the external diameter from the six study groups, were calculated and presented as mean (± standard deviation (SD)) respectively. Data from the six study groups were compared using the unpaired Student's *t*-test. P values ≤0.05 were considered statistically significant.

## Results

### *Functional analysis*

Pulmonary hypertension was induced by exposing rats to hypobaric hypoxia for 4 weeks. The extent of the pulmonary hypertension was determined by measurement of functional

aspects of pulmonary hypertension, such as the hematocrit, the right ventricular systolic pressure (RVSP) and the ratio of RV to LV + S weight.

The hypoxia group had a significant higher hematocrit level compared to control rats. The different recovery groups showed a gradual decrease in the hematocrit and after a recovery period of at least 2 weeks the hematocrit was no longer different compared to control animals, indicating a first sign of recovery in the adaptation response that was induced by hypoxia (Figure 1A).

The RVSP was increased after hypoxia, which gradually decreased upon recovery in normoxia. After at least 2 weeks of recovery the RVSP significantly decreased compared to the animals that did not have a recovery period (recovery 0 group). However, the RVSP remained significantly higher in all the recovery groups compared to the control animals, indicating that the hypoxia induced cardiac abnormality only partially recovered (Figure 1B).

The ratio of RV to LV + S weight, as a measurement of right ventricular hypertrophy (RVH) was significantly higher in hypoxic animals compared to controls (Figure 1C). As demonstrated for the hematocrit and RVSP, the same phenomenon of a significant gradual decrease in RVH was seen with recovery. Nevertheless, this ratio remained significantly increased compared to control animals after 6 weeks of recovery, as was observed for the RVSP measurements (Figure 1C).

Collectively, the functional analysis shows a (partial) reversal of hypoxia induced pulmonary hypertension after 6 weeks of recovery, although the RVSP had not returned to normal values and the RV remained hypertrophic.

*Morphometry*

Since the functional analysis of the rat groups revealed a partial reversal after 6 weeks of recovery, we next studied the morphometry and putative structural changes of the lung vasculature of the different groups. The media and adventitia of the pre-acinar vessels (50 – 150 μm) were significantly increased in the lungs of hypoxia treated animals, a histological sign of hypobaric hypoxia induced pulmonary hypertension (figure 2A and 2B respectively). Analysis of the recovery groups showed a gradual decrease of the medial and adventitial thickness during the prolonged periods of normoxia and eventually became indistinguishable from control animals (Figure 2A and 2B). In the intra-acinar arteries (<50 μm) significant changes in vessel wall thickness, as result of hypoxia and/or recovery could not be observed (data not shown).

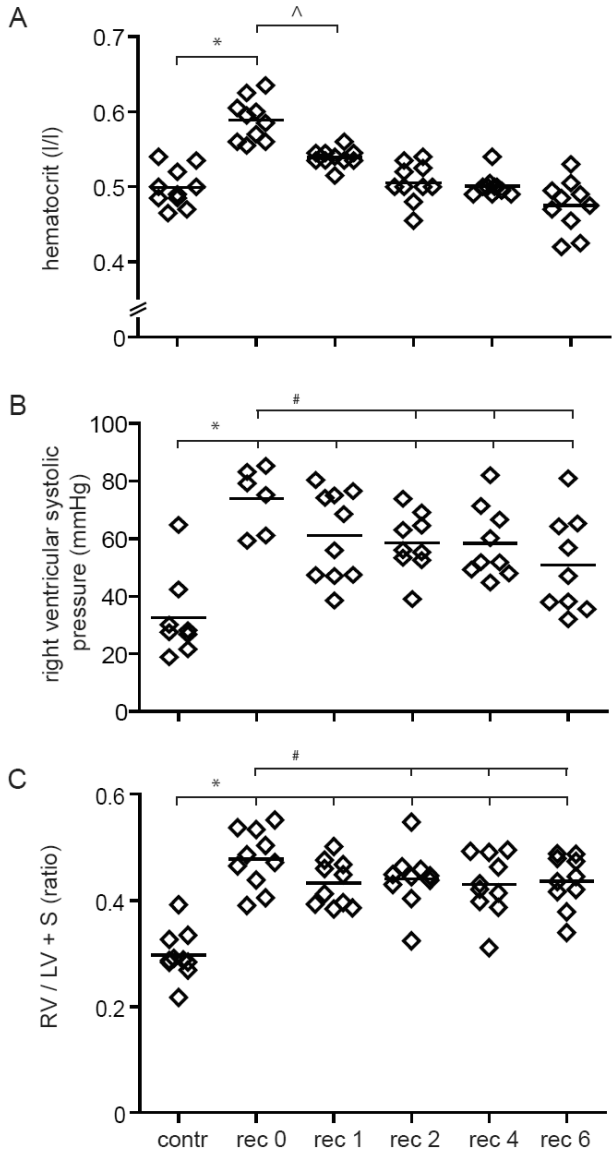


**Figure 1: Recovery of the functional aspects of pulmonary hypertension.** Different functional aspects of pulmonary hypertension are shown for the 6 different groups: (A) hematocrit, (B) right ventricular systolic pressure (RVSP) and (C) RV/(LV+S) ratio, abbreviated as right ventricle (RV) weight. The 6 different groups are: contr (control); rec 0 (exposed to hypoxia for 4 weeks); rec 1, 2, 4 and 6 (recovery in normoxia for 1, 2, 4 and 6 weeks after exposure to hypoxia for 4 weeks). All individual values per group are represented, the line indicates the mean. Statistical significance ($P<0.05$) is indicated above.

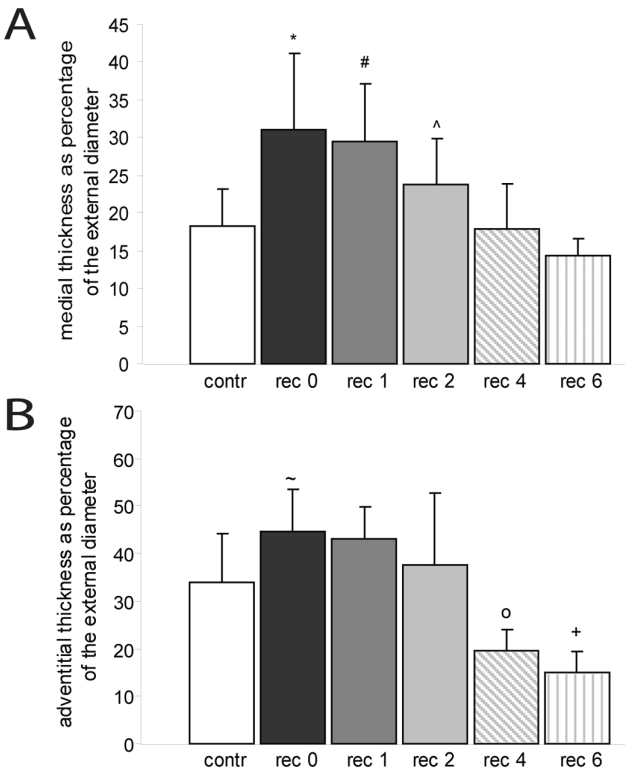


**Figure 2. Recovery of the hypoxia induced vascular abnormalities.** Morphometric analysis of the medial (A) and adventitial thickness (B) of the vasculature of control (contr), hypoxic (Rec 0; 4 weeks hypoxia) and recovery groups (rec 1, 2, 4 and 6; 1, 2, 4 and 6 weeks recovery in normoxia after 4 weeks exposure to hypoxia) Values are mean ± SD, based on 10 rats per group and on the measurements of at least 12 pre-acinar arteries per sample. Statistical significance ($P<0.05$) is indicated above the bars.

### *Smooth muscle marker expression*

The structural changes in the media and adventitia suggested possible alterations in the cellular composition. Therefore, we performed immunohistochemistry (IHC) to identify possible differences in the expression of SMC markers, which is associated with VSMC phenotype and thus the function of VSMCs. Analysis of the pre-acinar arteries (50 – 150 μm) showed that after 28 days of hypoxia the distribution of α-SMA was not changed (Figure 3A and 3B). However, in the intra-acinar arteries (<50 μm) of the hypoxia treated animals the expression of $\alpha$-SMA was observed in the most distal vessels, which normally are non-muscularized as shown in the controls (Figure 3C and 3D).

To discriminate between contractile and synthetic VSMCs, we analyzed the expression of markers specific for either contractile or synthetic VSMCs Markers of the contractile apparatus are SM-MHC (SM-MHC 1 and SM-MHC 2), Myl-9 and MLC1a (two regulatory units of myosin light chain).

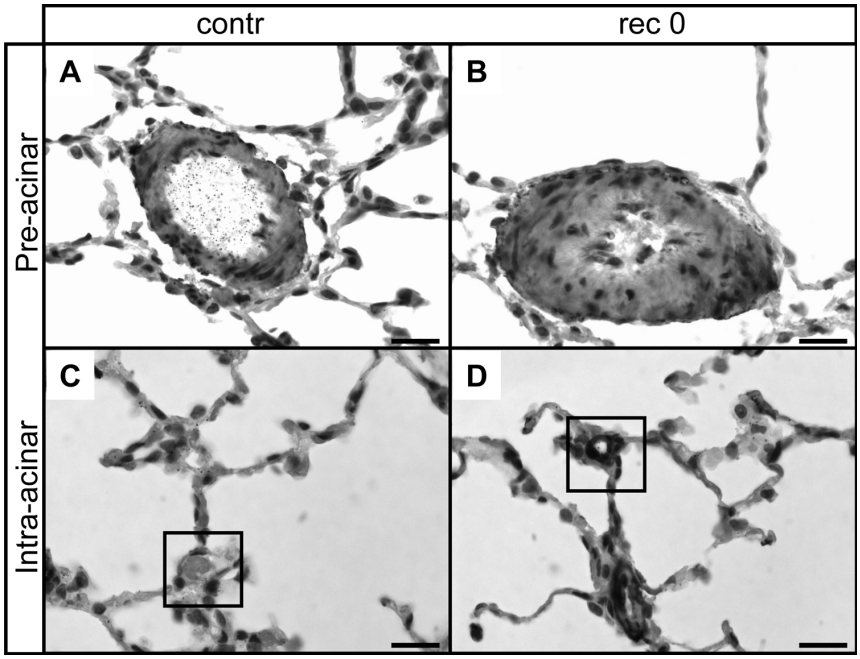


**Figure 3. Distribution of α-SMA expression in lung vessels of normal and hypoxic animals.** Representative pictures showing the distribution of α-SMA in contr (control) and rec 0 (after 28 days hypobaric hypoxia without recovery). The α-SMA distribution was not changed in the pre-acinar arteries (50 - 150μm) (upper panels), however in rec 0 the vessel morphology is completely changed; an increased vessel wall thickness with a small lumen. In the intra-acinar arteries (<50 μm) (bottom panels) α-SMA expression was induced by hypoxia (rec 0). Scale bar 20 μm.

Although α-SMA has been used previously as a marker for VSMCs, SM-MHC is currently a more specific marker for smooth muscle cells.[15] Calponin is expressed by more matured VSMCs that have a contractile phenotype.[51-53] VSMCs that have a synthetic phenotype express SMemb.[15]

SMemb positive VSMCs were observed in the vascular wall of both the pre-acinar arteries and the intra-acinar arteries in the lungs of the hypoxia group, indicating the presence of synthetic VSMCs (figure 4B). Moreover, hypoxia treated animals showed the emergence of contractile VSMCs in the intra-acinar arteries and the most distal vessels,

as shown by the expression of MLC1a, Calponin and SM-MHC 2 (for SM-MHC Figure 4E).

Lungs from animals that recovered for 6 weeks in normoxia almost completely lacked the presence of synthetic VSMCs in the pre-acinar and intra-acinar arteries, and contractile VSMCs in the intra-acinar arteries, as indicated by the loss of SMemb (Figure 4C), Calponin and MLC1a expression respectively (for overview, see table 2). The expression of $\alpha$-SMA, SM-MHC-2 (Figure 4F) and Calponin was also undetectable in the most distal vessels.

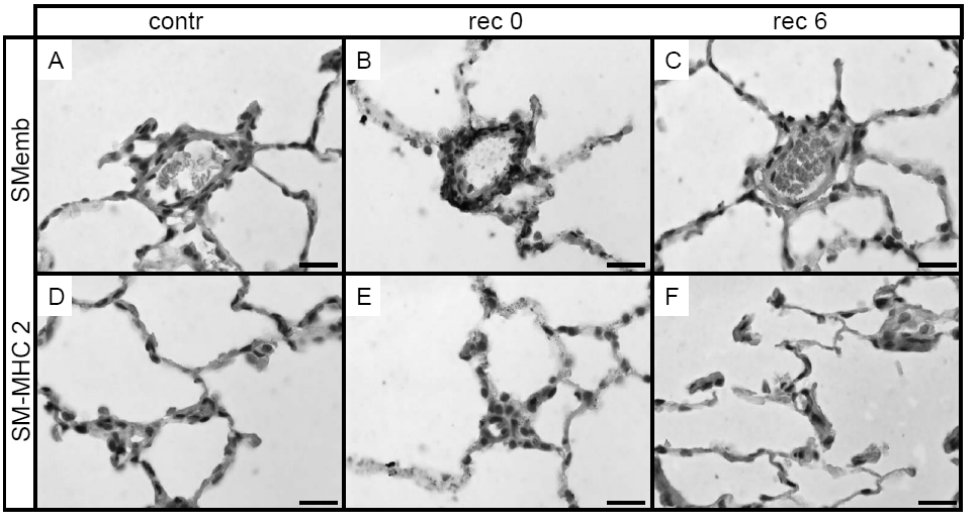


**Figure 4. Distribution of contractile and synthetic VSMCs in the lung vasculature of normal and hypoxic animals.** Representative pictures showing immunohistological staining for SMemb (A-C) and SM-MHC 2 (D-F) in lungs of control animals (contr; A,D), of animals exposed to hypoxia for 4 weeks (rec 0; B,E) and of animals recovered for 6 weeks in normoxia after exposure to hypoxia for 4 weeks (rec 6; C,F). The expression of SMemb in pre-acinar vessels is more widely expressed in the hypoxia treated animals (rec 0; B) compared to control (A) and is almost completely diminished after recovery in normoxia (rec 6; C). SM-MHC 2 is only expressed in the intra-acinar vessels of the hypoxic group (rec 0; E) and is undetectable in controls (D) and after recovery in normoxia (rec 6; F). Scale bars 20 µm.

These results show that the increased vessel wall thickness, induced by hypoxia, in the pre-acinar arteries is associated with an increase in the number of synthetic VSMCs, as indicated by the expression of that SMemb. No significant changes could be observed in the morphometry of the intra-acinar arteries. In addition, the hypoxia induced expression of $\alpha$-SMA, SM-MHC-2 and Calponin, in the most distal vessels, showed a transient differentiation of the cells in these most distal vessels, since after the recovery of 6

weeks in normoxia no expression of these markers could be identified. These molecular findings highly correspond with the functional analysis.

**Table 2. Analysis of smooth muscle marker expression**

| | α-SMA | SM-MHC1 | SM-MHC2 | Calponin | SMemb | Myl-9 | MLC1a |
|---|---|---|---|---|---|---|---|
| Pre-acinar | | | | | | | |
| Contr | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| Rec 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Rec 6 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| Intra-acinar | | | | | | | |
| Contr | 1 | N/A | 1 | 0 | 0 | 1 | 0 |
| Rec 0 | 1* | 1 | 1* | 1* | 1 | 1 | 1 |
| Rec 6 | 1 | N/A | 1 | 0 | 0 | 0 | 0 |

The expression of the different smooth muscle markers in pre-acinar arteries (50 - 150μm) and intra-acinar arteries (<50 μm) are represented for controls (contr), 4 weeks after hypobaric hypoxia without recovery (rec 0) and 6 weeks after recovery in normoxia (rec 6). Analysis was performed on 3 samples per group. 0 indicates no

## Discussion

Persistent pulmonary hypertension in newborns is a serious clinical condition responsible for considerable mortality and morbidity among newborns. The lungs in pulmonary hypertension display common structural changes existing of an increased number of cells that express α-SMA.[54] However, it is unknown to what extent these structural changes are reversible. Therefore, we investigated whether the physiological and molecular changes as result of pulmonary hypertension, induced by chronic hypobaric hypoxia, were reversible once the hypoxic stimulus was ended.

The lungs of newborn rats are in the saccular stage at birth and the airways and vasculature remodel significantly the first few weeks after birth to adapt to extra-uterine life. However, the human neonatal lung is already in the alveolar stage at birth. To eliminate complex influences from the physiological remodelling processes and to better compare the rat model with the human lung, we decided to analyse transient hypoxia-induced changes in the young adult rats as a model for neonatal pulmonary hypertension.

The physiological parameters that were tested; hematocrit levels, RVSP and RVH, all showed a significant increase after 28 days of hypoxia, indicating that the duration and amount of hypoxia were sufficient to induce significant clinical pulmonary hypertension. The hypoxia induced pulmonary hypertension model is used by several independent research groups to study the molecular pathways involved in vascular remodelling and to test possible therapeutic strategies. Different studies used different time periods of

hypobaric hypoxia to induce pulmonary hypertension, ranging from 7 to 28 days.[55-58] Pulmonary arterial hypertension was induced already one week after hypobaric oxygen, based on an increased PAP and RVH.[56] In addition, the medial and adventitial thickness increased significantly during the hypoxic period, which resembled the structural changes observed in human newborns.

To study the reversibility of the structural changes of hypoxia induced pulmonary hypertension, the rats were returned to normoxia for 1, 2, 4 and 6 weeks. Remarkably, the structural changes showed a decrease to control levels after the recovery of at least four weeks, which corresponds with previous results.[59] However, an increased RVSP and RVH remained present after 6 weeks of recovery. This can be linked to clinical observations, since some infants that suffered respiratory insufficiency due to pulmonary hypertension show clinical improvement with therapy, suggesting recovery of pulmonary hypertension. However, these infants are susceptible for right ventricular failure when hypoxic periods reoccur due to other clinical manifestations, such as pulmonary infection, sepsis or gastro-oesophageal regurgitation. A recurrent hypoxic insult causes an abnormal vaso-reactive response leading to an intolerance of the already compromised right ventricle in these infants.[60] As a consequence, long term clinical evaluation of infants suffering from pulmonary hypertension includes increasingly cardiac catheterisation in selected centers to observe the responsiveness of the pulmonary vasculature and the underlying cardiac abnormalities.[61-62]

The clinical reversibility of the hypoxia induced pulmonary hypertension was accompanied by the reversal of structural changes. Both medial and adventitial thickness were significantly increased by hypoxia and decreased gradually during the recovery period. After 4 to 6 weeks of recovery, the medial and adventitial thickness was comparable to those in the control group. The thickening of the arterial medial layer occurs throughout the pulmonary vasculature.[63] This muscular thickening of the media is reversible upon re-exposure to normoxia.[59,64] It was shown that the vascular wall contains different populations of VSMCs, but it remains unclear how these populations arise and how they are linked together.[65-66] Two possible mechanisms have been proposed, which are mutual non-exclusive and may be complimentary. The first, described by Owens et al., showed that during development of the systemic circulation VSMCs mature and differentiate into contractile cells as marked by the expression of genes specific for contractility and cytoskeleton.[15] However, the VSMCs displayed plasticity because they could adapt their phenotype in response to changing environmental factors.[15] So, differentiated VSMCs can switch from a contractile phenotype to a synthetic phenotype, as marked by the loss of expression of contractile and cytoskeletal genes and the subsequent gain of synthetic markers, like SMemb.[67-68] Secondly, Frid et al. described that the pulmonary vascular wall consists of several, distinct VSMC subpopulations characterized by their morphology and response to

external stimuli. Four phenotypically distinct VSMC subpopulations were identified in the media of bovine pulmonary arteries, two so-called non-muscle cell types and two smooth muscle cell types.[17] Based on morphology; rounded versus spindle-shaped, and expression of α-SMA and SM Myosin, it is suggestive that the non-muscle cells described by Frid et al. correspond to the synthetic VSMCs, as described by Owens, whereas the smooth muscle cells are having a contractile phenotype. Several studies showed a selective expansion of the non-muscle cells in response to pathological stimuli, such as hypoxia.[23] These two theories are not mutually exclusive and therefore, it is still possible that dedifferentiation of contractile VSMCs, as well as selective expansion of a subpopulation of VSMCs are processes that are involved in the development of the structural changes in pulmonary hypertension. Importantly these two theories are based on findings of vascular abnormalities of the systemic and pulmonary vasculature. In systemic vascular abnormalities the dedifferentiation of contractile VSMCs and re-differentiation of VSMCs was observed, showing the enormous plasticity of VSMCs to recover.[15] In the pulmonary vasculature of bovine, four different VSMC phenotypes were described of which the non-muscle cells responded with a selective expansion in response to growth factors *in vivo,* whereas the smooth muscle cells remained quiescent.[17] Lineage tracing with labelled VSMCs could give more information about the processes involved in the development of the vascular abnormalities.

In addition, circulating progenitor cells or resident vascular progenitor cells play a role in the development of the vascular remodelling in pulmonary hypertension.[28,34]

We showed the presence of SMemb in the medial layer of pre-acinar arteries in the hypoxia exposed lungs. Though α-SMA, SM-MHC2 and Calponin remained expressed as well. This suggests that there is a selective expansion of (sub) populations of VSMCs and based on the expression of SMemb these cells have a synthetic phenotype in the hypoxic lungs. Synthetic SMCs are involved in proliferation and migration of SMCs and the production of ECM proteins. These processes are responsible for the increase in vessel wall thickness in pulmonary hypertension.[69] However, it is also possible that the increase in SMemb expression by VSMCs is the result of the dedifferentiation of a subpopulation of VSMCs, as in control samples SMemb was not expressed by any cell type within the medial layer.

In the intra-acinar blood vessels, chronic hypoxia did not induce structural changes that lead to increased vessel wall thickness. However, in this part of the pulmonary vasculature neo-muscularization is characteristic for the pulmonary vascular remodelling in pulmonary hypertension.[10] The normal partial and non-muscular vessels become muscularized as the perivascular cells start to express α-SMA. It is thought that this is the result of either the differentiation of precursor cells, such as pericytes and intermediate cells,[59] or the differentiation of fibroblasts and circulating mononuclear mesenchymal precursors into smooth muscle cells.[70]

We showed that the expression of several smooth muscle cell markers in the intra-acinar vessels changed. In these small vessels there is an increase in the expression of SMemb, Calponin and MLC1a. Moreover, the smallest vessels (most distally) express α-SMA, SM-MHC2 and Calponin, suggesting that these cells differentiated either into a smooth muscle cell or into a smooth muscle like cell.

Previous observations of extensive muscularization of the most distal arteries was based on the expression of α-SMA.[59] Since research in the systemic circulation showed that some pericytes also express α-SMA, without being a VSMC,[71] SM-MHC is generally accepted to be restrictive for smooth muscle cells.[15,72] In our analysis we also found SM-MHC2 expressed by the smallest vessels, suggesting the perivascular cells in the smallest vessels indeed differentiate into a VSMC. After recovery we observed that the expression of α-SMA, SM-MHC2 and Calponin disappeared in the smallest vessels, suggesting that the change in phenotype induced by hypoxia is reversed.

VSMCs and pericytes are thought to originate from the same cell lineage and can be distinguished based on their location within the vascular wall.[13-14] VSMCs show an enormous plasticity to adapt their phenotype upon environmental changes, both under physiological and pathological conditions.[15] It has also been suggested that depending on environmental circumstances pericytes can give rise to VSMCs and the other way around.[73] Our results show that both the VSMCs and the perivascular cells in the smallest vessels changed their phenotype in response to hypoxia, based on the expression pattern of SMC markers. The different expression of SMC markers in hypoxia is completely reversed after recovery in normoxia, showing the enormous plasticity of perivascular cells.

Overall, we showed that the development of pulmonary hypertension is associated with an increase of cells with a synthetic phenotype in the pre-acinar arteries and a differentiation of the perivascular cells in the smallest intra-acinar arteries. These changes and the structural changes, including an increased vessel wall thickness, in the pulmonary vasculature are reversible with recovery in normoxia. Nevertheless the increased right ventricular systolic pressure and the right ventricular hypertrophy showed no complete reversal within 6 weeks. This forms a potential risk factor for circulatory failure in the future as documented in former premature infants with bronchopulmonary dysplasia[61,74] and infants with remaining subclinical pulmonary hypertension associated with for instance congenital diaphragmatic hernia.[75-76]

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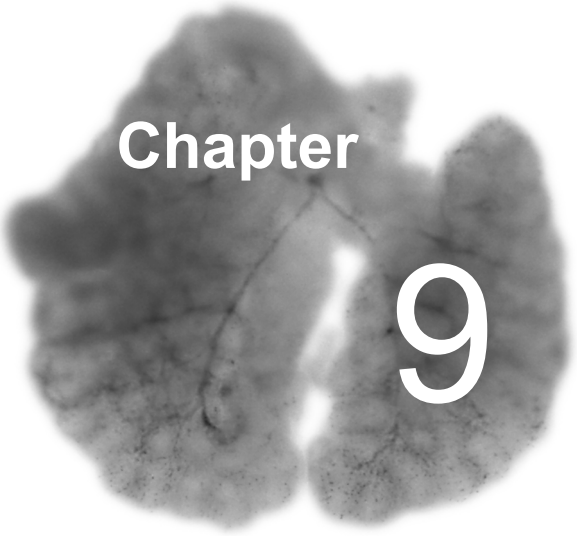
# General Discussion



# Chapter 9

## General Discussion

## Pulmonary vascular disease

Pulmonary hypertension (PH) is clinically defined as an increased pulmonary arterial pressure (in adults defined as a pulmonary arterial pressure of >25 mmHg in rest and >35 mmHg during exercise) and occurs as a symptom associated with a variety of disorders.[1] Pulmonary hypertension in newborns can be idiopathic, the persistent pulmonary hypertension of the newborn or related to an underlying cause such as asphyxia, infection, congenital diaphragmatic hernia (CDH) and congenital heart disease.[2-4] Pulmonary hypertension among newborns is characterized by respiratory insufficiency and hypoxemia as result of a failure of the pulmonary vascular resistance (PVR) to decrease after birth. Before birth, the PVR is high and most of the cardiac output from the right ventricle is shunted to the systemic circulation via the foramen ovale and the ductus arteriosus,[5-6] as the placenta is responsible for the gas exchange. After birth, a cascade of adaptations is induced to enable the lungs to perform their main function, the exchange of oxygen and carbon dioxide.

Pathophysiological PH is associated with functional and structural changes of the pulmonary vasculature, an increased vascular reactivity and remodeling of the vascular wall.[3,7-9] This is why PH is increasingly described nowadays as pulmonary vascular disease (PVD). If not treated appropriately, hypertrophy and failure of right ventricle (RV) develops, which is responsible for the significant mortality of pulmonary hypertension among newborns. The mortality rate range around 10 and 20%, and is related to the underlying cause.[10-11]

The current clinical classification of PH[1] is based on PH in adults and is not appropriate to classify PVD in neonates and children. Recently, the Pediatric Taskforce developed a new clinical classification for pediatric PVD (see table 1).[12] PVD in the neonatal period can be classified as either category 1; prenatal or developmental pulmonary hypertensive vascular disease or category 2; perinatal pulmonary vascular maladaptation, although a large overlap exists.

## Treatment strategies in pulmonary vascular disease

Treatment of pulmonary vascular disease (PVD) in neonates is still a major and in a great extent unsolved problem. Much of what we know or assume know about the pathophysiological mechanism of pulmonary vascular disease is derived from animal models to study pulmonary arterial hypertension (PAH) and human studies in adults that confirm these pathophysiological mechanisms.

**Table 1. The 10 basic categories of Pediatric Pulmonary Hypertensive Vascular Disease[12]**

| Category | Description |
|---|---|
| 1 | Prenatal or developmental pulmonary hypertensive vascular disease (PHVD) |
| 2 | Perinatal pulmonary vascular maladaptation |
| 3 | Pediatric cardiovascular disease |
| 4 | Bronchopulmonary dysplasia |
| 5 | Isolated pediatric PHVD (isolated pediatric PAH) |
| 6 | Multifactorial PHVD in congenital malformation syndromes |
| 7 | Pediatric lung disease |
| 8 | Pediatric thromboembolic disease |
| 9 | Pediatric hypobaric hypoxic exposure |
| 10 | Pediatric pulmonary vascular disease associated with other system disorders |

Vasoconstriction as result of endothelial dysfunction is thought to be an important key player in the pathophysiology of PAH in adults. Most of the therapies are based on inducing vasorelaxation of the pulmonary vasculature, either by activation of the NO-cGMP or the prostacyclin-cAMP pathway or by the inhibition the endothelin (ET-1) pathway, reviewed by Humbert.[13] The different drugs interfering in these pathways, such as inhaled nitric oxide, phosphodiesterase type 5 inhibitors, prostacyclin derivates and endothelin receptor antagonists improved the exercise capacity, at least in adults, and decreased the PVR and attenuated the morphological changes within the pulmonary vasculature. The last was extrapolated from observations in several animal studies.

Increasing knowledge about the understanding of the cellular and molecular processes involved in the development of PAH showed that increased proliferation and inflammatory processes are contributing to the structural changes within the pulmonary vasculature (remodelling).[14-18] The focus of the new therapeutic strategies are to restore the balance between proliferation/anti-proliferation and inflammation and anti-inflammation.

Effective therapeutic strategies in adults with PAH form the basis of treatment of PVD in children and neonates. Knowledge of these strategies in neonates is limited compared to adults. So far the only evidence based therapy for PVD in neonates is inhaled nitric oxide (iNO).[19]

Inhaled NO is a selective pulmonary vasodilator that has been proven to be effective in decreasing the PVR, improving the ventilation-perfusion ratio and reducing the right to left shunting.[20-21] In 2000, the use of iNO was approved in term and near-term (>34 weeks) infants with hypoxic respiratory failure.[22] Since then, several studies described the clinical benefits of the use of iNO.[21,23] However, the use of iNO in premature infants is not as beneficial as in term or near-term (> 34 weeks) infants. No difference in mortality or risk on the development of bronchopulmonary dysplasia among survivors was found in premature infants treated with iNO in compare with premature infants

treated without iNO.[24] Follow-up studies showed no differences in the long-term outcome, in terms of pulmonary morbidity and/or neurodevelopmental disabilities, between survivors treated with iNO or without iNO.[25-31] This all sounds very promising, however the access to iNO is limited due to its high costs.[23]

Another observation is that approximately 30% of the neonates do not respond to iNO.[19,32-33] At least a group of infants with CDH does not benefit from iNO.[32] A study showed that the use of iNO among infants with CDH did not affect the mortality and that the use of iNO was associated with an increased need for extracorporeal oxygenation (ECMO).[19,33]

Other therapeutic strategies based on adult PAH, the inhibition of phosphodiesterases, usage of prostaglandin analogues and selective and unselective endothelin receptor antagonists, are also used in neonates, but are only described in case-reports, case-series or as retrospective analysis.[34-41] These reports claim the potential benefits of the different drugs, however they all suggest that large randomized trials are needed to determine if the drug is really effective. To perform this kind of studies in neonates is difficult compared to adults. In adults PAH is clearly defined, the severity is classified according to an adapted WHO classification[42-44] and the 6 minutes walking test is used as a general accepted endpoint to determine drug efficacy,[45-46] these parameters are less clear in neonates. Current studies describing the effects of therapies used to treat PVD in neonates use different criteria for initiation of a drug. Most used is the oxygenation index (OI) however the threshold value varies between different studies. Originally the oxygenation index was used to determine the severity of acute respiratory failure in newborns[47] and currently still is used as criteria to start ECMO (OI > 40). The OI is dependent of the used ventilation setting, thus is remains questionable if OI alone is a good parameter to determine the initiation of a drug. Also different endpoints are described; however most studies use combined endpoints: survival and the need for ECMO. The lack of universality in these studies makes comparing the outcomes difficult.

One of the approved effective therapies for PAH in adults is the inhibition of phosphodiesterase 5 (PDE-5) by sildenafil or tadalafil.[48-49] PDE-5 is responsible for the conversion of cGMP into GMP. Inhibition of PDE-5 results in relaxation of smooth muscle cells (SMCs), because accumulation of cGMP activates protein kinases which decreases the cytosolic calcium level.[50] Studies in animal PAH models showed the increased PDE-5 expression and activity in pulmonary vascular SMCs[51-52] and treatment with sildenafil or another PDE-5 inhibitor resulted in a partial reversibility of the structural changes within the vasculature,[53-54] a reduction in RV hypertrophy and improved survival.[55-56]

In human lung and pulmonary vascular SMC PDE-5 mRNA is most abundantly expressed [57] and in PAH this expression of PDE-5 is increased.[58] Moreover, pharmacological studies in adults with PAH showed that inhibition of PDE-5 by sildenafil reduces the PVR[48] and has a similar vasodilator effect as iNO.[59] The Sildenafil Use in

PAH (SUPER-1) study, a multicenter, randomized, double-blind, placebo-controlled trial in which 278 adults with PAH (WHO classification II and III) were enrolled, showed the benefits of sildenafil. The use of sildenafil resulted in a dose-independent improved exercise capacity, functional class and hemodynamics.[48] Based on this study the use of oral sildenafil 20 mg three times a day became approved. Recently, sildenafil treatment was approved for children with PAH as well.[60-61]

Since 2002 sildenafil is used off-label in neonates to treat PVD. Several reports described the positive effects of sildenafil; improvement of pulmonary hypertension and prevention of rebound pulmonary hypertension during withdrawal of iNO.[62-65]

The current used dose of sildenafil in neonates, 0.5 – 2.0 mg/kg four times a day[66] is based on small pilot studies and case reports.[64,67-68] In Chapter 3 we analyzed the pharmacokinetics and exposure of oral sildenafil in post-ECMO infants and showed that an oral dose of 4.2 mg/kg/day results in a sildenafil exposure that is similar to effective dose of 3 x 20 mg in adults.[48] However, sildenafil exposure was showing large variability within and between patients. A quarter of the infants with a high dose of 7 mg/kg/day still had a low sildenafil plasma levels.

The degree of inter-patient variability is related to the postnatal age and will decrease with increase postnatal age.[69] Overall the variability between infants could be explained by differences in drug absorption, due to relatively immaturity of the gastro-intestinal tract and/or severity of disease.[70] Also the developmental maturation of the drug metabolizing enzymes which varies widely between neonates is responsible for the variability between infants.[71] Intravenous sildenafil is also used in some centers, in this way the differences in drug absorption is not an issue, but still variability between infants exist, based on the individual differences in the maturation of the metabolizing enzymes. This makes that sildenafil should be titrated carefully based on the efficacy and occurrences of periods of hypotension following individual dosing.

The first randomized controlled trials, analyzing the efficacy of sildenafil performed in patient groups that varied between 13 to 51 newborns,[68,72-73] are promising; sildenafil significantly reduced the mortality and improved the oxygenation, without clinical important side effects.[74] However these studies were performed in centers, which do not have access to iNO and ECMO. It could be that the infants that improved with sildenafil treatment were also improving with iNO. Recently, the authors of the Cochrane review suggest that large scale randomized trials comparing sildenafil with iNO are needed to assess the efficacy and safety of sildenafil.[74]

In chapter 4 we investigated the usage of sildenafil in infants with a CDH. Retrospective analysis showed that in the period 2006 – 2010, approximately 68 infants (23 percent) with an isolated CDH received sildenafil as therapeutic strategy. Our analysis reveals that sildenafil in CDH is not effective in high-risk infants that start sildenafil during ECMO treatment. It does not improve survival and it results in a delayed death. However, our

analysis has some limitations, first of all it is a retrospective analysis, thus infants that were treated with sildenafil were not randomized, but sildenafil was started as a rescue therapy and by compassionate use. Since the use of sildenafil was not protocolized, the start of sildenafil varied, some received the first dose of sildenafil early, just before or at the beginning of ECMO treatment, whereas others received the first dose of sildenafil at the end of ECMO treatment. It remains speculative, but probably in the last group the ECMO-flow could not be decreased without changes in hemodynamics. The individual dose regimen of sildenafil also varied enormous (1.5 mg/kg/day - 12 mg/kg/day). Moreover, we did not analyze the hemodynamic effects, in a protocolized manner (repeated cardiac ultrasound and measurement of pro-BNP) to see if sildenafil had any effects at all.

Noori et al. analyzed the cardiovascular effects in seven infants with a CDH, and showed that sildenafil effectively reduced the pulmonary hypertension and improved the respiratory insufficiency and oxygenation in infants treated with sildenafil.[67] Hunter et al. described the established use of sildenafil in CDH infants that suffer from severe pulmonary hypertension. Sildenafil was not associated with adverse effects. However, no differences in mortality was observed between the treatment groups and the control group.[75]

Overall, large randomized studies (RCT's) are needed to elucidate whether sildenafil is effective in CDH. Another fact that needs investigation is when to start sildenafil. Nowadays, most infants receive sildenafil as a rescue, when signs of unresponsiveness to iNO are present or when the oxygenation index is reaching levels that ECMO treatment is required. Probably the pulmonary vascular remodelling in these infants already resulted in a fixed pulmonary hypertension and the vasculature is no longer or less responsive to the vasodilatory effects of sildenafil. An earlier start with sildenafil could be more beneficial. The antenatal treatment with sildenafil in the nitrofen-induced CDH rat model showed that the lung structure improved with an increased density of pulmonary vessels. Also the postnatal vasodilatory response improved with antenatal sildenafil treatment.[76] The concept of antenatal therapy renders some promises. Individual identification of high risk patients remains the major limiting step nowadays for the conduction of RCT's.

In general, the treatment of PVD in neonates is largely based on "trial and error". Large randomized multi-centre studies are needed to determine if the different therapeutic strategies that are used without any evidence base so far are effective. To perform these studies universal definitions for PVD, drug initiation and efficacy endpoints are needed.

The current functional classification of pulmonary hypertension in children,[77] provides no clear classification in disease severity in neonates and a good diagnostic tool to determine PVD in neonates is missing as well. Cardiac ultrasound could provide signs of

pulmonary hypertension, however is semi-quantitative. The use of biomarkers such as ET1 or NT-pro-BNP, both are increased in PH and associated with the severity of PH in CDH,[78-79] or in neonatal PVD due to other causes,[80] could be potentially helpful in determining the severity of neonatal PVD.
To determine the efficacy of a certain drug the already combined endpoints; mortality and need for ECMO could be used. However, determining the hemodynamic changes in terms of improved micro-circulation and/or oxygenation and the cardiovascular effects, in terms of improved cardiac function could be used as an early predictor of the efficacy of a certain drug. Alternatively longitudinal evaluation of plasma levels of ET1, NT-pro-BNP etc could be used. So far reports are not available to support this approach.
Especially the treatment of PVD among infants with a CDH needs extra attention. Whereas first it was shown that these infants do not benefit of iNO; we retrospectively showed no positive effect of sildenafil in these infants. This indicates that the NO-cGMP pathway might not the major target for therapeutic intervention in infants with a CDH. More research is needed to identify which pathways are involved in the pathophysiology and determine the therapeutic strategies that could be beneficial in PVD among infants with CDH.

## Structural changes in pulmonary hypertension

The structural changes within the pulmonary vasculature are characterized by a thickened vascular wall and extensive muscularization of partial and non-muscular arterioles, together referred as vascular remodelling.[17,81-83]
Two aspects of PVD in neonates need to be taken in account;

1) Fetal or perinatal events result in structural changes within the vasculature that are responsible for a failure of the normal transition at birth.
2) Aggravation of these structural changes due to postnatal events; such as hypoxia or hyperoxia.

A better understanding of the processes, at the molecular and cellular level, involved in normal pulmonary vascular development are important to better understand the structural changes in PVD among neonates.

### *Pulmonary vascular development*

The lung buds form the anterior forgut and is the result of the interaction between the forgut endoderm and the surrounding mesenchyme. These interactions are necessary for a proper pulmonary development, both vascular and airway.[84] Signaling molecules between epithelium and mesenchyme are determining the branching morphogenesis.

Several studies in transgenic mice models have shown that the epithelial/mesenchymal interactions are very complex and that parts of these interactions are still unknown. Excellent reviews have described the epithelial/mesenchymal interactions involved in the development of the lung.[84-86] Whereas the epithelial aspects of pulmonary development are studied extensively, pulmonary vasculature research, in particular in human, is still in its infancy.

Already at the budding of the lung a capillary network is present in the surrounding mesenchyme.[87] Contrasting previous studies,[88-90] it is now well accepted that circulation is already present at the earliest stages of lung development and the lung vasculature primarily grows through angiogenesis.[87,91] The intimate interaction between the airways and vessels suggests their interdependence and several independent studies using different approaches have shown that the inhibition of vascular growth results in an epithelial branching defect, whereas the reverse is not occurring.[91-94]

This suggests that the vasculature directs the signals for epithelium to branch, and it was shown that parabronchial smooth muscle cells do secrete the branch inducing growth factor Fgf10.[95] Moreover, active circulation was not required for branching to commence.[96]

Pulmonary development is a dynamic process with initiated growth in the distal tip, resulting in a differentiation gradient within the lung along the proximodistal axis. The proximal parts which are formed earlier during development are more mature compared to the distal parts, that are formed later in development, and as a consequence are less mature.[87,97-98]

***Perivascular cells***

Perivascular cells, or mural cells, are recruited from the mesenchyme[97-98] and mesothelium,[99] to stabilize the primitive vascular tubes. They are important for the maturation of the newly formed vascular networks. Platelet derived growth factor B (PDGF-B) is secreted by endothelial cells and induces the migration and proliferation of mesenchymal cells that express PDGFrβ, such as perivascular cells.[100-103] Direct contact between ECs and mesenchymal cells results in the expression of TGF-β, which induces cellular changes of mesenchymal cells into perivascular cells. Additional signaling pathways, like sphingosine-1-phosphatase and angiopoietin-1 and their respective receptors play a role in the maturation and differentiation of mesenchymal cells as well.[104-105] Cell-cell and cell-matrix interactions within the vascular wall are required for the maturation of the complete vasculature.[106-109]

Perivascular cells represent a heterogeneous population of the SMC-lineage that forms a continuum along the proximodistal axis of the pulmonary vasculature. They can be classified as VSMCs or pericytes, based on the localization within the vasculature, their morphology and expression pattern of cytoskeletal markers and membrane

determinants.[106-107,110] Although pericytes and VSMCs are derived from the same perivascular lineage and express similar SMC associated markers,[111] some differences exists. VSMCs are separated from the ECs by the basement membrane, whereas the pericytes share the basement membrane with the ECs.[110] Furthermore, the expression of proteins, such as nestin, NG2 and aminopeptidase N is restricted to the pericyte populations, whereas other cytoskeletal markers and membrane receptor of pericytes, such as α-SMA, tropomyosin, desmin, non-muscle myosin, PDGFrβ, Tie2-receptor, endoglin, Notch, TGFrβ and VEGFr2,[107] are expressed by VSMCs as well. Most likely the differences between pericytes and VSMCs are caused by differences/changes in the micro-environment of the vessel.[112-114]

***Smooth muscle cells***

Smooth muscle cells are involved in the regulation of vascular tone and maintenance of the vasculature. Analysis of SMCs in the rat aorta showed that phenotypic different SMC populations exist[115-116] and which were associated at least *in vitro* with two main function; synthesis of extracellular matrix (synthetic) or vasoconstriction in response to agonists (contractile).[117] The contractile VSMCs express contractile and cytoskeletal proteins, suggesting that these cells were more matured and differentiated, whereas synthetic cells were immature. However, VSMCs are able to change their expression pattern in response to environmental changes[118] as shown by injury and recovery models in rats.[119-120] Injury induced the loss of contractile and cytoskeletal proteins, whereas recovery showed a re-expression of these proteins.[120-121] In addition, Frid et al. were able to distinguish four phenotypically different SMCs subpopulations in the medial layer of the bovine pulmonary vasculature.[122-123] Thus, in general, the media may exist of several SMC subpopulations with different phenotypic characteristics, ranging from synthetic to contractile.[124,125] The different phenotypes are based on localization, morphology, expression pattern of specific proteins and response to agonists.[123,126]

***Smooth muscle cells in CDH***

Since SMC phenotypes are developmental and environmental regulated and thus might play a role in vascular remodeling,[127] we analyzed the VSMCs in the pulmonary vasculature of CDH in human in Chapter 6.

Earlier research by our group and others have shown that the structural abnormalities already develop in utero[128-129] and the persistence of medial hyperplasia after ECMO treatment suggest unresponsiveness of the media in a subgroup of infants who died, indicative that VSMCs in CDH may already be different early in development.[130]

We showed that VSMCs in CDH are indeed intrinsically different as shown by VSMC specific staining and are already observed as early as 19 weeks gestation. Contrast the general accepted concept of delayed lung development in CDH, we show that the

VSMCs in the pulmonary vasculature in CDH display characteristics of an earlier differentiation into a mature, contractile phenotype. The functional consequence(s) of this early differentiation is unknown and requires further research to determine the responsible molecular mechanisms that are responsible for the differentiation.
In addition, the distribution of SM-MHC, as a marker for contractile VSMCs, was distributed more distally in human CDH lungs. This suggests an aberrant pulmonary vascular development and not a delayed development, as was suggested for the epithelial development in CDH.[131-133]

***Pericytes***

Originally, pericytes were defined as perivascular cells located in the non-muscular micro-vessels of the systemic circulation.[134] Recent literature describes pericytes as cells surrounding the endothelial cells of capillaries and micro-vessels.[135] Yet, others define the micro-vessels as pre-capillary arterioles and post-capillary venules.[106,111] Other groups use the term pericyte-like or introduced the term “intermediate” cell, a cell with a phenotype between SMCs and pericyte, to describe the cell type that was found the pre- and post capillary micro-vessels to distinguish them from the actual pericytes found in capillaries. Collectively, these studies are difficult to compare, because of the different definitions that individual groups use to describe pericytes in general, or pericytes and pericyte-like cells. In part this is caused by the lack of specific markers for pericytes. Most studies so far used only one or two markers to identify pericytes, in combination with the localization of the positive marker within the vasculature. Another problem is that the expression of the different markers, that nowadays are described as pericyte markers are developmental, tissue and species dependent.[111] (see table 1, Chapter 7)
In the lung, pericytes are localized in the distal parts of the pulmonary vasculature, which is also the site where neo-muscularization occurs in PH. Initially, the migration of muscle cells were assumed to cause the extensive muscularization,[136-137] but later it was shown that neo-muscularization resulted from an aberrant differentiation of pericytes and “intermediate” cells into mature SMCs.[82,138-139] The latter was confirmed by the expression of α-SMA and SM-B in the small vessels of hyperoxia induced pulmonary hypertension.[140-141] These markers were assumed to be specific for SMCs, but currently, α-SMA is considered to be a general marker for the SMC lineage derived cell types and less specific as a marker for differentiated SMCs.[118] Within this context, SM-B, an isoform of SM-MHC,[142] is considered a specific marker for contractile SMCs.[118]
In contrast to the pulmonary vasculature, pericytes and their role within the vasculature are studied extensively in the systemic circulation during normal development and tumor genesis. Some studies showed that pericytes are also able to express α-SMA,[143-144] which is associated with a matured pericyte.[143]

We analyzed the pericyte population in the pulmonary vasculature of rat lung in chapter 7. We distinguished the pericyte population during development using NG2 and PDGFrβ, combined with the localization of the positive cells within the vasculature. Moreover, the micro-vessels of nitrofen induced CDH rats clearly showed colocalization of α-SMA and NG2 in pericytes in contrast to control lungs. These results suggest that already before birth the smaller vessel of the pulmonary vasculature show signs of neo-muscularization.

***Smooth muscle cells in vascular remodeling and during recovery***

In chapter 8 we specifically analyzed the VSMCs in PH using a hypobaric hypoxia-induced rat model. We observed an increased thickening of the media and adventitial layer, in combination with an increased expression of the synthetic SMC marker SMemb. Moreover, extensive (neo)muscularization was observed by the more distal distribution of α-SMA, SM-MHC and Calponin. During recovery these structural changes completely reversed after a recovery period of up to six weeks in normoxia. Moreover, we showed that reversal of the structural changes is associated with cellular reversal.

Even though a neonatal model may mimic the PH in human newborns closer, we preferred to us the adult rat hypoxia model, since significant physiological changes occur within the pulmonary vasculature in the early periods after birth that influence the outcome of our experiments. The timeframe of our experiments was 10 weeks (4 weeks hypoxia and maximum recovery of 6 weeks) and at the end of the experiments the rats are no longer neonates, but adults.

Moreover, ultra-structural analysis of the postnatal changes within the pulmonary vasculature of pigs showed that hypoxia exposure immediately after births, as occurs most often in human newborns, is characterized with an unadapted pulmonary vasculature, whereas adaptations to extra uterine life first followed by hypoxia exposure, the approach in neonatal models, results in structural changes within the pulmonary vasculature, that might be comparable with the one that are found in adult rat models.[145]

All perivascular cells (VSMCs, “intermediate” cells and pericytes) seem to be derived from a general perivascular precursor cell.[110] During early vascular development, the perivascular precursor cell in the capillary networks express NG2 and PDGFrβ, markers assumed to be specific for pericytes. It could be that all perivascular cells originate from a primitive pericyte and that the local environment within the lung (localization along the vascular tree) determines further maturation and subsequent differentiation of the primitive pericyte. Along the vascular proximodistal axis within the lung VSMCs, “intermediate” cells and actual pericytes are found respectively.

In human CDH, we found already at 19 weeks gestation an early differentiation of VSMCs into a mature contractile phenotype and the presence of neo-muscularization in the micro-vessels, which combined suggests aberrant maturation and/or differentiation of

perivascular cells. This is usually reversible, given the high survival rate of CDH patients born with clinical signs of PH. However, a group of newborns exist that shows no clinical and/or hemodynamic improvement despite the current therapeutic strategies and they will die. Several reasons may underlie the unresponsiveness of this group of newborns, such as the presence of modifier genes or (epi)genetic factors (chromosomal anomalies, retinoic acid).

## Overall conclusion

In this thesis we focused on the therapeutic strategies and structural changes within the pulmonary vasculature of neonates with PVD, especially in neonates with PVD due to CDH. We showed that sildenafil as a therapeutic strategy is not affecting the outcome in the high-risk infants with a CDH. To our knowledge, in most cases these high risk infants are therapy resistant to the current drug therapies, such as the regular used iNO, and the experimental used endothelin antagonists and tyrosine kinase inhibitors. One might speculate that this unresponsiveness might be due to the different structural changes in the pulmonary vasculature, compared to those who respond to the current treatment strategies.

The perivascular cells are the major target of currently used pharmacological therapies. We hypothesized that VSMCs in the pulmonary vasculature of infants with CDH are different. We showed that the VSMCs in CDH showed an aberrant maturation and/or differentiation into a mature VSMC with a contractile phenotype, which occurs already early in development. This suggests no delay in vascular development in CDH, in contrast to the general concept of a delayed development in CDH based on analysis of the epithelium.

CDH is an anomaly characterized with a wide spectrum in clinical presentation. After birth, in general three main groups in terms of outcome can be distinguished. The first group consists of infants born with a diaphragmatic defect, which suffer no or mild respiratory insufficiency and have no signs of pulmonary hypertension on cardiac ultrasound. Those infants are the ones that survive almost without any morbidity. In the second group (moderate), infants suffer of respiratory insufficiency and do have clinical signs of pulmonary hypertension, confirmed with cardiac ultrasound. A part of these infants will survive, but suffer pulmonary morbidity and/or neurodevelopmental disabilities as described by van den Hout et al.,[146] the other part will die as result of iatrogenesis. An inflammatory response, as a consequence of, and in combination with ventilation induced injury, triggers the development of bronchopulmonary dysplasia, or chronic neonatal lung disease, which is also associated with pulmonary hypertension.[147-148] The ongoing respiratory insufficiency will result in the development of a fixed

pulmonary hypertension that is eventually no longer responding to the currently used therapeutic approaches. The third group exists of those that suffer severe respiratory insufficiency and pulmonary hypertension, which do not show any hemodynamic improvement on the current used therapeutic approaches including very expensive treatment modalities such as ECMO, the so-called non-responders, which will not survive.

A failure of the diaphragm to close between the 4$^{th}$ and 10$^{th}$ week of gestation, results in a diaphragmatic defect. The induction of lung budding and further branching starts around the same period (week 5). This indicates that already early in embryonic development a trigger results in both a diaphragmatic defect and the abnormal lung development. Since diaphragm and the pulmonary vasculature are both from mesodermal origin, we hypothesize that an early mesodermal hit results in the development of CDH. However, it is still unknown what causes the diversity in clinical course after birth. As suggested earlier, it could be that some infants have a (epi)genetic susceptibility that either prevent or aggravate the clinical course.

We have to realize that most of our knowledge about the morphology of the lung and vasculature is based on the analysis of the lungs of deceased infants, the ones that suffer the most severe forms of pulmonary hypertension. We do not know if the morphology of lung and vasculature are similar in the infants that suffer no or mild pulmonary hypertension or survive after moderate pulmonary hypertension as no lung tissue is available from these infants.

Until we provide answers what causes the diversity in clinical course, treatment of the individual patient remains “trial and error”. This stresses the importance of the conduction of properly designed RCTs as recently started within the framework of the CDH-EURO Consortium.[149]

## Pulmonary hypertension in lung hypoplasia (hypothesis)

Lung hypoplasia and pulmonary hypertension are responsible for the significant mortality and morbidity in congenital diaphragmatic hernia. The structural abnormalities within the pulmonary vasculature are the underlying cause for the pulmonary hypertension. Histologically, these vascular abnormalities are characterized by the increased thickening of the vascular wall and extensive muscularization of normal partial and non-muscularized arterioles.[17,81-83]

Retinoid acid (RA) signaling plays an important role in lung development and abnormal RA signaling is involved in the pathogenesis of CDH.[150] RA signaling is already active during late gastrulation[151] and is involved in the patterning of the prospective lung field. It is involved in the proliferation of mesodermal cells, induces the expression of fibroblast

growth factor 10 (Fgf10)[152] and prevents the expression of TGF-β.[153] Fgf10 is necessary and required to induce the formation of the primitive lung bud from the foregut endoderm.[154] After the initial budding of the lung, RA expression in the distal parts (the growing parts of the lung) decreases, necessary for further branching morphogenesis and the maintenance of Fgf10 expression is no longer RA-dependent.[152,155-156]

RA-deficiency during gastrulation results in a viable mesodermal cell population, which does not proliferate or properly transcribe Fgf10[152] and is known to result in unilateral or bilateral lung hypoplasia.[152] Moreover, RA signaling is essential for the expression of Sonic Hedgehog, which is involved in a the control of the spatial pattern of Fgf10 expression.[157]

We hypothesize that an early hit (genetic, environmental or combined) causes a relative RA deficiency during gastrulation, this leads to a reduced pool of mesodermal cells that will contribute to the future perivascular cells. Already at the emergence of the lung bud a capillary network is present and further growth of this vascular network is achieved by sprouting of endothelial cells, called distal angiogenesis. Initially, the early pulmonary vasculature will develop normal; the newly formed endothelial tubes become stabilized with perivascular cells from the pool of mesodermal cells. However, at a certain point no longer perivascular cells can be recruited, which limits the further growth of the vasculature. The relative RA deficiency also results into an increased TGF-β signaling which is responsible for the differentiation of perivascular cells. Moreover the Fgf10 expression is reduced which is necessary for branching morphogenesis.

In conclusion we consider that in CDH a relative RA deficiency results in a smaller pulmonary vascular network in which an abnormal TGF-β signaling results in an aberrant differentiation of perivascular cells (neo-muscularization) and decreased Fgf10 expression resulting in less pulmonary branching (lung hypoplasia).

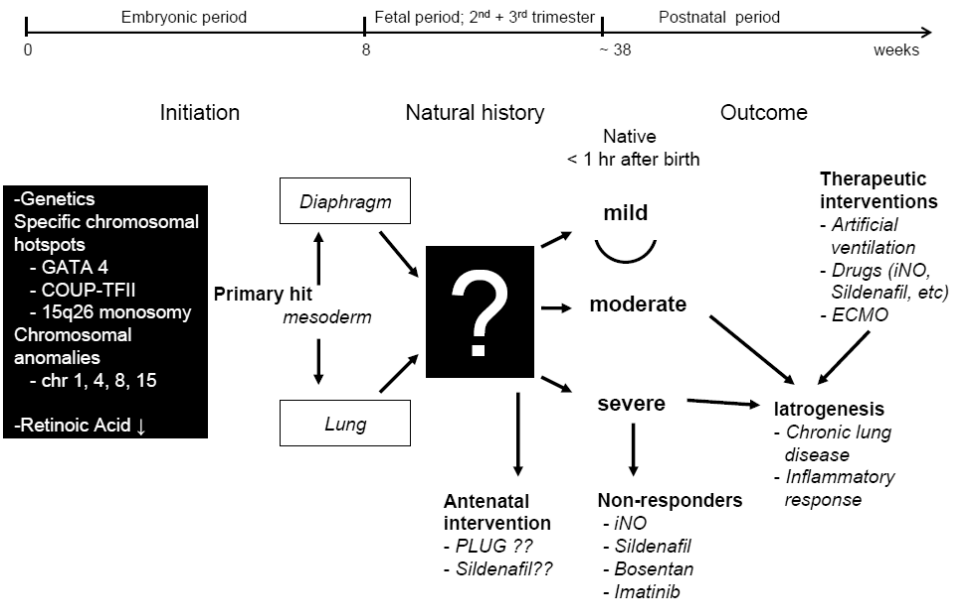



**Figure 1. Schematic overview of current knowledge of diversity in clinical course in CDH.**

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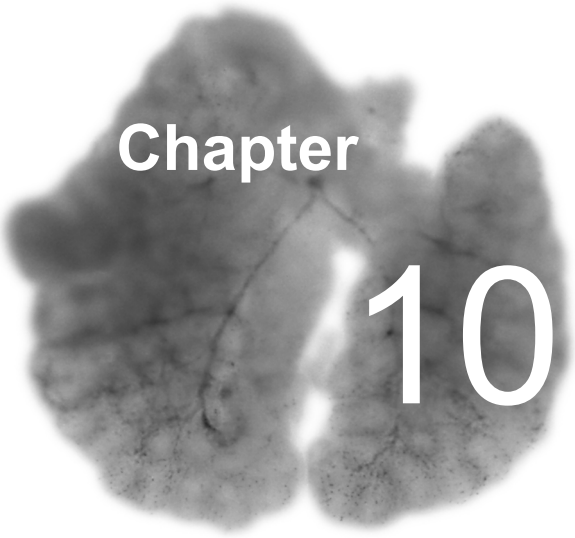

# Chapter 10

## Summary / Nederlandse samenvatting

## Summary

Pulmonary hypertension (PH) in newborns, also referred as pulmonary vascular disease (PVD), clinically results in severe respiratory insufficiency and hypoxemia, and is associated with structural (remodelling) and functional (abnormal vaso-reactivity) changes within the pulmonary vasculature.
Congenital diaphragmatic hernia (CDH) is a severe anomaly characterized by a diaphragmatic defect, lung hypoplasia and associated PH, which is responsible for the high mortality and morbidity among infants with CDH. Therapeutic approaches in neonates with PVD are largely experimental and based on therapeutic strategies used in adults with PH. Despite the increasing knowledge, the pathogenesis of CDH is still largely unknown. Although most studies performed to elucidate the cellular aspects involved in the pathogenesis of CDH focused on the lung hypoplasia and on the role of the airway epithelium, only a limited number of studies focused on the development of the pulmonary vasculature and the structural changes in PVD.

In ***chapter 1*** we reviewed that an increased understanding of CDH shifted the primary therapeutic target from emergency surgical repair towards non-operative emergency of the newborn treated by interdisciplinary teams. The improved therapeutic approaches reduced the mortality, but concomitantly resulted in increased morbidity. Randomized controlled trials are needed to determine the evidence base for the different therapeutic approaches.

Increased knowledge of the pathophysiological mechanisms that are contributing to PH in adults resulted in several pharmacological strategies. The main goal of these strategies is to induce pulmonary vasodilation and decrease the pulmonary vascular resistance. In ***chapter 2*** we review the current knowledge of these different pharmacological strategies used in newborns with PVD. Although proven to be effective in adults, most of these pharmacological strategies are used without any evidence-base in newborns. One such therapy is the use of sildenafil and we performed a pharmacokinetic study to elucidate the optimal dose regimen of oral sildenafil among newborns with PVD. As described in ***chapter 3*** we found that an oral sildenafil dose of 4 times 1 mg per day in newborns resulted in a sildenafil exposure that is similar to the proven to be effective oral dose of 3 times 20 mg sildenafil in adults. However, we also found a high variability in sildenafil exposure between infants, which might be explained by the individual differences in drug absorption and metabolism. This, in turn, may be related to the relative immaturity of the gastro-intestinal tract and the metabolizing enzymes among newborns. Therefore, careful dose titration of sildenafil based on efficacy and the occurrences of hypotension is recommended.

We expanded our study by retrospectively analyzing the use of sildenafil in infants with CDH, as described in ***chapter 4***. Sildenafil was used in high risk infants that suffer from severe pulmonary hypertension. Analysis showed that the start of sildenafil during extracorporeal membrane oxygenation did not influence the risk of mortality. Moreover, we did not find a difference in mortality rate between infants that received sildenafil compared with those that did not receive sildenafil, but sildenafil treatment resulted in a delayed death, 21 days versus 17 days.

PH is associated with structural changes within the vascular wall and in ***chapter 5*** we described the current knowledge of the pulmonary vascular abnormalities found in neonatal disorders associated with PVD. The perivascular cell population resides in the media of vessels and is likely involved in these structural changes. In normal conditions, the perivascular cells play an important role in the regulation of the vascular tone, the maintenance of vascular homeostasis and repair after injury. In ***chapter 6*** we described the analysis of the VSMC population, a perivascular cell subtype, in the pulmonary vasculature of infants with CDH. We found a different maturation and differentiation gradient of VSMCs in CDH. We showed that the normal gradient of differentiation of VSMCs along the proximodistal axis was different in CDH. Already early in vascular development, VSMCs in CDH lungs differentiated into mature cells with a contractile phenotype. This suggests that vascular development is not delayed in CDH, but rather appeared more mature compared to control lung. This is in sharp contrast with the idea that lung hypoplasia and associated PH in infants with CDH resulted from a delayed development, which is based on the analysis of the epithelium. Based on these findings we focused on the pericyte population, another perivascular cell subtype, in the micro-vessels of the lung, which is assumed to be a precursor for VSMCs. The muscularization of the normal non-muscular micro-vessels is a characteristic of the structural changes in PH. In ***chapter 7*** we described our analysis of pericytes in the nitrofen-induced CDH rat model and found that the pericytes in CDH rat lungs seems to be more mature compared to pericytes in control rat lungs. To study the reversibility of the structural changes, we analyzed these structural changes in the hypoxia induced PH rat model. Hypoxia is a trigger to induce vasoconstriction and chronic hypoxia leads to subsequent structural changes of the pulmonary vasculature resulting in pulmonary hypertension. In ***chapter 8*** we analyzed the hypoxia induced structural changes within pulmonary vasculature and showed that the morphological vascular changes are associated with cellular changes within the perivascular cell population. Recovery in normoxia resulted in a complete recovery of both the morphological and cellular changes within the vasculature.

The implications of our research are discussed in ***chapter 9*** and we propose that a relative short or local deficiency of retinoic acid during gastrulation results in the

abnormal expression of TGF-β. TGF-β inhibits either direct or indirect the mesodermal expression of the growth factor Fgf10, which results in reduced branching of the airways. We suggest that the abnormal TGF-β expression induce premature differentiation of perivascular cells (neo-muscularization), and may directly lead to the observed decreases Fgf10 expression and thus lung hypoplasia.

## Nederlandse samenvatting

Pulmonale hypertensie bij pasgeborenen leidt tot ernstige ademhalingsproblemen met een tekort aan zuurstof in het bloed. Het pulmonale vaatbed bij deze kinderen heeft een afwijkende structuur (een verdikte vaatwand) en verstoorde functie (een abnormale vaso-reactieve respons). Pulmonale hypertensie kan daarom ook als een pulmonale vasculaire ziekte worden gezien.
Congenitale hernia diafragmatica (CHD) is een ernstige aangeboren afwijking, waarbij er sprake is van een gat in het middenrif, hypoplasie van de longen en de daarbij behorende pulmonale hypertensie. De pulmonale hypertensie is verantwoordelijk voor de hoge mortaliteit en morbiditeit van CHD. De behandeling van pasgeborenen met pulmonale hypertensie is vooral experimenteel en gebaseerd op de behandeling van volwassen met pulmonale hypertensie. Er is al veel onderzoek gedaan, maar de oorzaak van CHD is nog steeds onduidelijk. Zo is bijvoorbeeld de rol van cellen in de long bestudeerd, echter met de nadruk op de hypoplasie van de longen en de functie van epitheel cellen in de long. Tot nu toe is er nog weinig onderzoek verricht naar de ontwikkeling van het pulmonale vaatbed en de structurele afwijkingen daarin.

In ***hoofdstuk 1*** beschrijven we dat de toegenomen kennis er toe heeft geleid de behandeling van CHD is veranderd van een acuut kinderchirurgisch probleem, sluiting van het gat in het middenrif, naar een aandoening is die gebaat is bij een multidisciplinaire aanpak. Verbeterde behandelingsstrategieën hebben geleid tot een grotere kans op overleving, maar daarmee is de morbiditeit toegenomen. Gerandomiseerde studies zijn nodig om te bepalen welke behandelingsstrategieën evidence-based zijn.

Onderzoek naar de pathofysiologische mechanismen bij het ontstaan van pulmonale hypertensie bij volwassenen heeft geleid tot verschillende medicamenteuze behandelingsstrategieën, die effectief zijn gebleken. Deze zijn vooral gericht op het induceren van pulmonale vasodilatatie en het verlagen van de pulmonale vaat weerstand. In ***hoofdstuk 2*** beschrijven we de toepassing van deze medicamenteuze behandelingen bij pasgeborenen met pulmonale hypertensie. Anders dan bij volwassenen, is er nog weinig tot geen bewijs voor de effectiviteit daarvan bij pasgeborenen. Een van deze behandelingsstrategieën is het gebruik van sildenafil. Onze studie om de juiste orale dosering van sildenafil bij pasgeborenen te bepalen is beschreven in ***hoofdstuk 3***. Een dosering van 4 keer daags 1 mg sildenafil (oraal) bij pasgeborenen geeft een sildenafil spiegel die bij volwassen wordt bereikt met een dosering van 3 keer daags 20 mg (oraal). Het is bewezen dat deze laatste dosering pulmonale hypertensie bij volwassenen vermindert. Echter, de sildenafil spiegels bij

pasgeborenen blijken enorm te verschillen onderling, als gevolg van individuele verschillen in de absorptie van sildenafil, die mede worden veroorzaakt door onrijpheid van het maag darm kanaal en de enzymen die sildenafil moeten metaboliseren. Daarom moet de dosering van sildenafil individueel aangepast worden aan de hand van effectiviteit en het uitblijven van systemische hypotensie. Daarnaast hebben we retrospectief het gebruik van sildenafil in pasgeborenen met CHD geanalyseerd en beschreven in ***hoofdstuk 4***. Vooral kinderen met ernstige pulmonale hypertensie kregen sildenafil toegediend. Verdere analyse toonde aan dat sildenafil geen invloed heeft op de kans op overlijden als het wordt gestart tijdens extracorporale membraan oxygenatie. Daarnaast vonden we geen verschil in sterfte tussen pasgeborenen met ernstige pulmonale hypertensie die wel sildenafil kregen toegediend en zij die het niet kregen toegediend. Echter, de overleden kinderen die met sildenafil waren behandeld overleden na gemiddeld 21 dagen; zij die geen sildenafil hadden gekregen na 17 dagen.

Pulmonale hypertensie gaat gepaard met structurele veranderingen van de vaatwand en in ***hoofdstuk 5*** beschrijven we dergelijke veranderingen bij aandoeningen die voorkomen bij pasgeborenen en gepaard gaan met pulmonale hypertensie. De veranderingen in de vaatwand gaan onder andere gepaard met veranderingen in de peri-vasculaire cellen. Onder normale omstandigheden spelen deze cellen een belangrijke rol in de regulatie van de vaattonus, maar zijn ze ook verantwoordelijk voor een goede homeostase in de vaatwand of voor herstel bij vaatschade. In ***hoofdstuk 6*** beschrijven wij de expressie patronen voor verschillende markers voor gladde spiercellen, een bepaald type peri-vasculaire cel, in de vaatwand van kinderen met een CHD. We vonden een afwijkende proximale-distale gradiënt in rijping en differentiatie van deze gladde spier cellen. Dit wijst er op dat al vroeg in de vasculaire ontwikkeling de gladde spiercellen differentiëren tot een rijpere gladde spiercel met een contractiel fenotype. Deze bevinding suggereert dat de vasculaire ontwikkeling niet vertraagd is in CHD. Dit is in scherp contrast met het op de analyse van epitheel cellen gebaseerde idee dat long hypoplasie en pulmonale hypertensie het resultaat zijn van een vertraagde ontwikkeling. Daarom hebben we ons gericht op de pericyten, een ander type peri-vasculaire cel in de kleine vaten in de long, die worden beschouwd als een voorlopercel voor gladde spiercellen. Bij pulmonale hypertensie zijn deze kleine vaten namelijk gemuscullariseerd, wat normaliter niet het geval is. In ***hoofdstuk 7*** beschrijven we het onderzoek naar deze pericyten in ratten met een CHD. De pericyten in de longen van deze dieren blijken al meer gerijpt te zijn dan de pericyten in longen van ratten zonder CHD. De mogelijke reversibiliteit van de veranderingen in de vaatwandstructuur is bestudeerd in een diermodel. Als gevolg van een gebrek aan zuurstof (hypoxie) ontstaat er vasoconstrictie van het pulmonale vaatbed. Aanhoudende hypoxie leidt uiteindelijk tot veranderingen in de vaatwandstructuur en tot pulmonale hypertensie. In ***hoofdstuk 8***

beschrijven we dat morfologische veranderingen in de vaatwand gepaard gaan met veranderingen van de peri-vasculaire cellen. Deze veranderingen herstellen volledig als het gebrek aan zuurstof verdwenen is.

In ***hoofdstuk 9*** wordt besproken wat al het onderzoek heeft opgeleverd. De bevindingen leidt tot de hypothese dat een gebrek aan vitamine A gedurende relatief korte tijd tijdens de gastrulatie, een zeer vroeg stadium in de embryonale ontwikkeling, leidt tot een abnormale expressie van de transcriptie factor, TGF-β. Dit heeft direct of indirect een verminderde expressie van Fgf10 in het mesoderm tot gevolg, waardoor de luchtwegen zich minder vertakken. We stellen dat de abnormale TGF-β expressie leidt tot een vroege differentiatie van peri-vasculaire cellen en een verminderde expressie van Fgf10 welke leidt to hypoplasie van de longen.

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## Curriculum Vitae

Ilona Sluiter was born on August 3rd 1978 in Almelo, the Netherlands. After completing MAVO and HAVO at the Thomas a Kempis College, Zwolle, and VWO at the Deltion College, Zwolle, she started her medical training at the University of Groningen in 1998. After her graduation in 2005, she worked as a resident at the pediatric departments of Streekziekenhuis Midden-Twente, Hengelo (2005) and Medisch Spectrum Twente, Enschede (2006) and the pediatric intensive care unit of the Erasmus Medical Centre – Sophia Children's Hospital, Rotterdam (2006-2007). In 2007 she started working as a research fellow at the department of pediatric surgery and intensive care medicine (Prof. dr. D. Tibboel) on the project that is described in this thesis.
Ilona lives together with Robert van Utteren.

## PhD Portfolio

**Summary of PhD training and teaching**

| | |
|---|---|
| Name PhD student | Ilona Sluiter |
| Erasmus MC Department | Intensive Care and Pediatric Surgery / Cell Biology |
| Research School | Molecular Genetic Center |
| PhD period | September 2007 – February 2012 |
| Promotor's | Prof. Dr. D. Tibboel<br>Prof. Dr. I.K.M. Reiss |
| Supervisor | Dr. R.J. Rottier |

| PhD training | Year | Workload | |
|---|---|---|---|
| | | Hours | ETCS |
| **General courses** | | | |
| - Biomedical English Writing and Communication | 2008-2009 | | 4 ETCS |
| - Laboratory animal science (art 9) | 2007 | | 3 ETCS |
| - Statistics (Classical Methods for Data analysis | 2009 | 160 hrs | |
| **Specific courses (e.g. Research school, Medical Training)** | | | |
| - Molecular and Cell Biology | 2008-2009 | 168 hrs | |
| - Cell and Developmental Biology | 2010 | | 3 ETCS |
| - Biochemistry and Biophysics | 2010 | | 3 ETCS |
| - Safely working in the laboratory | 2010 | 8 hrs | |
| - Development, Stem Cell and Disease | 2011 | 32 hrs | |
| **Seminars and workshops** | | | |
| - Symposium perinatal lung development (Leuven, Belgium) | 2007 | 8 hrs | |
| - MGC workshop Koln, Germany (oral presentation) | 2010 | 32 hrs | |
| - Photoshop and Illustrator CS5 workshop | 2010 | | 0.3 ETCS |
| - Postgraduate Course CDH Euro-Consortium Meeting (Rome, Italy) | 2011 | 8 hrs | |

| | Year | Workload | |
|---|---|---|---|
| | | Hours | ETCS |
| **Presentations** | | | |
| - Monday Morning Meetings | 2007-2011 | 320 hrs | |
| - Grant Application Sophia Foundation for Medical Research | 2009 | 10 hrs | |
| - Grant Application Sophia Foundation for Medical Research | 2010 | 10 hrs | |
| - Grant Application Sophia Foundation for Medical Research | 2011 | 10 hrs | |
| **(Inter)national conferences** | | | |
| - ESPNIC Verona, Italy (oral presentation) | 2009 | | 1 ETCS |
| - International CDH Meeting Rome, Italy (oral presentation) | 2011 | | 1 ETCS |
| **Other: Writing Grant Applications** | | | |
| - Pulmonary vascular smooth muscle cell heterogeneity determines pulmonary hypertension of the newborn | 2009 | 28 hrs | |
| - The central role of pericytes in neonatal pulmonary hypertension | 2010 | 28 hrs | |
| **Supervising practicals and excursions, Tutoring** | | | |
| - students Minor Pediatric Surgery | 2011 | 10 hrs | |

## List of publications

**I. Sluiter**, C.P van de Ven, R.M. Wijnen, D. Tibboel. Congenital Diaphragmatic Hernia: Still a moving target. *Semin Fetal Neonatal Med 2011;16(3):139-44 (review)*

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**I. Sluiter**, U. Kraemer, T. Schaible, I. Reiss, D. Tibboel. Sildenafil treatment in congenital diaphragmatic hernia: End of another saga? *Submitted Pediatric Pulmonol*

**I. Sluiter**, I. Reiss, U. Kraemer, R. Krijger, D. Tibboel, R.J. Rottier. Vascular abnormalities in newborns with pulmonary hypertension. *Expert Rev Respir Med 2011;5(2):245-56 (review)*

**I. Sluiter**, I.W.J.M van der Horst, P. van der Voorn, A. Boerema-de Munck, M. Buscop-van Kempen, R.R. de Krijger, D. Tibboel, I.K.M. Reiss, R.J. Rottier. Premature differentiation of vascular smooth muscle cells in human congenital diaphragmatic hernia. *Submitted*

**I. Sluiter**, A. van Heijst, R. Haasdijk, A. de Munck, I. Reiss, D. Tibboel, R.J. Rottier Reversal of pulmonary vascular changes after recovery from chronic hypoxic pulmonary hypertension in rats. *Submitted Histopathology*

L. van den Hout, **I. Sluiter**, S. Gischler, A. de Klein, R. Rottier, H. IJsselstijn, I. Reiss, D. Tibboel. Can we improve outcome of congenital diaphragmatic hernia? The CDH Euro-Consortium. *Pediatric Surg Int 2009;25(9):733-43 (review)*

E.D. Wilschut, R. Keijzer, R.J. Houmes, C.P. van de Ven, L. van den Hout, **I. Sluiter**, P. Rycus, N.M.A. Bax, D. Tibboel. Congenital Diaphragmatic Hernia: to repair on or off ECMO: that is the question. *Accepted.*

## List of abbreviations

Ang-1 or 2 – Angiopoietins 1 or 2
α-SMA – smooth muscle actin
ASD – arterial septal defect
AT – adventitial thickness
$AUC_{24\ (SIL+DMS)}$ – average plasma concentration area under the curve over 24 hrs
BMP – bone morphogenetic protein
BNP – b-type naturetic peptide
BPD – bronchopulmonary dysplasia
cAMP – cyclic adenosine monophosphate
CDH – congenital diaphragmatic hernia
cGMP - cyclic guanosine monophosphate
CL – clearance
CLD – chronic lung disease
CRBP-1 – cellular retinol binding protein
CV – conventional ventilation
CYP3A – cytochrome P450, family 3, subfamily A
DMS – desmethylsildenafil
EC – endothelial cell
ECM – extracellular matrix
ECMO – extracorporeal membrane oxygenation
EGM2MV – microvascular endothelial cell growth medium 2
eNOS – endothelial nitric oxide synthase
ET-1/ ET1 – endothelin
ET-A – endothelin receptor A
ET-B – endothelin receptor B
F – absolute bioavailability
FBS – fetal bovine serum
FETO – fetal tracheal occlusion
FGF-2 – fibroblast growth factor 2
Fgf10 – fibroblast growth factor 10
GER – gastro-esophageal reflux
HFO – high frequency oscillation

ICU – intensive care unit
iNO – inhaled nitric oxide
LC-MS/MS – liquid chromatography-mass spectrometry/mass spectrometry
LHR – lung to head ratio
LV + S – left ventricle + septum
MAS – meconium aspiration syndrome
MLC – myosin light chain
MLCP – myosin light chain kinase
MMPs – metalloproteinases
MRI – magnetic resonance imaging
MT – medial thickness
MVOF – minimum value of objective function
Myl-9 – regulator part myosin light chain
NO – nitric oxide
O/E LHR – observed versus expected lung to head ratio
OI – oxygenation index
PAH – pulmonary arterial hypertension
PAP – pulmonary arterial pressure
PBS – phosphate buffered saline
PDE – phosphodiesterase
PDE5 – phosphodiesterase 5
PDGF – platelet derived growth factor
PDGFrβ – platelet derived growth factor receptor β
PF – prophylactic fundoplication
PFTE – polytetrafluorethylene
$PGI_2$ - prostacyclin
PH – pulmonary hypertension
PHN – pulmonary hypertension of the newborn
PK – pharmacokinetic
PNA – postnatal age
PPHN – persistent pulmonary hypertension of the newborn
PROM - premature rupture of membranes
PS – pencillin-streptomysin
PVD – pulmonary vascular disease

pVHL - von Hippel-Lindau protein
PVR: pulmonary vascular resistance
RA – retinoid acid
Raldh2 – retinaldehyde dehydrogenase 2
RCT – randomized controlled trial
RV – right ventricle/ right ventricular
RVH – right ventricular hypertrophy
RVSP – right ventricle systolic pressure
SHH – sonic hedgehog
SIL – sildenafil
SMC – smooth muscle cell
SM-MHC – smooth muscle myosin heavy chain
TGA – transposition of the great arteries
TGF-β – transforming growth factor B
TIMPs - tissue inhibitors of metalloproteinases
TMA – tissue microarray
V – volume of distribution
VEGF – vascular endothelial growth factor
VSD: ventricular septal defect
VSMC – vascular smooth muscle cell
WHO – world health organization

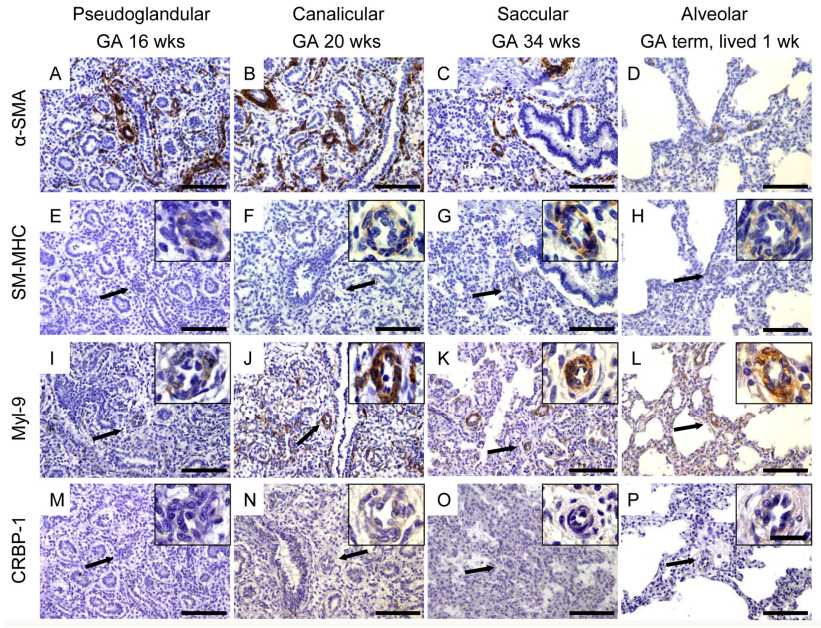


**Figure 2. Expression pattern of SMC markers during normal development.** (page 103)

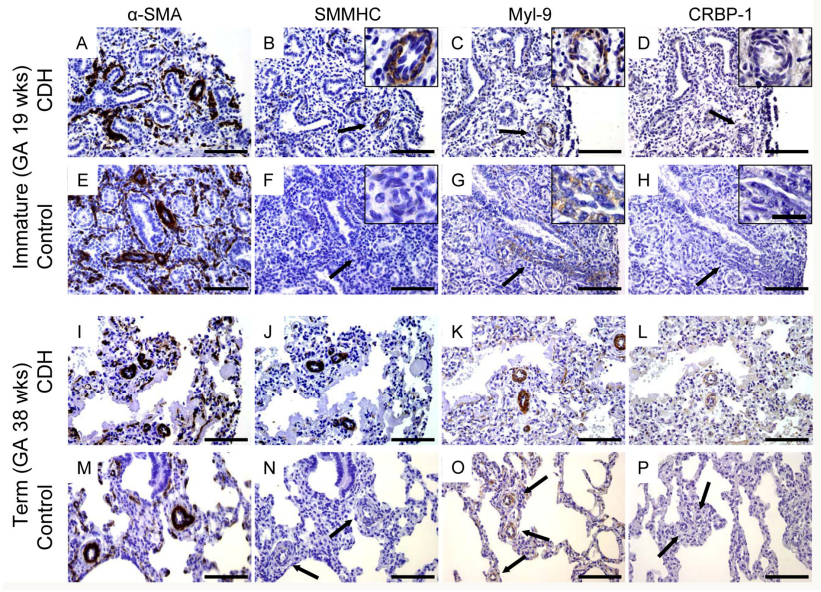


**Figure 3. Expression pattern of SMC markers in immature / premature and term CDH and age-matched controls.** (page 104)

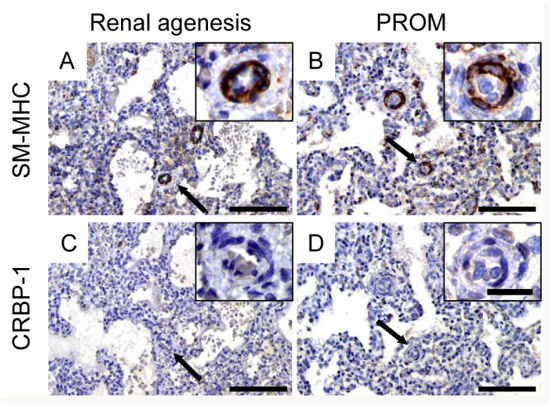


**Figure 4. Expression of SMC markers SMMHC and CRBP-1 in lung hypoplasia unrelated to CDH.** (page 105)

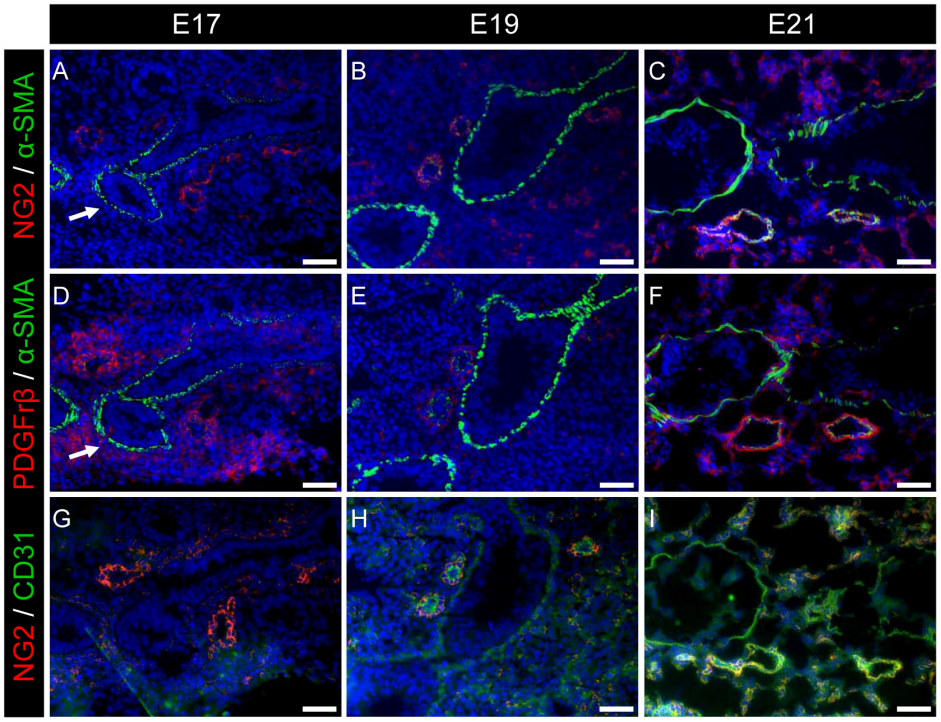


**Figure 1. Developmental expression pericyte markers control rat lung.** (page 116)

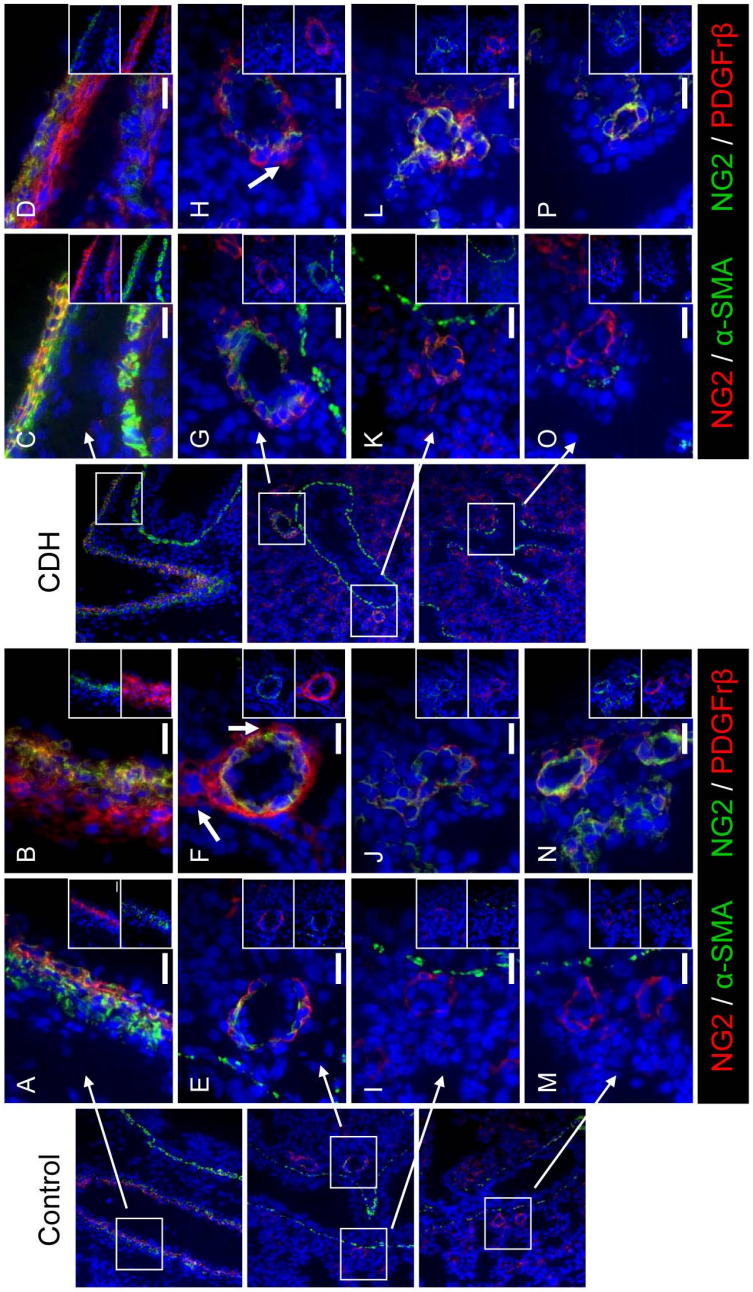


**Figure 2. Comparison pericyte population control rat lung versus CDH rat lung.** (page 117)

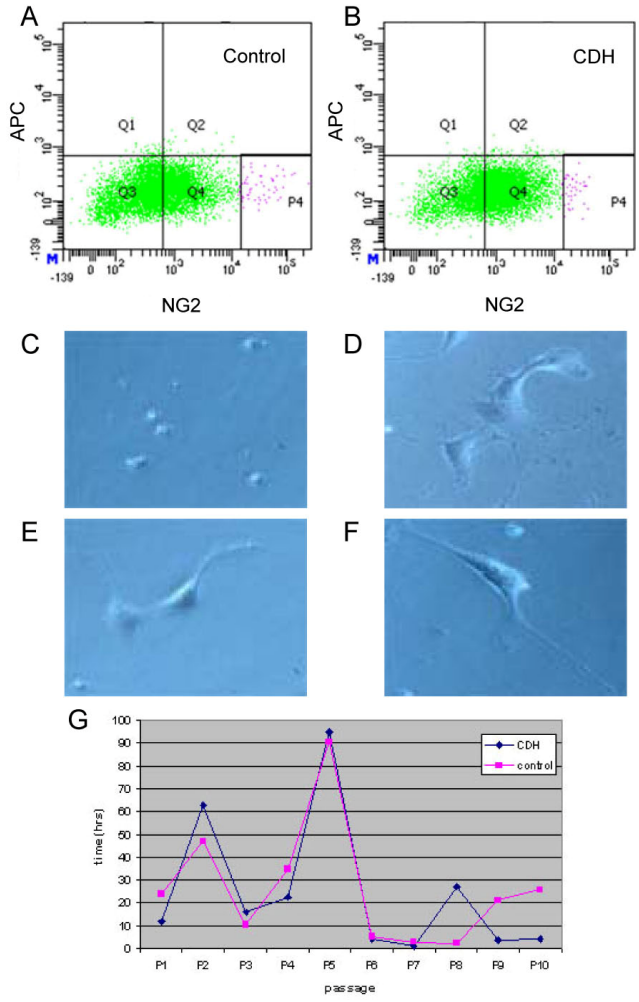


**Figure 3. Analysis of control and CDH NG2 positive cell population.** (page 119)

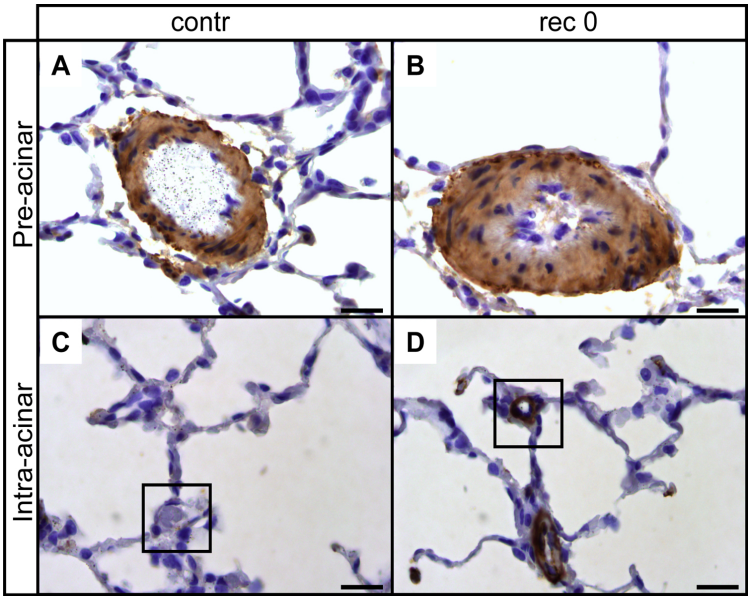


**Figure 3. Distribution of α-SMA expression in lung vessels of normal and hypoxic animals.** (page 137)

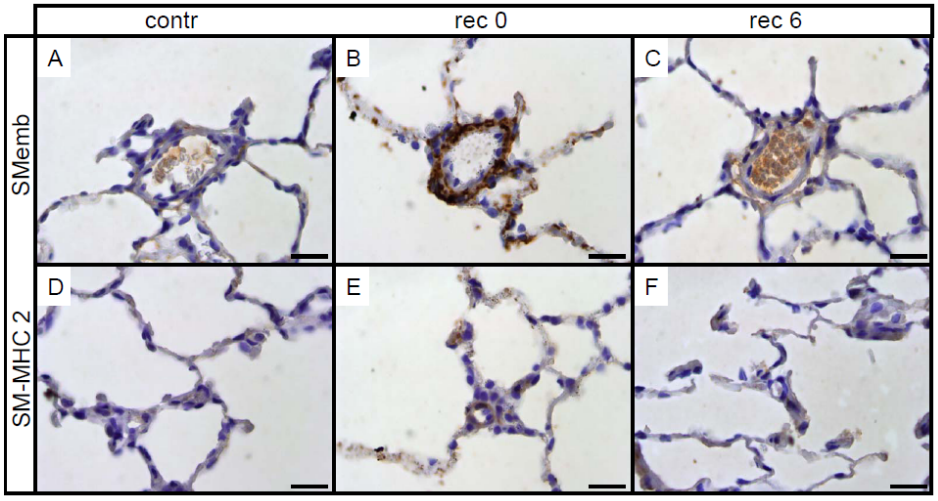


**Figure 4. Distribution of contractile and synthetic VSMCs in the lung vasculature of normal and hypoxic animals.** (page 138)