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THERAPEUTIC POTENTIAL OF ALL-*TRANS* RETINOIC ACID TO ATTENUATE PULMONARY HYPOPLASIA IN AN EXPERIMENTAL RAT MODEL OF CONGENITAL DIAPHRAGMATIC HERNIA

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DOCTORAL DISSERTATION

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To my late father, who I dearly miss.

Abstract

Background: Congenital diaphragmatic hernia (CDH) is a prenatal defect in the integrity of the developing diaphragm, which results in pulmonary hypoplasia (PH) with alveolar immaturity. PH leads to life-threatening respiratory insufficiency at birth, thus remaining a major cause of neonatal mortality and long-term morbidity in CDH. Lipid-containing interstitial fibroblasts (LIFs) are critically important for fetal lung growth by stimulating alveolarization and surfactant phospholipid production in alveolar epithelial cells type II (AECII), which in turn increases alveolar maturation. Thymocyte antigen 1 (Thy-1) is a strongly expressed cell surface protein in LIFs, which plays a key role in alveolar lipid homeostasis by upregulating adipocyte differentiation-related protein (Adrp). Adrp is necessary for the intracellular uptake of neutral lipids into LIFs and their transport to AECII. Furthermore, LIFs express leptin (Lep), which binds to its receptor (Lep-R) on AECII, thus stimulating *de novo* synthesis and secretion of surfactant proteins.

Objectives: As *Thy-1*$^{-/-}$ knockouts show a phenotype similar to PH in human CDH with impaired alveolar development and reduced proliferation of LIFs, one objective of this study was to identify disruptions in *Thy-1* signaling in hypoplastic rat lungs with toxicological induced CDH, which may have an adverse effect on the expression and lipid content of pulmonary LIFs. In addition, as it has been demonstrated that retinoids positively affect the proliferation of LIFs and expression of Lep and Lep-R in developing rat lungs, another objective was to investigate if prenatal administration of all-*trans* retinoic acid (ATRA) may have the potential to attenuate PH in this rodent CDH model by improving fetal alveolarization and surfactant production.

Material and methods: Time-mated rats received either nitrofen or vehicle via oral-gastric lavage on embryonic day 9.5 (E9.5). For the first objective of this work, fetuses were sacrificed on E21.5, and dissected lungs were divided into controls (*n*=28) and CDH-associated PH (*n*=28). For the second objective, control and nitrofen-exposed dams were randomly assigned to either intraperitoneal ATRA (5 mg/kg/d) or placebo administration on E18.5, E19.5 and E20.5. Fetal lungs were harvested on E21.5, and divided into Control+Placebo (*n*=32), Control+ATRA (*n*=32), Nitrofen+Placebo (*n*=32) and Nitrofen+ATRA (*n*=32). Pulmonary gene expression levels of *Thy-1*, *Adrp*, *Lep* and *Lep-R* were determined by qRT-PCR. Adrp, Lep and Lep-R immunohistochemistry was combined with oil red O staining to assess

pulmonary protein expression and lipid content. Immunofluorescence double staining was performed to evaluate pulmonary LIF expression and localization by confocal laser scanning microscopy. Alveolarization was investigated using stereo- and morphometric analysis techniques. Surfactant phospholipid synthesis was analyzed by labeling for surfactant protein B (SP-B).

Results: Relative mRNA expression of *Thy-1* and *Adrp* was significantly downregulated in hypoplastic rat lungs with nitrofen-induced CDH. Confocal laser scanning microscopy confirmed markedly decreased Thy-1 immunoflurescence in pulmonary LIFs of nitrofen-exposed fetuses with CDH, which was associated with markedly reduced cytoplasmatic lipid inclusions. Adrp immunoreactivity was clearly diminished in specimens with CDH-associated PH, which was accompanied by impaired alveolar mesenchymal cell differentiation and overall reduction of LIFs. Maternal application of ATRA resulted in a significantly increased lung-to-body weight ratio of nitrofen-exposed fetuses, which was associated with upregulation of pulmonary *Adrp* transcripts and corresponding protein expression. Immunofluorescence double staining demonstrated markedly increased LIFs in interstitial compartments of distal alveolar walls in Nitrofen+ATRA, which was accompanied by an overall increase of lipid droplets in LIFs. A significantly enhanced radial alveolar count and decreased mean linear intercept was detected in nitrofen-exposed fetuses after prenatally administered ATRA. Relative mRNA expression of *Lep* and *Lep-R* was significantly upregulated in hypoplastic rat lungs following maternal ATRA treatment. Light microscopy showed notably increased Lep and Lep-R immunoreactivity in interstitial and alveolar epithelial cells of nitrofen-exposed fetuses that received ATRA application shortly before birth. Immunoflurescence revealed markedly increased alveolar SP-B protein expression in hypoplastic rat lungs after prenatal administration of ATRA.

Conclusions: Disruption of the *Thy-1*/*Adrp* signaling cascade in hypoplastic rat lungs leads to a reduction of pulmonary LIFs with fewer lipid inclusions and impaired alveolar mesenchymal cell differentiation, which may contribute to decreased alveolar development and PH in the nitrofen-induced CDH model. Prenatal treatment with ATRA may therefore have a therapeutic potential in attenuating CDH-associated PH by increasing the expression of LIFs and thus *Lep*-mediated surfactant phospholipid synthesis, which in turn stimulate fetal alveolarization, distal airway maturation and *de novo* surfactant production.

Table of contents

List of original publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Friedmacher F, Hofmann AD, Takahashi H, Takahashi T, Gosemann JH, Puri P. Disruption of *Thy-1* signaling in alveolar lipofibroblasts in experimentally induced pulmonary hypoplasia. Pediatr Surg Int 2014;30:129-135.

II. Friedmacher F, Gosemann JH, Fujiwara N, Takahashi H, Hofmann A, Alvarez LA, Puri P. Evidence for decreased lipofibroblast expression in hypoplastic rat lungs with congenital diaphragmatic hernia. Pediatr Surg Int 2014;30:1023-1029.

III. Friedmacher F, Fujiwara N, Hofmann A, Takahashi H, Alvarez LAJ, Gosemann JH, Puri P. Prenatal retinoic acid increases lipofibroblast expression in hypoplastic rat lungs with experimental congenital diaphragmatic hernia. J Pediatr Surg 2014;49:876-881.

IV. Friedmacher F, Hofmann AD, Takahashi H, Takahashi T, Kutasy B, Puri P. Prenatal administration of all-*trans* retinoic acid upregulates leptin signaling in hypoplastic rat lungs with experimental congenital diaphragmatic hernia. Pediatr Surg Int 2014;30:1183-1190.

V. Friedmacher F, Pakarinen MP, Rintala RJ. Congenital diaphragmatic hernia: a scientometric analysis of the global research activiy and collaborative networks. Pediatr Surg Int 2018;34:907-917.

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Abbreviations

αSMA	Alpha smooth muscle actin
AD	Anno Domini (in the year of the Lord)
Adrp	Adipocyte differentiation-related protein
AECII	Alveolar epithelial cells type II
ANOVA	Analysis of variance
AST	Alveolar septal thickness
ATRA	All-*trans* retinoic acid
BC	Before Christ
CDH	Congenital diaphragmatic hernia
Coup-TFII	Chicken ovalbumin upstream promoter transcription factor 2
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
E	Embryonic day
ECMO	Extracorporeal membrane oxygenation
FETO	Fetal endoscopic tracheal occlusion
Fog2	Friend of Gata2
Gata4/6	GATA-binding protein 4 and 6
Gli2/3	Glioma-associated oncogenes 2 and 3
Lep	Leptin
Lep-R	Leptin receptor
LIF	Lipid-containing interstitial fibroblast
kDA	Kilodalton
Kif7	Kinesin family member 7
Lox	Lysyl oxidase
MLI	Mean linear intercept
NCRC	National Children's Research Centre
Ob	Obese
ORO	Oil red O
PBS	Phosphate buffered saline
Pdgfrα	Platelet-derived growth factor receptor alpha
PFA	Paraformaldehyde
PH	Pulmonary hypoplasia
PPF	Pleuroperitoneal fold

PPHN	Persistent pulmonary hypertension of the newborn
PPM	Pleuroperitoneal membrane
PTHrP	Parathyroid hormone-related protein
qRT-PCR	Quantitative real-time polymerase chain reaction
RAC	Radial alveolar count
RARs	Retinoic acid receptors
RNA	Ribonucleic acid
Robo1	Roundabout guidance receptor 1
Shh	Sonic hedgehog
Slit3	Slit homolog 3
SP-B	Surfactant protein B
SEM	Standard error of the mean
ST	Septum transversum
Thy-1	Thymocyte antigen 1
Wt1	Wilms Tumor 1

1 Introduction

Congenital diaphragmatic hernia (CDH) is characterized by a spectrum of developmental defects in the forming diaphragm, which are caused by disordered embryogenesis [Kluth et al., 1996a]. These malformations lead to an incomplete fusion of the diaphragmatic leaflets and subsequently to a hole in the diaphragm [Clugston and Greer, 2007]. As a consequence of this abnormal opening, the developing abdominal organs can protrude into the thoracic cavity, occupying space normally reserved to accommodate the growing lungs [Greer, 2013]. Because the process of CDH herniation occurs at the same time as bronchial subdivision and alveolarization, normal lung development is severely affected, resulting in pulmonary hypoplasia (PH) and persistent pulmonary hypertension of the newborn (PPHN) [Keijzer and Puri, 2010; Rottier and Tibboel, 2005]. Depending on the extent of this unfortunate combination, CDH patients often suffer from life-threatening respiratory distress at birth [Losty, 2014; Tovar, 2012]. Despite significant improvements in postnatal resuscitation and modern lung-protective strategies, CDH remains one of the major therapeutic challenges in modern neonatal intensive care, causing high mortality and long-term morbidity for survivors [Rocha et al., 2012; Peetsold et al., 2009].

Most of our current knowledge about the pathogenesis of CDH and the related morphological changes in hypoplastic lungs has originated from various experimental animal models [Chiu 2014; van Loenhout et al., 2009; Mortell et al., 2006]. Abnormal differentiation and malfunctioning of alveolar epithelial cells type II (AECII) have been demonstrated in the nitrofen-induced CDH model, which contribute to the development of PH [Brandsma et al., 1994]. However, the exact molecular and cellular mechanisms causing impaired alveolarization and distal airway formation in hypoplastic lungs with CDH remain unknown.

Lipid-containing interstitial fibroblasts (LIFs) are critically important for fetal lung growth by stimulating differentiation of AECII and surfactant phospholipid production, which in turn increases alveolar maturation [Torday et al., 2003; McGowan and Torday, 1997]. Thymocyte antigen 1 (Thy-1) is a strongly expressed cell surface protein in LIFs, which plays a key role in alveolar lipid homeostasis by upregulating adipocyte differentiation-related protein (Adrp) [Varisco et al., 2012; McGowan and Torday 1997]. Adrp is necessary for the intracellular uptake of neutral lipids into LIFs and their subsequent transport to AECII [Schulz et al., 2002; Brasaemle et al., 1997]. Furthermore, LIFs express leptin (Lep), which binds to its receptor (Lep-R) on AECII, thus stimulating *de novo* synthesis and secretion of pulmonary surfactant proteins [Torday et al., 2002].

As *Thy-1*$^{-/-}$ knockout mice show a phenotype similar to PH in human CDH with impaired alveolar development and reduced proliferation of LIFs [Nicola et al., 2009], one objective of this study was to investigate the hypothesis that *Thy-1* signaling is disrupted in hypoplastic rat lungs with toxicological induced CDH, which potentially may have an adverse effect on the expression and lipid content of pulmonary LIFs. In addition, as it has been demonstrated that retinoids (derivates of vitamin A) positively affect the proliferation of LIFs and expression of Lep and Lep-R in developing rat lungs [McGowan et al., 1995], another objective of this study was to investigate the hypothesis that prenatal *in vivo* administration of all-*trans* retinoic acid (ATRA) may have the potential to attenuate PH in this rodent CDH model by enhancing fetal alveolarization, which in turn may increase pulmonary LIF expression and thus surfactant production. The first indication that diaphragmatic defects may be associated with disruptions in retinoid signaling came from newborn rats with CDH whose mothers were bred on a vitamin A-deficient diet [Andersen, 1941]. Interestingly, the rate of CDH was found to be decreased when vitamin A was reintroduced into the maternal diet during midgestation [Wilson et al., 1953; Andersen, 1949]. Subsequently, similarities between retinoic acid receptor (RAR) double mutant mice and nitrofen-exposed fetal rats have suggested that nitrofen may interfere with the retinoid pathway [Mendelsohn et al., 1994].

Further evidence for this theory was derived from the observation that the administration of vitamin A or ATRA to pregnant rats that had previously received nitrofen treatment showed a reduced incidence and severity of CDH in their offspring [Babiuk et al., 2004; Thebaud et al., 1999]. A series of *in vivo* and *in vitro* experiments have indicated that nitrofen may inhibit the activity of retinal dehydrogenase 2, a key enzyme necessary for the synthesis of ATRA that is expressed in the developing diaphragm and various other components of this important signaling pathway [Noble et al., 2007; Chen et al., 2003; Mey et al., 2003]. In newborn infants, diaphragmatic defects have been associated with low retinol and retinol-binding protein levels, independent of their maternal retinol status [Beurskens et al., 2013; Beurskens et al., 2010; Major et al., 1998], supporting the idea that human CDH is linked with abnormal retinoid homeostasis.

2 Review of the literature

2.1 Historical overview

The diaphragm and allied defects have been a source of fascination for scientists and clinicians for many centuries [Puri and Wester, 1997; Irish et al., 1996]. The ancient Greeks already recognized the diaphragm as a distinct anatomical structure in the human body. One of the earliest known literary references was by Homer in the 9th century BC, precisely describing wounds suffered by warriors during the Trojan War [Derenne et al., 1994]. At that time, however, the diaphragm was not tied to any particular physical function and was associated with the region of the body responsible for thought. The initial physiological explanations of respiration by Empedocles (490-430 BC) as well as the concepts introduced by Plato (428-348 BC) and Hippocrates (460-370 BC) did not include any significant participation of the diaphragm [Potter, 2010; Bury, 1929; Leonard, 1908]. Aristotle (384-322 BC) was the first to link respiration to a particular organ and a specific movement of the thorax, but in his theory the diaphragm was just a barrier separating the thorax from the abdomen and played no role in respiration [Peck, 1970]. Herophilus (335-280 BC) and his contemporary Erasistratus (304-250 BC), who systematically performed scientific dissections of human cadavers and animal experiments at the medical school in Alexandria, demonstrated for the first time that the diaphragmatic muscle fibers were the agents of respiratory movement [Von Staden, 1989]. Galen of Pergamon (129-200 AD) developed the concept of interaction between ribcage and abdominal muscles in maintaining the position of the diaphragm, showing a clear understanding of the principle that the diaphragm can move upward during an isovolume maneuver as long as the ribcage is allowed to expand [Derenne et al., 1995].

The term diaphragmatic hernia was introduced in 1575 by the French surgeon Ambroise Paré, who described two autopsy cases of traumatic diaphragmatic defects [Paré, 1575]. Following a post mortem examination of a 24-year-old man, the French physician Lazare Rivière discovered the first case of CDH, which he published in 1679 [Bonet, 1679]. In 1701, Sir Charles Holt reported the first pediatric case of CDH [Holt, 1701]. A detailed description of the gross anatomy and pathophysiology associated with CDH was presented in 1754 by the Scottish physician George Macaulay, who noted a right-sided defect in a newborn child that died 1.5 hours after birth due to severe breathing difficulties [Cullis and Davis, 2018; Macaulay, 1754]. The Italian anatomist Giovanni Battista Morgagni differentiated in his monograph from 1761 various subtypes of CDH, including the anterior defect that bears his name [Zani and Cozzi, 2008; Morgagni, 1761]. In 1819, the French physician René Laennec demonstrated that the diagnosis of CDH could easily be made by chest auscultation and also suggested that laparotomy might be the correct approach for hernia repair [Laennec, 1819]. Sir Astley Paston Cooper published in 1827 the first comprehensive report on classification, symptoms and pathology of CDH [Cooper, 1827]. The first cohort series of patients with CDH was collected by Henry Bowditch in 1847 at the Massachusetts General Hospital in Boston, emphasizing the clinical criteria for diagnosis [Bowditch, 1853]. In 1848, the Czech anatomist Vincent Alexander Bochdalek accurately described a posterolateral defect in the diaphragm, which carries his name today [Bochdalek, 1848]. However, his understanding of the embryological pathogenesis of CDH was incorrect as he speculated that the hernia resulted from an intrauterine rupture of the membrane in the lumbocostal triangle. The Swedish surgeon Gustaf Naumann proposed in 1888 a 2-cavity approach after unsuccessfully operating on a 19-year-old patient with an infarcted bowel that had herniated through a diaphragmatic defect [Naumann, 1888]. The first, but unsuccessful repair in a 3-year-old infant with CDH was published in 1889 by the American physician Joseph O'Dwyer, precisely describing a thoracic approach [O'Dwyer, 1889].

The first successful CDH operation was performed in 1902 by the German surgeon Lothar Heidenhain, who corrected a diaphragmatic defect in a 9-year-old boy with a favorable 18-year follow-up [Aue, 1920; Heidenhain, 1905]. In a comprehensive review from 1925, the American surgeon Carl Hedblom showed that 75% of untreated cases with CDH died in the newborn period, suggesting that an earlier intervention might improve survival [Hedblom, 1925]. After that, Bettman and Hess presented in 1929 the youngest patient with incarcerated CDH, who had successfully been operated on aged 3.5 months [Bettman and Hess, 1929]. Greenwald and Steiner reviewed symptoms of infants and children with CDH, concluding that this condition might not be as infrequent as it was generally believed [Greenwald and Steiner, 1929]. Surgical repair of CDH remained often unsuccessful until 1940, when Ladd and Gross reported 9 of 16 patients surviving surgery, the youngest being 40 hours old [Ladd and Gross, 1940]. Robert Gross subsequently performed in 1946 the first successful repair in a neonate with CDH less than 24 hours after birth [Gross, 1946]. In 1950, Koop and Johnson proposed a transthoracic approach as a means of closing the CDH under more direct vision [Koop and Johnson, 1952]. As the surgical expertise improved further, several innovative techniques [Holcomb Jr, 1962; Neville and Clowes Jr, 1954] were developed to address large diaphragmatic defects, including the use of pedicled abdominal muscle flaps [Simpson and Gassage, 1971; Rosenkrantz and Cotton, 1964], reverse latissimius dorsi flaps [Bianchi et al., 1983] and prosthetic patches [Geisler et al., 1977; Shaffer, 1964]. Extracorporeal membrane oxygenation (ECMO) was introduced in 1976 by Robert Bartlett and successfully applied in neonates with CDH for the management of respiratory insufficiency [German et al., 1977; Bartlett et al., 1976]. In 1990, Michael Harrison and his team performed the first successful *in utero* repair in a 24 1/2-weeks-old male fetus with severe CDH using a Gore-Tex patch [Harrison et al., 1990].

Charles Stolar and co-workers presented in 1995 the concept of “gentle” ventilation in the management of CDH, characterized by preservation of spontaneous ventilation, permissive levels of hypercapnea and avoidance of high inspiratory airway pressures. This novel approach has subsequently reduced iatrogenic lung injury from barotrauma in CDH and resulted in improved survival with decreased need for ECMO (Wung et al., 1995). The Dutch surgeons van der Zee and Bax reported in 1995 the first laparoscopic closure of a left-sided posterolateral CDH in a 6-month-old boy [van der Zee and Bax, 1995]. In order to stimulate the prenatal growth of hypoplastic lungs in fetuses with CDH, additional sophisticated strategies were developed: placement of external metal clips on the fetal trachea by means of open hysterotomy [VanderWall et al., 1997; Harrison et al., 1996] or fetoscopic neck dissection [Harrison et al., 1998] and internal tracheal occlusion with a detachable silicone balloon that was placed through a single uterine port using fetal bronchoscopy [Harrison et al., 2001]. A French team led by Francoise Becmeur published in 2001 the first thoracoscopic CDH repair in three infants [Becmeur et al., 2001]. Although there is currently insufficient evidence to recommend fetal endoscopic tracheal occlusion (FETO) as a part of routine clinical practice [Grivell et al., 2015], a few specialized fetal medicine centers in Europe, North and South America successfully perform this procedure [Persico et al., 2017; Belfort et al., 2017; Ruano et al., 2012; Dekoninck et al., 2011]. FETO has recently been reported to improve neonatal survival in CDH fetuses with severe PH compared with standard perinatal management, resulting in a survival rates of 50% to 60% [Araujo Júnior et al., 2017; Al-Maary et al., 2016; Deprest et al., 2014a]. Further results from ongoing international randomized trials are anticipated in the near future [Deprest et al., 2014b].

Historically, a CDH that was diagnosed in the newborn period was almost uniformly fatal. The plethora of scientific discoveries and technical advances has contributed to the significant reduction in mortality of infant children with CDH.

2.2 Congenital diaphragmatic hernia (CDH)

2.2.1 Definition

CDH is a prenatal defect in the integrity of the developing diaphragm that occurs during embryogenesis [Kluth et al., 1996b]. It is characterized by defective formation of the pleuroperitoneal membranes (PPMs) and/or incomplete fusion with the septum transversum (ST) [Clugston and Greer, 2007]. In humans, closure of the pleuroperitoneal canal normally takes place around week eight of gestation by formation of a primordial diaphragm structure, which eventually separates the thoracic from the abdominal cavity prior to the major period of internal organ growth [Kluth et al., 1993]. However, in the instance of CDH, a significant proportion of the diaphragm is absent [Greer, 2013]. The resulting opening allows intrathoracic herniation of the abdominal viscera, causing mediastinal displacement to the contralateral side and thus severely compromises pulmonary development [Keijzer and Puri, 2010; Rottier and Tibboel, 2005].

2.2.2 Classification

Traditionally, CDHs have been classified according to their presumed anatomical location and three different types of hernia can be distinguished [Pober, 2007]:

Posterolateral (or Bochdalek) hernia

This is the most common hernia type, comprising approximately 90-95% of all CDHs. About 80% are left-sided, 19% right-sided and 1% bilateral [Veenma et al., 2012; Clark et al., 1998]. Varying degrees of deficiency can be observed: an extremely large defect or complete absence of the hemi-diaphragm including the posterior muscle rim is called diaphragmatic agenesis [Tsang et al., 1994; Bingham, 1959].

Attempts of the CDH Study Group to more accurately categorize the severity of the defect has led to the introduction of a classification system that is based on intraoperative findings, comprising the anatomic spectrum from small defects (A), which can be repaired primarily to total diaphragmatic agenesis (D) [Tsao and Lally, 2008].

Anterior retrosternal or parasternal (or Morgagni-Larry) hernia

This hernia type is located in the most anterior portion of the diaphragm and results from a failure of the crural and sternal portions to fuse. It is usually accompanied by a hernia sac and comprises approximately 2% of all CDHs. Although some patients are asymptomatic with incidental discovery, acute or subacute pulmonary and gastrointestinal symptoms can indicate this diagnosis [Loong and Kocher, 2005].

Central (or septum transversum) hernia

This is a very rare diaphragmatic defect, which primarily involves the non-muscular or central tendinous portion of the diaphragm with presence of the entire muscular rim.

Detailed examination of 181 autopsy records of children with congenital diaphragmatic defects at Boston Children's Hospital, however, has recently demonstrated wide phenotypic variations in size, shape, location and extent of organ displacement [Ackerman et al., 2012], suggesting that a clear distinction among the different subtypes of CDH can be problematic.

2.2.3 Epidemiology

With an estimated incidence of 1 in 2,000 to 4,000 newborns, CDH is a relatively common birth defect that accounts for approximately 8% of all major congenital malformations [Stege et al., 2003; Skari et al., 2000; Langham Jr et al., 1996]. Recent population-based cohort studies from the USA [Shanmugam et al., 2017; Balayla and Abenhaim, 2014; Yang et al., 2006; Dott et al., 1999; Torfs et al., 1992] and Western Australia [Colvin et al., 2005] have found prevalence rates ranging between 1.93 and 3.8 cases per 10,000 total births. Similar prevalence rates were reported by European registry-based studies, currently affecting between 2.3 and 2.7 cases per 10,000 births [McGivern et al., 2015; Loane et al., 2011; Gallot et al., 2007]. However, the incidence of CDH in stillborns and therapeutic abortions seems to be less well documented. It can be estimated that roughly one third of all infants with CDH are stillborn, which is mainly a result of the associated fatal congenital anomalies [Stolar and Dillon, 2012] and adds a “hidden mortality” to the operative and postoperative deaths [Harrison et al., 1978]. A Swedish cohort study has observed an increasing number of terminations of CDH pregnancies of up to 23% [Burgos and Frenckner, 2017], which further supports the theory that the current mortality rate actually remained unchanged since the 1990s [Stege et al, 2003]. Overall, the true incidence of CDH is most likely considerably higher than seen in the neonatal surgical practice [Brownlee et al., 2009, Mah et al., 2009]. When stillborns are counted with live births, females appear to be more commonly affected than males [Stolar and Dillon 2012].

2.3 Normal embryological development of the diaphragm

Due to the large number of complex spatiotemporal processes involving multiple cellular and tissue interactions, normal embryological development of the diaphragm remains incompletely understood [Merrell and Kardon, 2013]. In humans, the diaphragm starts to develop at approximately four weeks of gestation, which is equivalent to embryonic day 12.5 in rats [Kluth et al., 1993]. Based merely on historic post mortem examinations of perinatal human specimens [Wells, 1954], it was believed over decades that the fully developed, dome-shaped, musculotendinous diaphragm is a composite structure that derives from the following four distinct embryonical components:

2.3.1 Septum transversum

The anterior central tendon forms from an infolding of the ventrolateral body wall also known as ST, which is first seen as a thick mesodermal plate cranial to the pericardial cavity between the base of the thoracic cavity and the stalk of the yolk sac. The ST does not separate the thoracic and abdominal cavities entirely, but after the head folds ventrally around week four of gestation, it becomes a thick incomplete partition between the cavities with an opening on each side. Closure of these paired pericardioperitoneal canals normally occurs by week eight of gestation through fusion with the caudal PPMs and the primitive mediastinal mesenchyme ventral to the esophagus. Hereby, the right side usually closes before the left side [Moore et al., 2015; Wells, 1954].

2.3.2 Pleuroperitoneal membranes

These membranes arise from an infolding of the posterolateral body wall. Although they constitute large portions within the early fetal diaphragm, they represent only the relatively small dorsolateral portions of the fully developed structure. The PPMs merge with the dorsal mesentery of the esophagus and

with the dorsal part of the ST, which completes the partition between the thoracic and abdominal cavities and forms the primordial diaphragm [Moore et al., 2015; Wells, 1954].

2.3.3 Dorsal mesentery of the esophagus (or Mesoesophagus)

The ST and the PPMs fuse with the dorsal mesentery of the esophagus and eventually form the median portion of the diaphragm. The crura of the diaphragm, a leg-like pair of diverging muscle bundles that cross in the median plane anterior to the aorta, evolve from myoblasts that grow into the dorsal esophageal mesentery [Moore et al., 2015; Wells, 1954].

2.3.4 Muscular ingrowth from the lateral body walls

Between gestational week 9 and 12, the lungs and pleural cavities enlarge significantly, thus invading into the lateral body walls. During this process, the body wall tissue splits into an internal layer that contributes to the peripheral parts of the diaphragm and subsequently forms its muscular portion [Moore et al., 2015; Wells, 1954].

The advent of labeling techniques for developmentally regulated molecules and the use of transgenic mouse models has made it possible to systematically elucidate multiple stages of diaphragm myogenesis during normal diaphragmatic development and in CDH models [Greer, 2013; Babiuk et al., 2003; Greer et al., 1999]. Hereby, the mesenchymal-derived pleuroperitoneal fold (PPF) has recently been identified as a key structure [Sefton et al., 2018; Merrell et al., 2015; Clugston et al., 2010a; Greer et al., 2000; Allan and Greer, 1997]. However, it remains controversial if the muscle precursor cells and phrenic axons, which originate from the PPF, form the diaphragm alone or how much the posthepatic mesenchymal plate contributes to the closure of the pleuroperitoneal canal [Mayer et al., 2011; Babiuk and Greer, 2002].

2.4 Normal prenatal development of the lung

Although starting from different pathways and anlages, diaphragm and lung morphogenesis are interrelated [Kays, 2006]. In humans, normal airway formation begins around week four of gestation with outgrowth of two small buds of endodermally derived foregut cells and can be divided into five overlapping stages: embryonic, pseudoglandular, canalicular, saccular and alveolar [Smith et al., 2010; Rottier and Tibboel, 2005]. During these individual phases, which each involve coordinated growth and differentiation of the epithelial and mesenchymal components of the immature lung, the pulmonary system develops by dichotomous branching from two primitive endodermal buds to a functional organ with a large surface area and highly differentiated alveoli [Morrisey and Hogan, 2010]:

2.4.1 Embryonic stage

This is the stage of actual organogenesis. The embryonic lung and trachea originate from the caudal end of the laryngotracheal diverticulum by formation of an initial respiratory bud, which divides in humans at the end of the fourth week into two outpouchings [Burri, 1984]. These primary bronchial buds enlarge early in week five to form a primordial main bronchus and by the end of the sixth week they have grown laterally into the pericardioperitoneal canals as defined lobar and segmental portions of the airway tree [Burri, 1984]. At seven weeks, subsegmental branching is evident, which is driven by signals from the surrounding mesenchyme [Alescio and Cassini, 1962].

2.4.2 Pseudoglandular stage

During this stage, cellular airway differentiation begins in a proximodistal pattern [Merkus et al., 1996]. Epithelial tubes lined with cuboidal epithelial cells undergo peripheral branching and lengthening, thus resembling an

exocrine gland with marked widening of distal airspaces at the expense of intervening mesenchyme. By 16 weeks, the branching morphogenesis is completed and 20 generations of conducting airways are laid down [Kitaoka H et al., 1996]. However, this fluid-filled primitive respiratory tree structure is too immature to support efficient gas exchange.

2.4.3 Canalicular stage

This period partially overlaps the pseudoglandular stage because cranial parts of the lung mature faster than caudal ones. Bronchi and terminal bronchioles further expand in diameter and length, which is accompanied by vascularization along the airways and multiplication of capillaries [deMello et al., 1997]. At 24 weeks, each terminal bronchiole has given rise to two or more respiratory bronchioles, each of which has divided into three to six crude alveolar air sacs [Burri, 1984]. Histologically, airway epithelial cells are differentiated into peripheral squamous cells and proximal cuboidal cells.

2.4.4 Saccular stage

During this period, many more alveolar sacs develop with substantial thinning of the interstitium during the terminal saccular stage. This results from apoptosis as well as ongoing differentiation of mesenchymal cells [Hashimoto et al., 2002]. In addition, the capillary network proliferates rapidly and begins to sprout into the mesenchyme around these sacs. By 26 weeks, the terminal sacs are mainly lined with differentiated squamous epithelial cells, called type 1 pneumocytes [Mercurio and Rhodin, 1976], and scattered precursors of AECII [Otto-Verberne et al., 1988], which ultimately become responsible for surfactant production.

2.4.5 Alveolar stage

At the beginning of the alveolar stage, each respiratory bronchiole terminates in a cluster of thin-walled alveolar sacs, separated from one another by loose connective tissue. Sacs analogous to primordial alveoli appear at 32 weeks and represent future alveolar ducts [Burri, 1984]. The epithelial lining of these sacs attenuates to a thin squamous epithelial layer and type I pneumocytes become so thin that the adjacent capillaries bulge into the alveolar airspaces. By the late fetal period, true alveoli are present and the lung is capable of respiration because the alveolocapillary membrane is thin enough to allow sufficient gas exchange with mature AECII secreting pulmonary surfactant [Merkus et al., 1996].

2.5 Pathogenetical and -physiological aspects of CDH

The etiology of the diaphragmatic defect is identified in less than 50% of patients with CDH, although numerous chromosomal aberrations and gene mutations have been linked to this congenital malformation [Slavotinek, 2014; Longoni et al., 2014; Wynn et al., 2014]. From a surgical perspective, it is relatively easy to repair the defect in the diaphragm either by primary closure or reconstruction using a patch, but the main problem remains the associated disturbed lung development, resulting in severe PH and PPHN [Ameis et al., 2017; Harting, 2017]. Both conditions occur to a variable extent in patients with CDH and due to the absence of sufficient lung-protective strategies, most of the newer treatment modalities such as exogenous surfactant, inhaled nitric oxide, high-frequency oscillation and ECMO have replaced high mortality rates with significant long-term morbidity in survivors, including bronchopulmonary dysplasia, chronic lung disease, gastroesophageal reflux, scoliosis and various neurodevelopmental deficits [Puligandla et al., 2015; Kotecha et al., 2012; van den Hout et al., 2011; Antonoff et al., 2011; Tsao and Lally, 2008]. Prenatally diagnosed CDH is often associated with larger defect sizes compared to those with a postnatal diagnosis, and consequently have higher morbidity and mortality [Burgos et al., 2018]. Moreover, a significant hidden mortality exists in CDH due to termination of pregnancy and intrauterine fetal demise, with an overall mortality rate of 45% in a recent population-based study from Sweden [Burgos and Frenckner, 2017]. Therefore, CDH represents one of the major therapeutic challenges not only in modern neonatal intensive care units, but also for other specialties involved [Danzer and Hedrick, 2014; Tovar, 2012].

In the past, it has been assumed that the different parts of the diaphragm fail to fuse properly [Broman, 1901]. Consequently, the pleuroperitoneal canal does not close and PH was believed to be the result of mechanical compression by herniation of abdominal viscera into the thoracic cavity [Wells, 1954]. It has also been suggested that a primary disturbance of the pulmonary development might negatively influence the formation of the fetal diaphragm, thereby causing CDH [Iritani, 1984].

However, it has been demonstrated by combining teratogenic and transgenic animal models that these hypotheses are not true [Guilbert et al., 2000].

Moreover, *Fgf10* knockout mice do not have any lungs but show normal diaphragmatic development [Sekine et al., 1999]. The so-called "*dual-hit hypothesis*" implicates a primary disruption in bilateral lung development before closure of the diaphragm combined with a second ipsilateral insult caused by intrathoracic herniation and thus interference with fetal breathing movements [Keijzer et al., 2000; Jesudason et al., 2000]. Recently, a proliferative abnormality of the PPF has been postulated for the insufficient formation of the diaphragm [Clugston et al., 2010a; Greer et al., 2000; Allan and Greer, 1997]. Surprisingly, the origin of the diaphragmatic defect appears to lie in the mesenchymal component of the PPF, supporting the hypothesis that CDH occurs independently of myogenesis and lung formation [Babiuk RP 2002]. Likewise, Gata4 mosaic mutations in PPF-derived muscle connective tissue fibroblasts result in the development of localized amuscular regions that are biomechanically weaker and more compliant, leading to CDH [Merrell et al., 2015]. Also, the recruitment of muscle progenitors from cervical somites to the nascent PPFs is uniquely mammalian and a key developmental innovation essential for the evolution of the muscularized diaphragm [Sefton et al., 2018]. Regardless of all these theories, the exact pathogenesis of CDH and associated pulmonary malformation remains unclear.

Hypoplastic lungs in CDH are characterized by immaturity and smaller size with a significantly decreased number of terminal airway generations, thickened alveolar walls, increased interstitial tissue, diminished alveolar airspaces and reduced gas-exchange surface area [Sabharwal et al., 2000; Brandsma et al., 1994]. In addition, abnormal differentiation and malfunctioning of AECII have been reported in a rat model of CDH-associated PH, which in turn leads to surfactant deficiency [Brandsma et al., 1994]. Yet, controversy remains whether surfactant synthesis and maturation are also disrupted in human neonates with CDH [Janssen et al., 2009; Boucherat et al., 2007; Janssen et al., 2003]. Nevertheless, data from the CDH Study Group has suggested that surfactant replacement for newborns

with diaphragmatic defects does not actually provide significant outcome benefits in respect to survival rate, length of ECMO course, length of intubation, or subsequent need for supplemental oxygen [Colby et al., 2004; Van Meurs, 2004; Lally et al., 2004].

Apart from the gas-exchange layer, well-documented changes are present in the vascular components consisting of arterial media hyperplasia, peripheral muscularization of smaller pre-acinar vessels and adventitial thickening [Sluiter et al., 2011; Taira et al., 1998; Yamataka and Puri, 1997].

2.6 Animal models of CDH

Most of our current knowledge about the structural and morphological changes in hypoplastic lungs associated with CDH has originated from experimental animal models, in which the diaphragmatic defect is either surgically, transgenically or toxicologically created [Chiu, 2014; van Loenhout et al., 2009; Clugston et al., 2006; Mortell et al., 2006].

2.6.1 Surgical models

Surgically created diaphragmatic defects in fetal lambs and rabbits are useful for investigating interventional treatment strategies such as *in utero* repair and tracheal occlusion [Jelin et al., 2011; Lipsett et al., 2000; De Paepe et al., 1999; Hedrick et al., 1994], but are less helpful in studying the earlier pathogenesis of PH in CDH as well as being more expensive and time-consuming compared to rodent models.

2.6.2 Genetic models

Over the last two decades, several knockout models for genes involved in embryonic mouse development, such as for *Wt1* [Moore et al., 1998; Kreidberg et al., 1993], *Shh* [Pepicelli et al., 1998], *Gli2*/*Gli3* [Motoyama et al., 1998], *Slit3* [Liu et al., 2003; Yuan et al., 2003], *Fog2* [Ackerman et al., 2005], *Gata4*/*Gata6* [Merrell et al., 2015; Jay et al., 2007; Molkentin, 2000], *Coup-TFII* [You et al., 2005], *Pdgfrα* [Bleyl et al., 2007], *Kif7* [Coles and Ackerman, 2013], *Lox* [Hornstra et al., 2003; Mäki et al., 2002], *RARs* [Mendelsohn et al., 1994], *Robo1* [Xian et al., 2001] and *Sox7* [Wat et al., 2012] have been established, which show various phenotypes of CDH and disruption in lung branching morphogenesis. However, until now only the *Fog2* gene mutation has been identified in a patient with non-syndromic CDH [Bleyl et al., 2007].

2.6.3 Nitrofen model

Administration of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) to pregnant rodents has been found to cause different developmental anomalies in heart, lung and diaphragm [Ambrose et al., 1971]. Maternal exposure during midgestation results in CDH in approximately 70% and PH in 100% of the offspring [Noble et al., 2007]. Therefore, the toxicologically introduced nitrofen model has widely been used to investigate the CDH-associated anomalies, as the timing of the diaphragmatic insult and bilateral PH are remarkably similar to the human situation [van Loenhout et al., 2009; Beurskens et al., 2007]. Nevertheless, the significance of these potential teratogenic effects has never been proven in humans so far.

Although none of the available animal models for CDH are perfect in mimicking the human diaphragmatic defect, they all have provided new insights into the underlying pathogenesis and associated pathophysiological alterations in pulmonary development.

2.7 Involvement of all-*trans* retinoic acid (ATRA) and retinoid signaling during fetal lung development

ATRA, one of the most biologically active metabolites of vitamin A, is an essential component of the complex gene network that regulates growth of several organ systems, including lung morphogenesis and formation of the diaphragm [Ross et al., 2000]. During fetal lung development, retinoids are crucial in each of the developmental stages [Montedonico et al., 2008b]. ATRA is known to be critically involved in the saccular phase through stimulation of AECII proliferation [Nabeyrat et al., 1998]. A previous research study has reported a 50% increase in the number of pulmonary alveoli in newborn rats after treatment with ATRA, suggesting an important role of retinoids during alveolarization [Massaro and Massaro, 1996]. Recent findings from animal experiments have revealed that disruption of retinoid signaling contributes to the formation of CDH and associated lung hypoplasia [Clugston et al., 2010b; Greer et al., 2003]. Furthermore, it has been demonstrated that prenatal administration of ATRA during late gestation upregulates pulmonary expression of several genes involved in the retinoid signaling pathway [Doi et al., 2009]. It has also been shown that ATRA reduces the severity of PH in nitrofen-induced hypoplastic lung explants [Montedonico et al., 2006] and rescues PH in calorie-restricted developing rat lungs [Londhe et al., 2013]. Additional evidence that prenatal administration of ATRA stimulates alveolarization in hypoplastic lungs has been provided by *in vivo* studies in rats with nitrofen-induced CDH [Montedonico et al., 2008a], indicating that ATRA may have a therapeutic potential in attenuating CDH-associated PH. However, further research is needed to establish the exact molecular and cellular effects of ATRA treatment on fetal alveolar development.

2.8 Lipid-containing interstitial fibroblasts (LIFs) and their role in fetal lung development

LIFs, also known as lipofibroblasts, because of their characteristic lipid inclusions, play an essential role in fetal lung development by inducing alveolar epithelial cell proliferation, growth and differentiation [McGowan and Torday, 1997]. Pulmonary LIFs, which are mainly expressed in the alveolar interstitium in close contact to AECII, have been shown to actively absorb, store and transport neutral lipids [McGowan and Torday, 1997]. Due to this capability, they are not only critically involved in the regulation of alveolar lipid homeostasis and differentiation of adjacent AECII, but also in the *de novo* production of surfactant phospholipids by supplying one of the key components for their synthesis [Torday et al., 2003; McGowan and Torday, 1997; Nunez and Torday, 1995]. Moreover, it has been revealed that proliferation of AECII and associated formation of alveoli depends on the amount of ATRA in LIFs [Dirami et al., 2004; Okabe et al., 1984]. In rodents, LIFs are first evident during the late canalicular stage of lung development with a significant increase over the last few days of gestation [Torday et al., 1995; Tordet et al., 1981]. Thy-1 is a highly expressed cell surface protein in this specific subset of pulmonary fibroblasts, which are important for fetal alveolarization and alveolar maturation [Varisco et al., 2012]. The expression of Thy-1 has been found to increase continuously during the neonatal period, coinciding with the onset of alveolar formation [Varisco et al., 2012]. Interestingly, *Thy-1*$^{-/-}$ mice exhibit impaired alveolar development and reduced proliferation of pulmonary LIFs, which results in a PH-similar phenotype [Nicola et al., 2009]. It has also been reported that Thy-1 has the ability to enhance the lipid content of LIFs by upregulation of Adrp [McGowan and Torday, 1997]. Adrp is in turn necessary for the alveolar lipid homeostasis and associated pulmonary surfactant production by mediating the intracellular uptake of neutral lipids into LIFs and their subsequent transport to AECII [Schultz et al., 2002]. This functional lipogenic molecular marker, which clearly characterizes pulmonary LIFs, shows a significant increase in expression immediately before birth [Brasaemle et al., 1997].

The paracrine stimulation of alveolar epithelial cell maturation also requires the expression of Lep by LIFs, a 16 kDa peptide product of the *ob* gene and its corresponding receptor Lep-R on AECII [Torday JS 2002]. Lep and Lep-R are strongly expressed by these two types of distal alveolar cells during the saccular stage of fetal lung development with a 7- to 10-fold increase prior to term [Henson et al., 2004; Torday et al., 2002]. *Lep*-deficient mice showed decreased alveolarization with reduced pulmonary surfactant phospholipid synthesis [Tankersley et al., 1996], similar to human and nitrofen-induced PH. **Figure 1** displayes a schematic diagram of the relevant components in this signaling pathway. In addition, it has been demonstrated that Lep and Lep-R expression in developing lungs is regulated by retinoid signaling [McGowan et al., 1995], suggesting a therapeutic potential of ATRA in attenuating CDH-associated PH by stimulating alveolar maturation through an increased expression of pulmonary LIFs and thus proliferation of AECII.

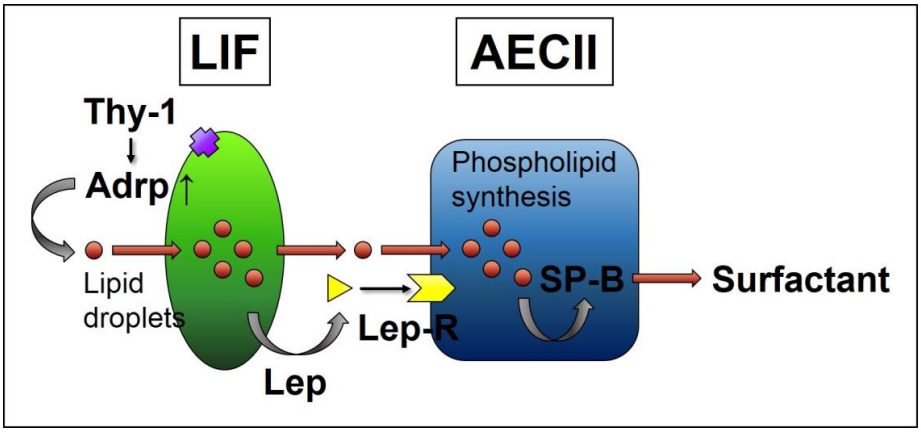

Figure 1 Schematic diagram summarizing how Thy-1 increases the lipid content of alveolar LIFs by upregulation of the lipogenic marker Adrp, which in turn stimulates the intracellular uptake of neutral lipids into LIFs. Lep and Lep-R then mediate the transport of these lipid droplets to AECII, which is necessary for phospholipid synthesis and subsequent pulmonary surfactant production.

3 Aims and objectives of the study

The overall aim of this dissertation was to investigate the underlying molecular and cellular effects of prenatally administered ATRA in alveolar LIFs and AECII and its impact on fetal lung development and surfactant synthesis in the nitrofen-induced rat model of CDH. Based on previous studies [Montedonico et al., 2008a; Montedonico et al., 2006], it can be assumed that ATRA may have the ability to enhance alveolarization in fetal rats with experimentally induced diaphragmatic defects by increasing pulmonary LIFs and thus to attenuate CDH-associated PH. In order to prove this hypothesis, *in vivo* treatment studies with maternal application of ATRA shortly before birth were performed and effects on fetal alveolar maturation were assessed by using molecular genetic, immunohistochemical/-fluorescence and stereo-/morphometric analysis techniques.

The specific objectives of this work were:

1. To identify potential disruptions of *Thy-1* signaling in hypoplastic rat lungs with nitrofen-induced CDH, which may have an adverse effect on the lipid content of pulmonary LIFs and thus form the basis for a therapeutic approach with ATRA at the end of gestation.

2. To analyze the pulmonary Adrp expression levels in control- and nitrofen-exposed fetuses, which may be accompanied by an overall reduction of LIFs in hypoplastic rat lungs with diaphragmatic defects that can potentially be reversed by ATRA.

3. To evaluate the impact of prenatal ATRA application on the expression of pulmonary LIFs in hypoplastic rat lungs with nitrofen-induced CDH, which in turn may stimulate fetal alveolarization in PH.

4. To examine if prenatally administered ATRA has the ability to upregulate *leptin* signaling in nitrofen-exposed rat fetuses with diaphragmatic defects, which may result in a significant increase of *de novo* surfactant production.

As CDH continues to be a relatively complex and rare birth defect with often unpredictable outcome, there is urgent need to foster further research activities in this field. An appreciation of CDH literature and scientific progress is therefore essential for both clinicians and basic scientists to plan future research projects. Until now, no detailed study has systematically analyzed the immense number of publications relating to CDH research and the true extent of the scientific output in this area remains unclear.

Hence, an additional objective of this work was:

5. To assess the global CDH research activity using a combination of validated scientometric methods and noval visualization techniques, which may help to establish future collaborations and thus to advance patient care.

4 Material and methods

4.1 Animal model, drugs and experimental design (I-IV)

4.1.1 Animal protocol and observational studies (I-IV)

Pathogen-free Sprague-Dawley® rats (Harlan Laboratories, Shardlow, UK) were kept in a well-controlled environment (50-55% humidity, 19-21°C, 12-h light period, food and water ad libitum). Following acclimatization, animals were mated overnight and females were checked daily for the presence of spermatozoids in the vaginal smear. The day of plugging was defined as embryonic day 0.5 (E0.5) and timed-pregnant subjects were randomly divided into two experimental groups ("Nitrofen" and "Control"). On E9.5, dams were briefly anesthetized with 2% volatile isoflurane (Piramal Healthcare Ltd, Morpeth, UK) and 100 mg nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) (Wako Chemicals GmbH, Neuss, Germany) was administered in 1 ml olive oil via oral-gastric lavage, whereas control animals received vehicle alone.

For all following studies (irrespective of the experimental design), fetuses were delivered via caesarean section under anesthesia and sacrificed by decapitation on the selected end point E21.5 (alveolar phase). After laparotomy, diaphragms were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for diaphragmatic defects and whole lungs were microdissected via thoracotomy under sterile conditions. Specimens for stereo-/morphometric, lipid and immunohistochemical/-fluorescence analysis were fixed in 4% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc, Santa Cruz, USA) diluted in phosphate buffered saline (PBS) (Oxide Ltd, Basingstoke, UK) for 24 hrs, whereas specimens for RNA isolation and subsequent quantitative real-time polymerase chain reaction (qRT-PCR) were snap-frozen in liquid nitrogen (and stored at -80°C) until further processing was carried out.

In order to obtain a representative number of samples for the observational studies, fetal lungs in each experimental group originated from at least four different dams (6-8/litter). Hence, a total of 56 specimens were used, which can be divided into Controls (n = 28) and nitrofen-induced CDH-associated PH (n = 28).

4.1.2 *In vivo* treatment studies with ATRA (III, IV)

On E18.5, control and nitrofen-treated rats were randomly assigned to one of the four treatment groups: Control+Placebo, Control+ATRA, Nitrofen+Placebo and Nitrofen+ATRA. Based on previous studies, 5 mg/kg ATRA (Sigma Aldrich, Saint Louis, USA) was dissolved in 1 ml cottonseed oil and injected intraperitoneally under short anesthesia once daily on E18.5, E19.5 and E20.5 [Montedonico et al., 2008] to achieve sufficient tissue levels in the fetuses without exposing them to toxicological dosages [Morriss-Key, 1999; Chen et al., 1995], whereas control animals received dissolvent alone. Dams were anesthetized on E21.5 and body weight of delivered fetuses was measured before their whole lungs were dissected (**Figure 2**). Moreover, all specimens were weighed before further processing was accomplished.

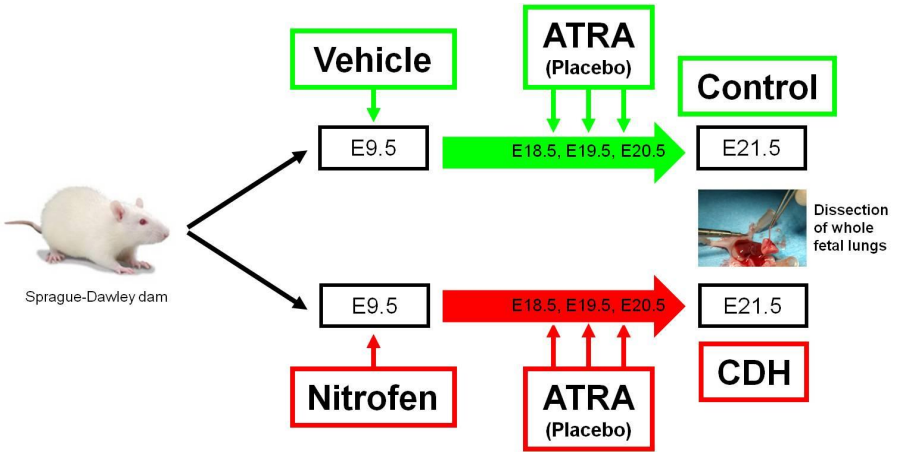

Figure 2 Experimental design for *in vivo* treatment studies with ATRA and Placebo.

Fetuses for the *in vivo* treatment studies originated from at least six different dams (4-6/litter). In total, 128 fetal lung samples were used, which can be divided into Control+Placebo (*n* = 32), Control+ATRA (*n* = 32), Nitrofen+Placebo (*n* = 32) and Nitrofen+ATRA (*n* = 32).

4.2 Preparation of fetal lung specimens for stereo-/ morphometric, lipid and immunohistochemical/-fluorescence analysis (I-IV)

The PFA-fixed lung specimens were washed overnight in ice-cold PBS to remove exterior debris, embedded in O.C.T. compound mounting medium (VWR International Ltd, Dublin, Ireland) and snap-frozen in liquid nitrogen. Frozen blocks were stored at -80°C until cryosectioning was performed. All lung specimens were cut transversely at a thickness of 10 µm using a CM1900 cryostat (Leica Microsystems GmbH, Nussloch, Germany) at -20°C and serial sections were mounted on SuperFrost® Plus microscopy glass slides (VWR International Ltd, Dublin, Ireland).

4.3 Molecular genetic analysis (I-IV)

4.3.1 Total RNA isolation (I-IV)

After thawing and homogenization, total RNA was extracted from snap-frozen lung specimens with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol® reagent (Invitrogen™ by Life Technologies™, Carlsbad, USA) according to the manufacturer's protocol. Concentration and purity of total RNA was determined with a NanoDrop ND-1000 UV-vis® spectrophotometer system (Thermo Scientific Fisher, Wilmington, USA).

4.3.2 Complementary DNA synthesis (I-IV)

Reverse transcription of 1 µg total RNA was carried out with a LightCyler® 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany) at 85°C for 3 min (denaturation), at 44°C for 60 min (annealing), and at 92°C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit® (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The resulting complementary DNA was then used for qRT-PCR.

4.3.3 Quantitative real-time polymerase chain reaction (I-IV)

Pulmonary gene expression of *Thy1*, *Adrp*, *Lep* and *Lep-R* was quantified with a LightCyler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany) using a LightCyler® 480 SYBR® Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Primer sequences for target and reference genes were designed with the online tool Primer3 (http://primer3.ut.ee) using rat nucleotide sequences from the open access GenBank® database (http://www.ncbi.nlm.nih.gov/genbank). A genomic service provider (Eurofins MWG Operon, Ebersberg, Germany) synthesized all selected primers.

Gene-specific primer sequences are listed in **Table 1**. After an initialization phase at 95°C for 5 min, 55 amplification cycles were carried out. Each cycle included an initial denaturation step at 95°C for 10 sec, an annealing step at 60°C for 15 sec and an elongation step at 72°C for 10 sec. The final elongate temperature was 65°C for 1 min. Relative mRNA expression levels were determined using the comparative cycle threshold method and results were normalized to the expression of our housekeeping gene *β-actin*. All qRT-PCR experiments were run in duplicate for each sample and primer pair.

Table 1 Gene-specific primer sequences for quantitative real-time polymerase chain reaction.

Gene	Sequence (5'-3')	Product size (bp)
Thy-1		174
Forward	TTG CCT TCT AAG CCA GAT GC	
Reverse	AGC AGC GCT CTC CTA TCT TG	
Adrp		171
Forward	CTC TCG GCA GGA TCA AAG AC	
Reverse	CGT AGC CGA CGA TTC TCT TC	
Lep		168
Forward	ATG GGA CAG CCA AAC AAA AG	
Reverse	TCC TGA GCC ATC CAG TCT CT	
Lep-R		181
Forward	TGA CAC CAA AAC CCT CAT CA	
Reverse	ATG AAG TCC AAA CCG GTG AC	
β-actin		108
Forward	TTG CTG ACA GGA TGC AGA AG	
Reverse	TAG AGC CAC CAA TCC ACA CA	

4.4 Oil red O staining (I-III)

Lipid content in pulmonary tissue was assessed by staining with oil red O (ORO). Thawed, frozen sections were immersed in 100% propylene glycol (Sigma Aldrich, Saint Louis, USA) for 5 min before ORO staining was performed. The ORO solution was prepared by slowly dissolving 0.7 g ORO powder (Sigma Aldrich, Saint Louis, USA) in 100 ml propylene glycol, while heating to 100°C for a few minutes. The resulting solution was filtered twice and cooled down before further use. Sections were immersed in ORO solution for 7 min, followed by 3 min in 85% propylene glycol. After counterstaining with hematoxylin for 30 sec, sections were coverslipped using Mowiol® (Sigma Aldrich, Saint Louis, USA) and independently evaluated by two investigators who were unaware of the respective experimental group.

4.5 Immunohistochemistry (I-IV)

4.5.1 Immunohistochemical staining (I-IV)

Localization of alveolar LIFs was ascertained by labeling for Adrp, a functional lipogenic marker protein characterizing this specific subset of lung fibroblasts and downstream target of Thy-1. The distribution of alveolar Lep and Lep-R proteins was evaluated by specific immunohistochemical staining in order to localize their exact cellular expression. Thawed frozen sections were incubated with PBS containing 1.0% Triton X-100 (Sigma Aldrich, Saint Louis, USA) for 20 min to improve cell permeabilization. In order to avoid masking of antigenic sites, sections were immersed in heated Target Retrieval Solution® (DAKO Ltd, Cambridgeshire, UK) in a microwave oven at 750 W for 15 min. Endogenous peroxidase activity was blocked using Peroxidase Block® (DAKO Ltd, Cambridgeshire, UK) according to the manufacturer's protocol for 5 min. To prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by incubation with affinity-purified rabbit polyclonal anti-Adrp (sc-32888, 1:50) (Santa Cruz Biotechnology Inc, Santa Cruz, USA), anti-Lep (ab3583, 1:100) and anti-Lep-R (ab5593, 1:250) antibodies (Abcam plc, Cambridge, UK) at 4°C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (K4011, 1:100) (DAKO Ltd, Cambridgeshire, UK) at room temperature for 30 min. The antibody-antigen complexes were then visualized by staining with diaminobenzidine (DAB) + Substrate Buffer® and DAB + Chromogen® (DAKO Ltd, Cambridgeshire, UK) for 30 sec. After counterstaining with hematoxylin (Sigma Aldrich, Saint Louis, USA) for 10 sec, sections were coverslipped using DPX Mountant for histology (Sigma Aldrich, Saint Louis, USA). All sections were independently evaluated by two investigators who were unaware of the respective experimental group with a Leica DM LB research microscope (Leica Microsystems GmbH, Wetzlar, Germany) using the image and data management software Leica IM50, version 1.20 (Leica Microsystems AG, Heerbrugg, Switzerland).

4.5.2 Immunofluorescence double staining (I-III)

The distribution of pulmonary LIFs was evaluated by immunofluorescence double staining for alpha smooth muscle actin (αSMA), which is known to be absent in this specific subset of lung fibroblasts, and lipid droplets. In order to localize Thy-1 expression and to determine lipid content in alveolar LIFs, immunoflurescence double staining with specific Thy-1 antibodies and ORO was performed. Thawed frozen sections were blocked with 10% normal goat serum for 30 min, followed by incubation with either affinity-purified mouse anti-αSMA (M0851, 1:500) (DAKO Ltd, Cambridgeshire, UK) or anti-Thy-1 (ab225, 1:100) (Abcam plc, Cambridge, UK) antibodies and ORO solution at 4°C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with Alexa Fluor® 488 goat anti-mouse secondary antibodies (A11029, 1:100) (Bio-Sciences Ltd, Dun Laoghaire, Ireland) for 30 min. The sections were counterstained with DAPI (10236276001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min to visualize double-stranded DNA. Following coverslipping with fluorescent mounting medium (DAKO Ltd, Cambridgeshire, UK), two investigators unaware of the respective experimental group independently evaluated all sections with a LSM 700 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). The acquired images were meticulously analyzed using the image processing and analysis software program ZEN (Carl Zeiss MicroImaging GmbH, Jena, Germany).

4.6 Assessment of fetal lung stereo-/morphometry (IV)

Two independent-blind investigators unaware of the experimental group performed fetal lung stereo-/morphometry, which was objectively assessed by determining radial alveolar count (RAC) and mean linear intercept (MLI) on hematoxylin- and eosin-stained (Sigma Aldrich, Saint Louis, USA) sections. Fifty randomly selected, non-overlapping fields from serial sections were investigated under a Leica DM LB research microscope (Leica Microsystems GmbH, Wetzlar, Germany). Each field was viewed at 40-fold magnification, and the image was digitized and projected on a computer screen using a Leica DC300F digital camera (Leica Microsystems AG, Heerbrugg, Switzerland). For each field, the number of alveoli was counted visually and RAC was performed by identifying respiratory bronchioles, as previously described [Randell et al., 1989]. Briefly, the number of distal air sacs that were transacted by a line drawn from a terminal respiratory bronchiole to the nearest pleural surface was counted. No counts were made if the respiratory bronchiole was nearer to the edge of the slide than to the nearest connective tissue septum. The MLI represents the average alveolar diameter, alveolar septal thickness (AST) and tissue density, which is the proportion of the field occupied by tissue (area occupied by tissue/area occupied by lung tissue + alveoli). All images were independently analyzed by two investigators who were unaware of the respective experimental group with ImageJ 1.47a (National Institute of Health, Bethesda, USA), a public domain, Java™-based image processing and analysis software program.

4.7 Determination of fetal surfactant phospholipid synthesis (IV)

Fetal surfactant phospholipid synthesis was determined by labeling for surfactant protein B (SP-B), which plays an essential role in alveolar stability and thus contributes to the biophysiological function of the lung [Weaver and Conkright, 2001; Hawgood et al., 1998]. Thawed frozen sections were incubated with PBS containing 1.0% Triton X-100 (Sigma Aldrich Ltd, Arklow, Ireland) for 20 min to improve cell permeabilization. In order to prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by incubation with affinity-purified rabbit polyclonal anti-SP-B antibodies (sc-7702-R, 1:100) (Santa Cruz Biotechnology Inc, Santa Cruz, USA) at 4°C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with Alexa Fluor® 647 goat anti-rabbit secondary antibodies (A21244, 1:200) (Bio-Sciences Ltd, Dun Laoghaire, Ireland) at room temperature for 30 min. The sections were counterstained with DAPI (10236276001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min to visualize double-stranded DNA. Following coverslipping with fluorescent mounting medium (DAKO Ltd, Cambridgeshire, UK), two investigators unaware of the respective experimental group independently evaluated the sections with a LSM 700 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). All images were analyzed with ZEN (Carl Zeiss MicroImaging GmbH, Jena, Germany), an image processing and analysis software program.

4.8 Statistical analyses (I-IV)

Data were analyzed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, USA) and tested for Gaussian distribution with a Kolmogorov-Smirnov test. All results are presented as means ± standard error of the mean (SEM). Statistical differences between two experimental groups were compared using an unpaired Student's *t* test when the data had normal distribution or a Mann-Whitney *U* test when the data deviated from normal distribution. In order to determine any statistical differences between four experimental groups, one-way ANOVA with Tukey's test for post-test analysis was performed. A P value < 0.05 was considered as statistically significant.

4.9 Ethical considerations (I-IV)

All animal procedures were carried out according to the current guidelines for management and welfare of laboratory animals. The experimental protocol was approved by the research ethics committee of the Royal College of Surgeons in Ireland (Ref. REC668b) as well as by the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005).

4.10 Information source and literature-based search (V)

In order to identify all peer-reviewed scientific publications relating to CDH research, a comprehensive search strategy was designed for the Web of Science™ database (Clarivate Analytics, Boston, USA). This online subscription-based research platform, which provides temporal coverage from the year 1900 to present, was accessed on 20 June 2017. The following linked search terms were used taking into account alternative nomenclature for CDH:"*congenital** *diaphragm** *hernia**" OR "*congenital** *diaphragm** *defect**" OR "*fetal** *diaphragm** *hernia*" OR "*pediatric** *diaphragm** *hernia**" OR "*foetal** *diaphragm** *hernia*" OR "*paediatric** *diaphragm** *hernia**" OR "*agenes** *diaphragm**" OR "*agenes** *hemidiaphragm**" OR "*Bochdalek** *hernia**" OR "*Morgagni** *hernia**". A "title" rather than "topic" search was performed to determine only the most relevant research items. No language restrictions were imposed. Results from 2017 were excluded from the search to ensure complete data acquisition because the incorporation of several parameters into the database requires a certain time.

4.10.1 Selection categories and data analysis (V)

The retrieved data on CDH-related publications was critically evaluated and classified with regard to subject category, document type, journal title, publication date and language. Total research output of countries, institutions, individual authors and collaborative networks was determined and systematically analyzed. The "*citation report*" function was applied to assess semi-qualitative research aspects including citation rate and *h*-index. The *h*-index is an established metric, which incorporates a citation index and the overall scientific output of authors or institutions, thus quantifying importance, impact and significance of individual research contributions [Hirsch, 2005]. It can be calculated, if *h* of all publications received at least *h* citations each. In this study, the *h*-index has also been used to estimate to productivity of publishing countries.

The relationship of two or more authors from different countries, who contributed to a joint publication, was defined as a cooperation article. Complete bibliographic data was downloaded as *plain text* files and extracted into an electronic datasheet in a standardized manner. Choropleth mapping (i.e. differences in color values to represent geographical data) and network diagrams were employed to visualize results.

5 Results

5.1 Observational studies (I, II)

5.1.1 Relative mRNA expression levels of *Thy-1* and *Adrp* in fetal rat lungs (I, II)

As *Thy-1* and its downstream target *Adrp* are both molecular markers characterizing LIFs, pulmonary gene expression levels of *Thy-1* and *Adrp* were analyzed by qRT-PCR. Relative mRNA expression of *Thy-1* and *Adrp* was significantly downregulated in hypoplastic rat lungs with nitrofen-induced CDH on E21.5 compared to controls (**Figure 3**).

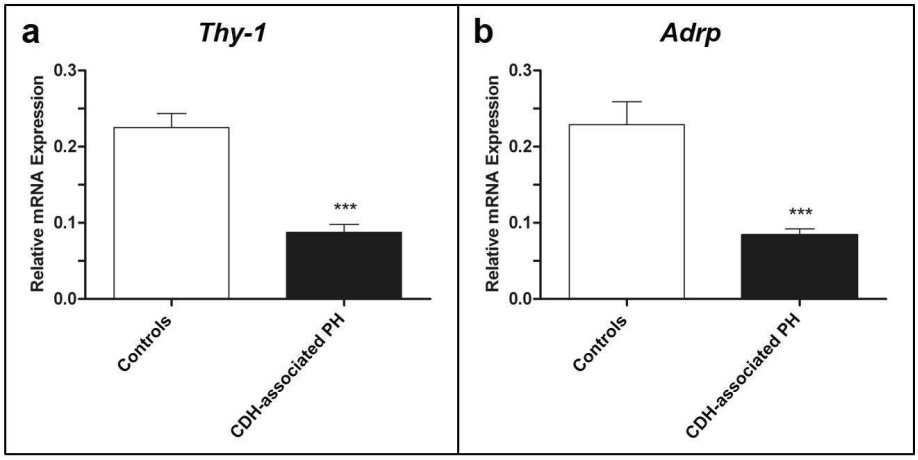

Figure 3 Relative mRNA expression of *Thy-1* (**a**) and *Adrp* (**b**) was significantly downregulated in fetal rat lungs with CDH-associated PH compared to control lungs (0.09 ± 0.02 *vs.* 0.22 ± 0.04; ***P = 0.0002 and 0.08 ± 0.01 *vs.* 0.23 ± 0.09; ***P = 0.0009, respectively).

5.1.2 Thy-1 protein expression in pulmonary LIFs of fetal rats (I)

In order to confirm the qRT-PCR results, immunoflurescence double staining with Thy-1 and ORO was performed to evaluate Thy-1 protein expression in pulmonary LIFs on E21.5. Confocal laser scanning microscopy confirmed markedly decreased Thy-1 immunoflurescence in this specific subset of alveolar fibroblasts in hypoplastic rat lungs with diaphragmatic defects compared to controls (**Figure 4**).

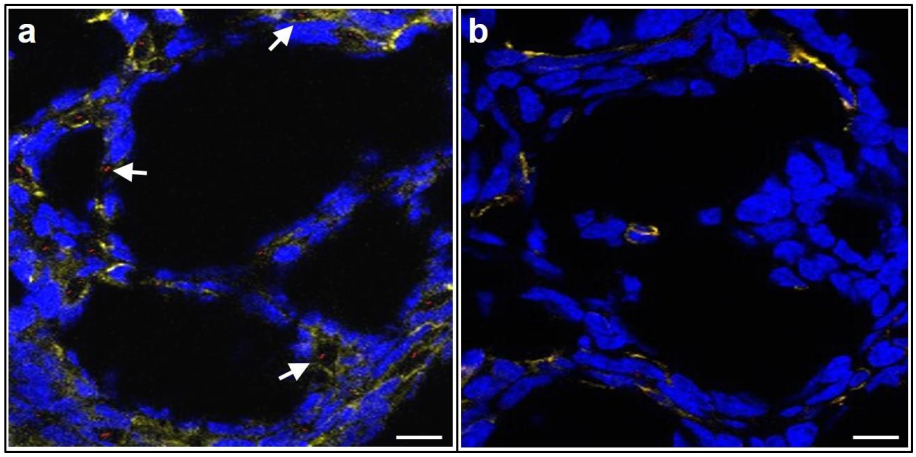

Figure 4 Thy-1 expression and lipid content in pulmonary LIFs of fetal rat lungs. Immunofluorescence double staining with Thy-1 and ORO demonstrated markedly decreased Thy-1 expression and lipid inclusions in this specific subset of alveolar fibroblasts (*arrows*) in fetal rat lungs with CDH-associated PH (**b**) compared to control lungs (**a**). Representative cryostat sections of fixed lung tissue stained with specific Thy-1 antibodies (*yellow staining*), ORO (*red staining*) and DAPI (*blue staining*) are shown (Magnification x40; Scale bars = 75 µm).

5.1.3 Adrp protein expression and cytoplasmatic lipid content in pulmonary LIFs of fetal rats (I, II)

To further validate these findings and to identify lipid inclusions in pulmonary LIFs, their expression was ascertained on E21.5 by labeling for the functional lipogenic marker protein Adrp combined with specific ORO lipid staining. Adrp immunoreactivity was strikingly diminished in alveolar interstitial cells of lungs with CDH-associated PH compared to controls, which coincided with impaired alveolar mesenchymal cell differentiation (**Figure 5**). Moreover, this was associated with fewer cytoplasmatic lipid droplets in interstitial alveolar compartments of nitrofen-exposed rat fetus with diaphragmatic defects (**Figure 6**).

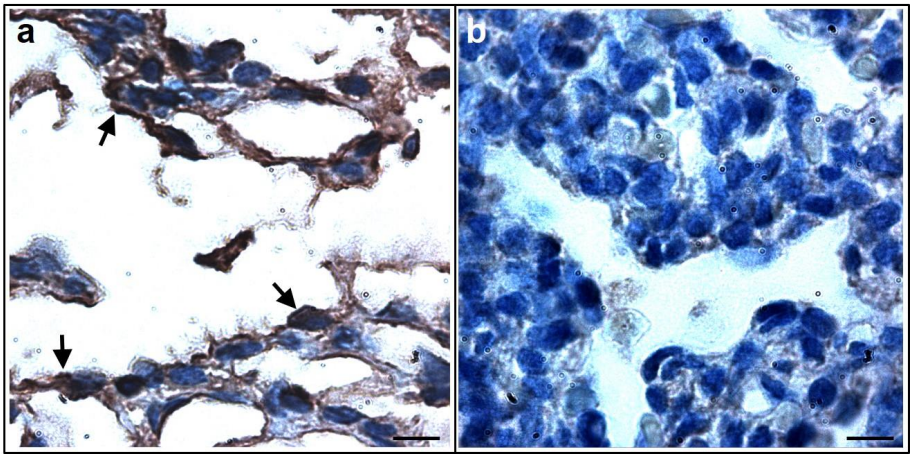

Figure 5 Adrp protein expression in fetal rat lungs. Adrp immunohistochemistry (*arrows*) was markedly diminished in alveolar interstitial cells of fetal rat lungs with CDH-associated PH (**b**) compared to control lungs (**a**), as determined by specific labeling for the functional lipogenic marker Adrp, which coincided with impaired alveolar mesenchymal cell differentiation. Representative cryostat sections of fixed lung specimens labeled with specific Adrp antibodies (*brown staining*) and hematoxylin (*blue staining*) are shown (Magnification x40; Scale bars = 50 µm).

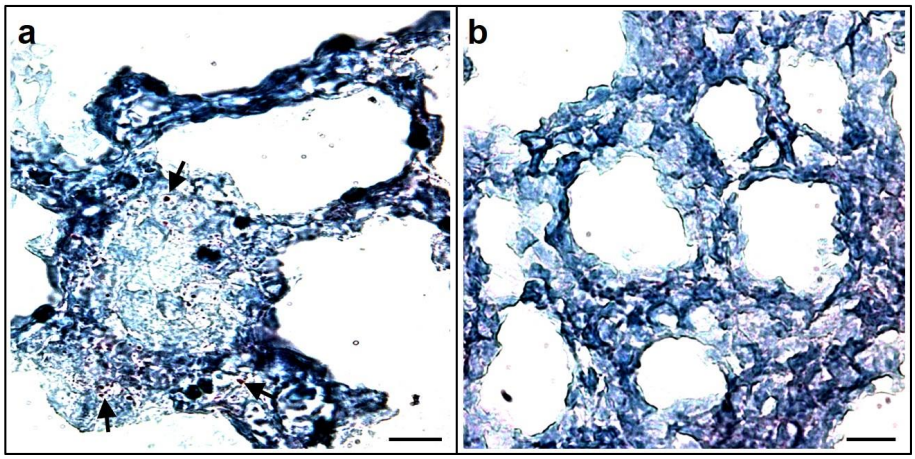

Figure 6 Cytoplasmatic lipid content in fetal rat lungs. Specific lipid staining with ORO showed markedly reduced lipid droplets (*arrows*) in the alveolar interstitium of fetal rat lungs with CDH-associated PH (**b**) compared to control lungs (**a**). Representative cryostat sections of fixed lung tissue are shown (Magnification x40; Scale bars = 50 µm).

5.1.4 Pulmonary LIF expression in fetal rat lungs (II)

Following immunofluorescence double staining, confocal laser scanning microscopy showed absence of αSMA expression as well as markedly reduced lipid inclusions in pulmonary LIFs on E21.5, thus clearly demonstrating notably decreased expression of this specific subset of fibroblasts in the alveolar interstitium of hypoplastic rat lungs with CDH compared to controls (**Figure 7**).

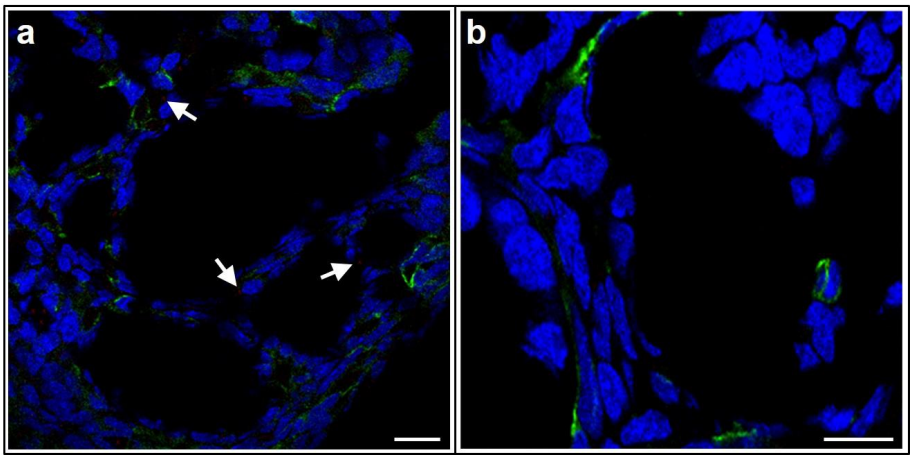

Figure 7 Pulmonary LIF expression in fetal rat lungs. Confocal laser scanning microscopy revealed absence of αSMA expression with markedly reduced lipid inclusions in pulmonary LIFs, thus demonstrating a markedly decreased expression of this specific subset of fibroblasts (*arrows*) in alveolar interstitium of CDH-associated PH (**b**) on E21.5 compared to controls (**a**). Representative cryostat sections of fixed lung specimens double stained with αSMA (*green staining*), ORO (*red staining*) and DAPI (*blue staining*) are shown (Magnification x40; Scale bars = 50 µm).

5.2 *In vivo* treatment studies with ATRA (III, IV)

5.2.1 Effect of prenatally administered ATRA on fetal lung-to-body weight ratio (IV)

As fetal body and lung weight is a reflection of overall development, the *in vivo* effect of maternal ATRA application on body and lung weight was examined in E21.5 rat fetuses. In nitrofen-exposed fetuses, there was a significant increase in lung-to-body weight ratio after prenatal administration of ATRA shortly before birth compared to the placebo group (2.14 ± 0.03% *vs.* 1.78 ± 0.05%; $P < 0.01$) (**Figure 8**). The difference in lung-to-body weight ratio between control and nitrofen-exposed fetuses that only received placebo treatment was also statistically significant (2.21 ± 0.03% *vs.* 1.78 ± 0.05%; $P < 0.0001$).

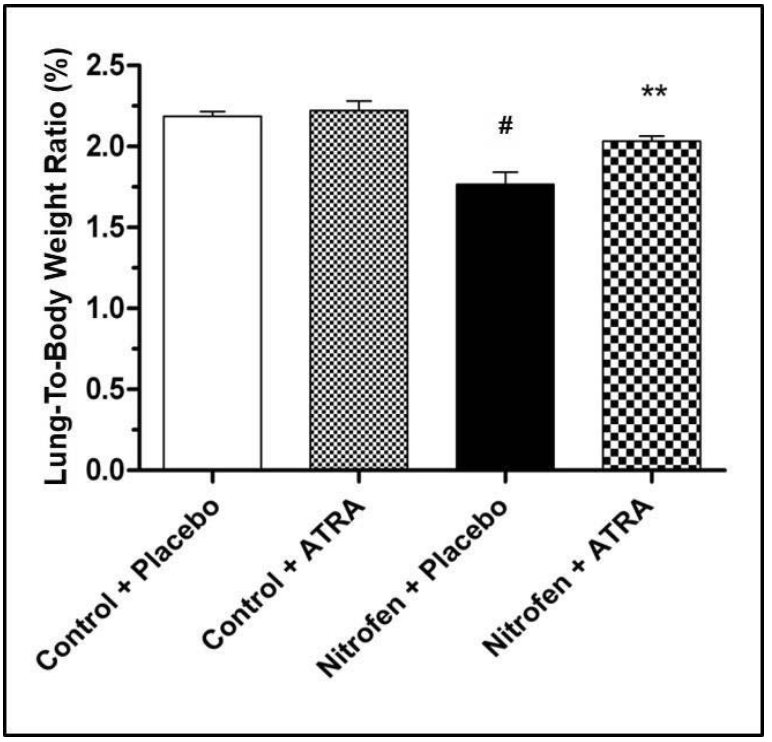

Figure 8 Effect of maternal ATRA application on fetal lung-to-body weight ratio. Prenatal administration of ATRA resulted in nitrofen-exposed fetuses in a significantly increased lung-to-body weight ratio compared to the placebo group (**$P < 0.01$, *vs.* Nitrofen+Placebo; $^{\#}P < 0.0001$, *vs.* Control+Placebo).

5.2.2 Effect of prenatally administered ATRA on pulmonary gene expression levels of *Adrp* in fetal rats (III)

As *Adrp* is a downstream target of *Thy-1* signaling and known functional lipogenic marker characterizing LIFs [Schultz et al., 2002], pulmonary gene expression levels of *Adrp* were analyzed on E21.5 by qRT-PCR. Relative mRNA expression of pulmonary *Adrp* was significantly increased in Nitrofen+ATRA compared to Nitrofen+Placebo (0.31 ± 0.02 *vs.* 0.08 ± 0.01; $P < 0.0001$), whereas there were no significant differences between Control+ATRA and Control+Placebo (0.24 ± 0.01 *vs.* 0.21 ± 0.02; $P = 0.1319$).

5.2.3 Effect of prenatally administered ATRA on alveolar protein expression of Adrp in fetal rats (III)

In order to determine whether the increased amounts of *Adrp* transcripts after prenatal treatment with ATRA were also translated to the protein level, Adrp expression and distribution were investigated on E21.5 by specific labeling with Adrp antibodies directly on pulmonary tissue sections. Light microscopy confirmed the qRT-PCR results showing markedly increased Adrp immunoreactivity mainly in distal alveolar interstitium of Nitrofen+ATRA compared to Nitrofen+Placebo, whereas there were no differences between Control+ATRA and Control+Placebo (**Figure 9**).

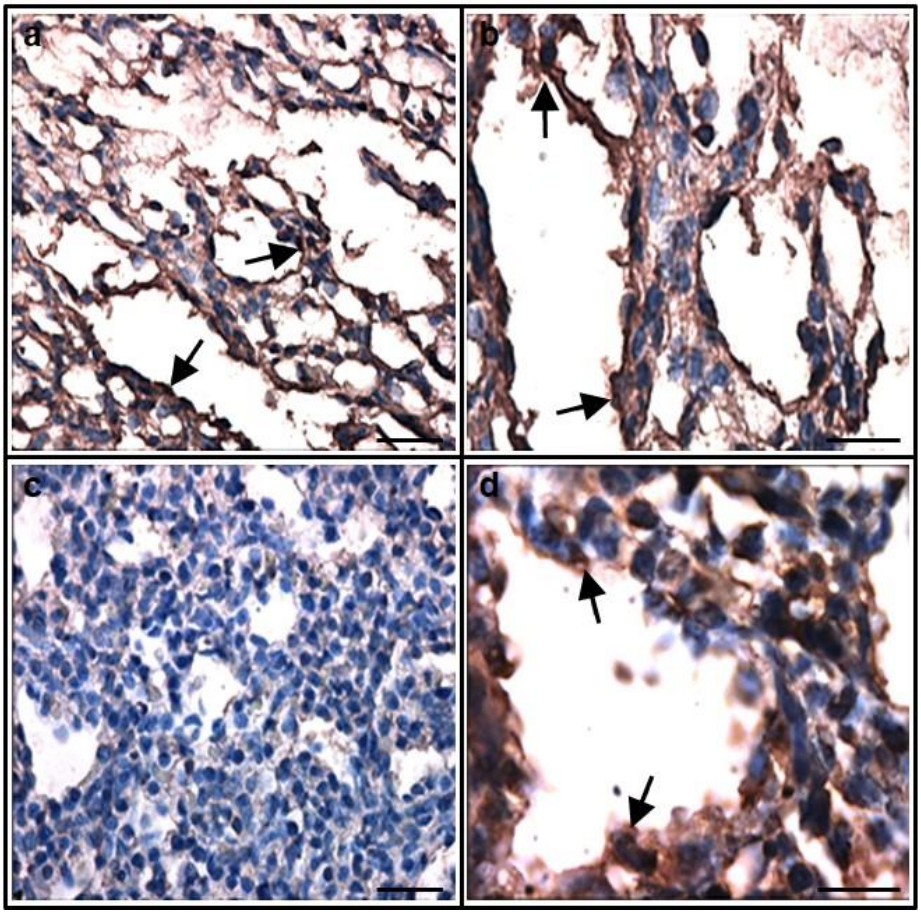

Figure 9 Effect of maternal ATRA application on alveolar protein expression of Adrp in fetal rats. Adrp immunoreactivity (*arrows*) was markedly increased in distal alveolar interstitium of Nitrofen+ATRA (**d**) compared to Nitrofen+Placebo (**c**), whereas there were no differences between Control+ATRA (**b**) and Control+Placebo (**a**). Representative cryostat sections of fixed lung tissue stained with specific Adrp antibodies (*brown staining*) and hematoxylin (*blue staining*) are shown (Magnification x40; Scale bars = 100 µm).

5.2.4 Effect of prenatally administered ATRA on lipid content and LIFs in fetal rat lungs (III)

The presence of lipid droplets, which are characteristic for pulmonary LIFs [Brasaemle et al., 1997], was evaluated by ORO staining directly on tissue sections of E21.5 rat lungs. Light microscopy indicated a notably increased expression of cytoplasmatic lipid inclusions in alveolar interstitial cells of Nitrofen+ATRA compared to Nitrofen+Placebo, whereas there were no differences between Control+ATRA and Control+Placebo (**Figure 10**).

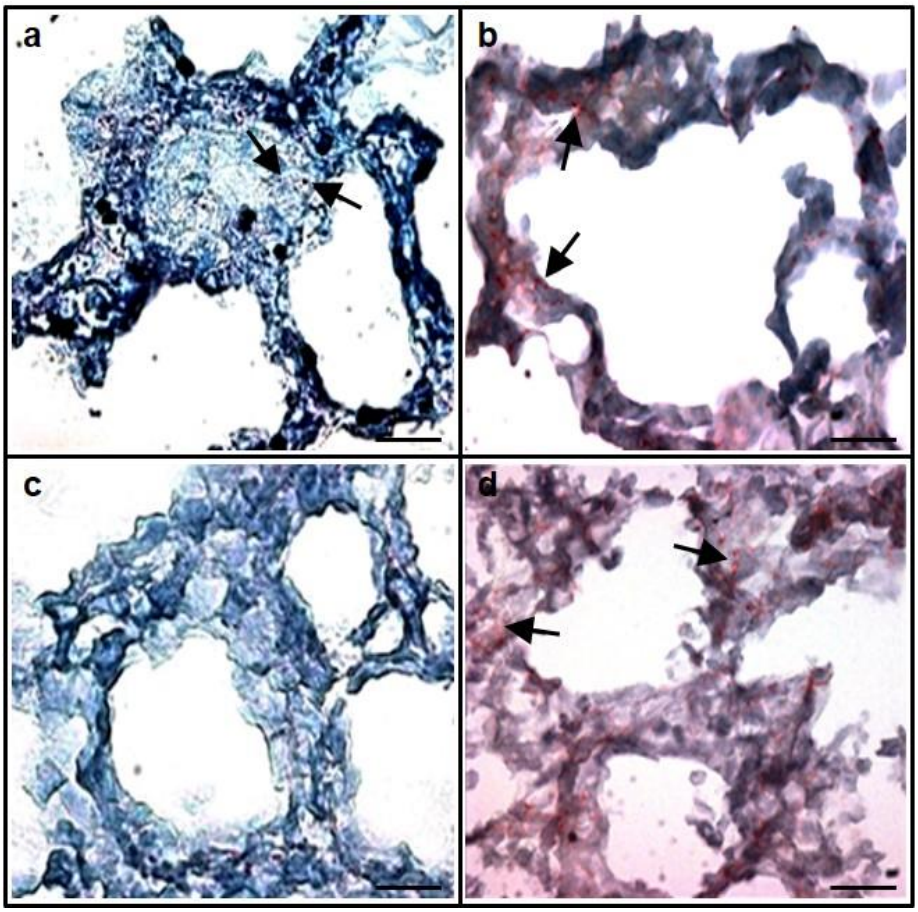

Figure 10 Effect of maternal ATRA application on lipid content in fetal rat lungs. Expression of cytoplasmatic lipid droplets (*arrows*) was markedly increased in alveolar interstitial cells of Nitrofen+ATRA (**d**) compared to Nitrofen+Placebo (**c**), whereas there were no differences between Control+ATRA (**b**) and Control+Placebo (**a**). Representative cryostat sections of fixed lung specimens stained with ORO are shown (Magnification x40; Scale bars = 50 µm).

Immunofluorescence double staining for αSMA, which is characteristically absent in LIFs [Torday et al., 2003], and ORO was used to assess pulmonary LIF expression and localization in E21.5 rat fetuses. Confocal laser scanning microscopy demonstrated markedly increased LIFs in interstitial compartments of distal alveolar walls of Nitrofen+ATRA compared to Nitrofen+Placebo, whereas there were no differences between Control+ATRA and Control+Placebo (**Figure 11**).

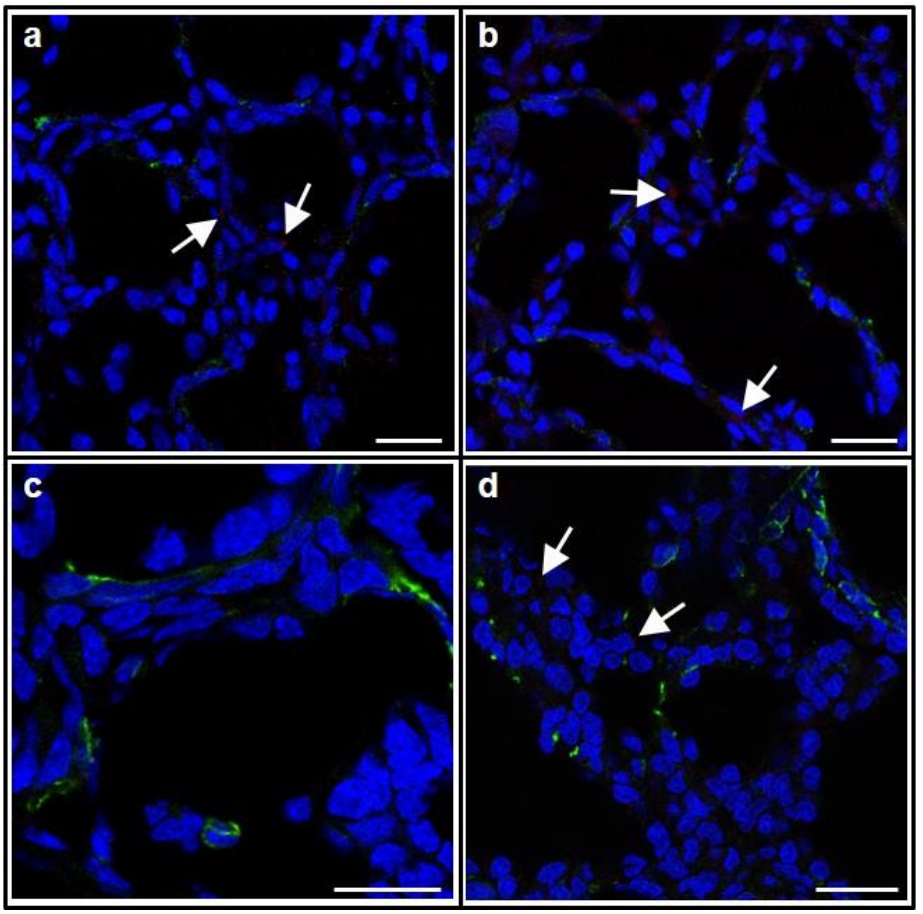

Figure 11 Effect of maternal ATRA application on pulmonary LIFs in fetal rats. The expression of this specific subset of lung fibroblasts (*arrows*) was markedly increased in interstitial compartments of distal alveolar walls of Nitrofen+ATRA (**d**) compared to Nitrofen+Placebo (**c**), whereas there were no differences between Control+ATRA (**b**) and Control+Placebo (**a**). Representative cryostat sections of fixed lung tissue double stained with αSMA (*green staining*), ORO (*red staining*) and DAPI (*blue staining*) are shown (Magnification x40; Scale bars = 75 µm).

5.2.5 Effect of prenatally administered ATRA on alveolarization in fetal rat lungs (IV)

Stereo-/Morphometric analysis of fetal rat lungs revealed a significant progression in alveolar development after prenatal administration of ATRA. Nitrofen-exposed fetuses that received ATRA application shortly before birth showed enhancement of alveolarization on E21.5 (**Figure 12**), which was expressed in a significant increase in RAC (6.66 ± 1.3 per mm^2 *vs.* 5.70 ± 1.2 per mm^2; $P < 0.0001$) and decrease in MLI (42.44 ± 1.5 µm *vs.* 45.06 ± 1.3 µm; $P < 0.0001$) compared to Nitrofen+Placebo, whereas there was no significant differences between Control+Placebo and Control+ATRA (**Figure 13**). The differences in RAC (10.05 ± 1.4 per mm^2 *vs.* 5.70 ± 1.2 per mm^2; $P < 0.0001$) and MLI (41.23 ± 1.6 µm *vs.* 45.06 ± 1.3 µm; $P < 0.0001$) between control and nitrofen-exposed fetuses that only received placebo treatment were each statistically significant.

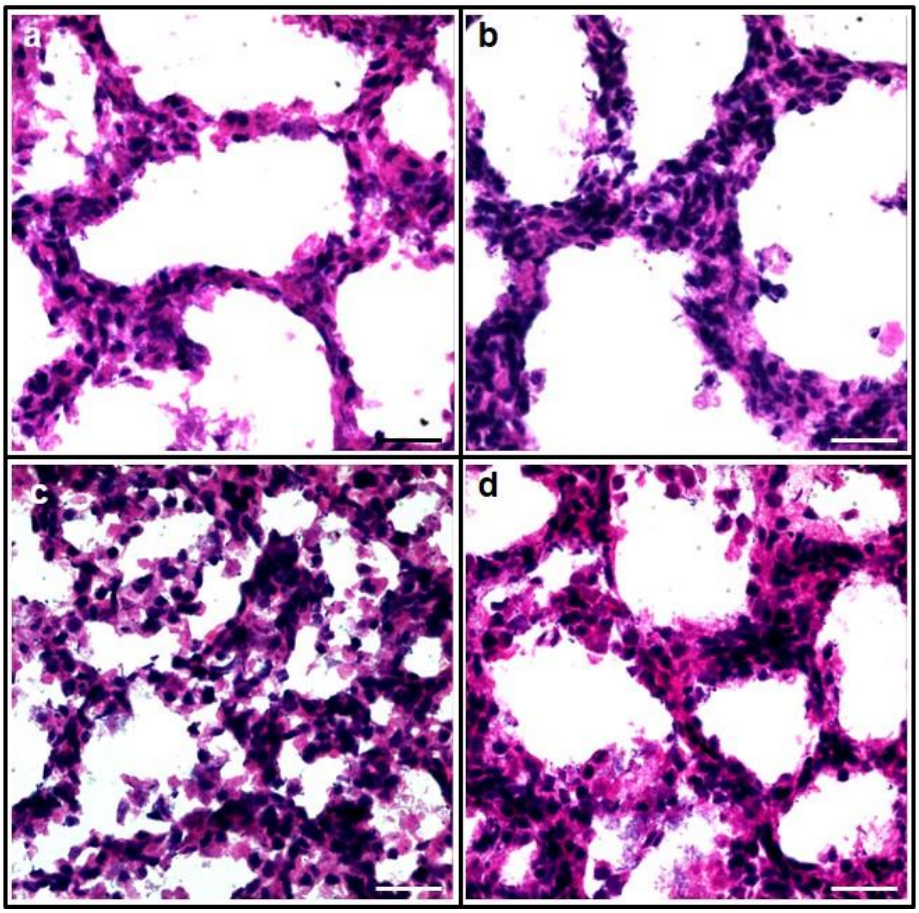

Figure 12 Effect of maternal ATRA application on alveolarization in fetal rat lungs. Prenatal administration resulted in a marked increased alveolarization in Nitrofen+ATRA (**d**) compared to Nitrofen+Placebo (**c**), whereas there were no differences between Control+ATRA (**b**) and Control+Placebo (**a**). Representative cryostat sections of fixed lung specimens stained with hematoxylin (*blue staining*) and eosin (*pink staining*) are shown (Magnification x40; Scale bars = 100 µm).

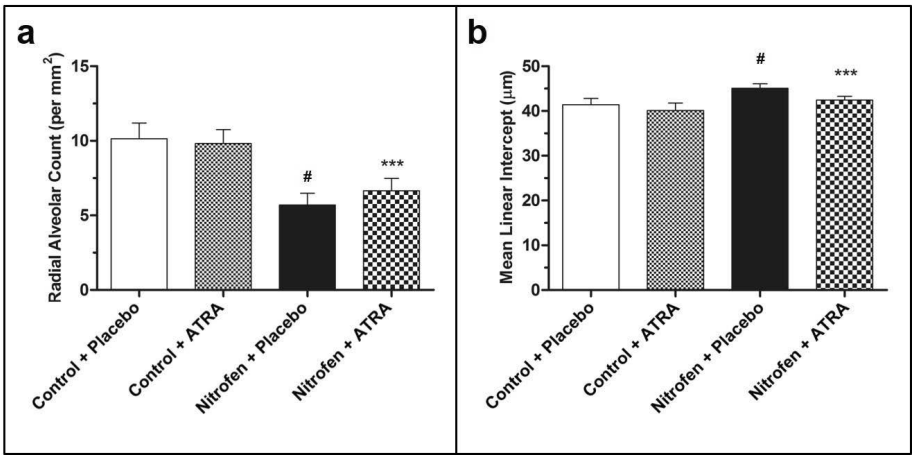

Figure 13 Effect of maternal ATRA application on lung stereo-/morphometry in fetal rats. Prenatal administration of ATRA resulted in nitrofen-exposed fetuses in a significantly increased radial alveolar count (**a**) and decreased mean linear intercept (**b**) on E21.5 compared to placebo-treated lungs (***$P < 0.0001$, *vs.* Nitrofen+Placebo; #$P < 0.0001$, *vs.* Control+Placebo).

5.2.6 Effect of prenatally administered ATRA on pulmonary gene expression levels of *Lep* and *Lep-R* in fetal rats (IV)

As *Lep* and its receptor *Lep-R* are both molecular markers characterizing alveolar maturation, pulmonary gene expression levels of *Lep* and *Lep-R* were analyzed by qRT-PCR on E21.5. Nitrofen-exposed lungs of rat fetuses that received ATRA application shortly before birth exhibited a significantly increased relative mRNA expression of *Lep* (4.65 ± 0.67 *vs.* 2.38 ± 0.67; $P < 0.05$) and *Lep-R* (1.73 ± 0.10 *vs.* 0.83 ± 0.12; $P < 0.05$) compared to Nitrofen+Placebo.

5.2.7 Effect of prenatally administered ATRA on alveolar protein expression of Lep and Lep-R in fetal rats (IV)

To further validate whether the increased amounts of pulmonary *Lep* and *Lep-R* mRNA transcripts after prenatal treatment with ATRA were also translated to the protein level, alveolar Lep and Lep-R protein expression was evaluated in fetal rat lungs on E21.5 by labeling with specific Lep and Lep-R antibodies. Light microscopy confirmed the qRT-PCR results showing markedly increased Lep and Lep-R immunoreactivity in interstitial and alveolar epithelial cells of Nitrofen+ATRA compared to Nitrofen+Placebo (**Figure 14**).

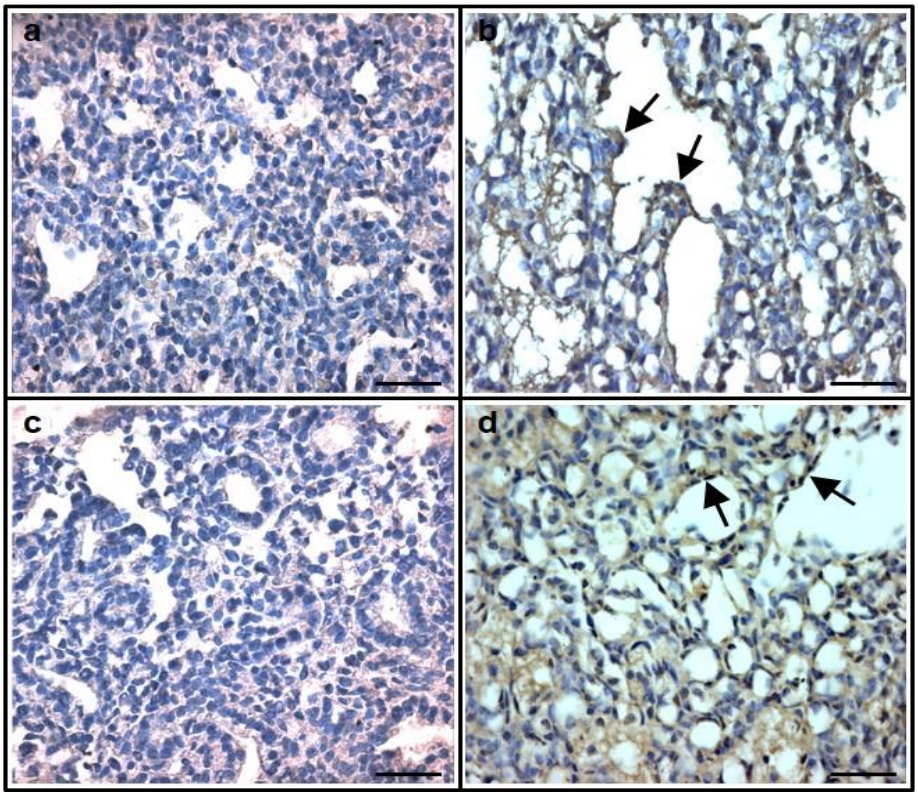

Figure 14 Effect of maternal ATRA application on alveolar protein expression of Lep and Lep-R in fetal rats. Prenatal administration of ATRA resulted in nitrofen-exposed fetuses in a markedly increased Lep (**b**) and Lep-R (**d**) immunoreactivity in interstitial and alveolar cells (*arrows*) compared to placebo-treated lungs (**a** and **c**, respectively). Representative cryostat sections of fixed lung tissue stained with specific Lep or Lep-R antibodies (*brown staining*) and hematoxylin (*blue staining*) are shown (Magnification x40; Scale bars = 100 µm).

5.2.8 Effect of prenatally administered ATRA on surfactant phospholipid synthesis in fetal rat lungs (IV)

Immunoflurescence staining with SP-B was performed to examine pulmonary surfactant production in E21.5 rat fetuses. Confocal laser scanning microscopy demonstrated notably increased alveolar SP-B protein expression (**Figure 15**) and significantly increased SP-B count (**Figure 16**) after prenatal administration of ATRA in Nitrofen+ATRA compared to Nitrofen+Placebo.

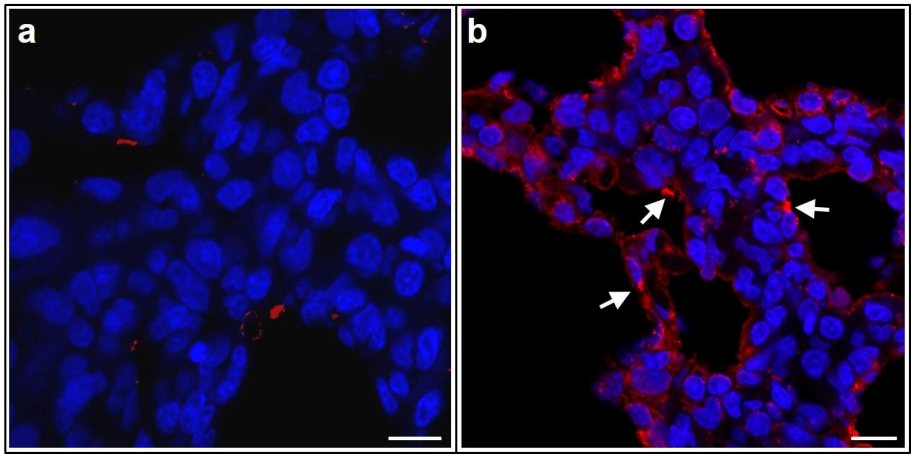

Figure 15 Effect of maternal ATRA application on surfactant phospholipid synthesis in fetal rat lungs. Prenatal administration of ATRA resulted in nitrofen-exposed fetuses (**b**) in a markedly increased alveolar SP-B staining on E21.5 compared to placebo treatment (**a**), as determined by specific labeling for the surfactant phospholipid marker SP-B. Representative cryostat sections of fixed lung specimens stained with SP-B antibodies (*red staining*) and DAPI (*blue staining*) are shown (Magnification x40; Scale bars = 50 µm).

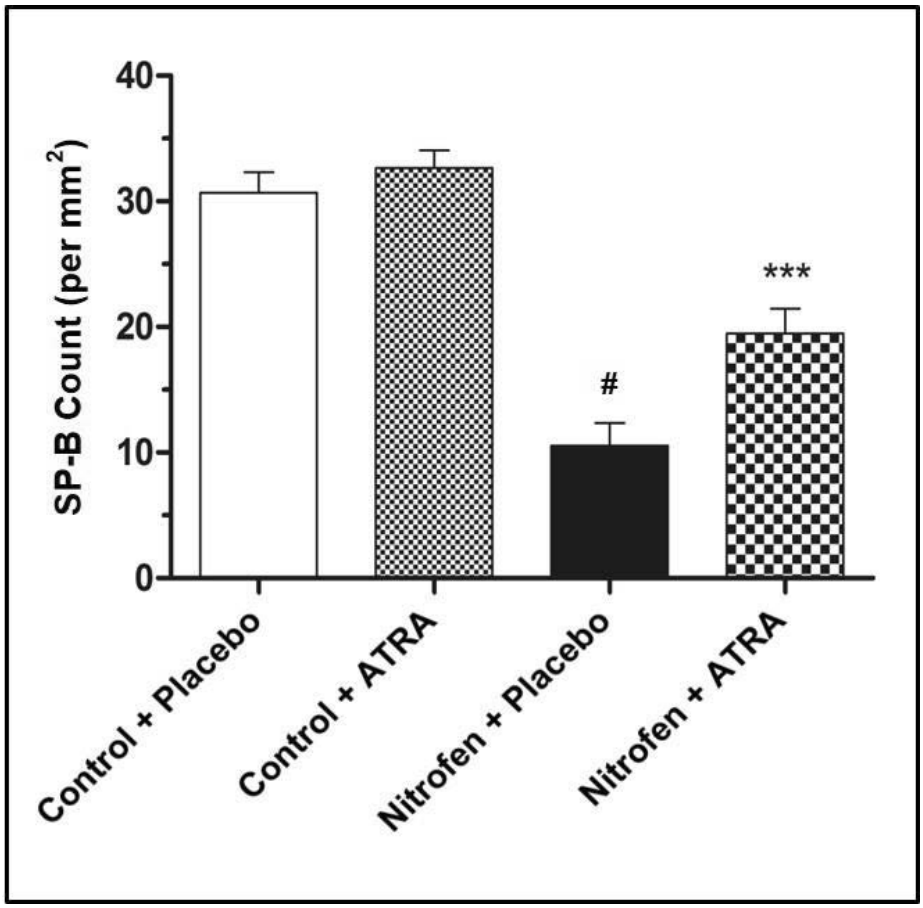

Figure 16 Effect of maternal ATRA application on SP-B count in fetal rat lungs. Prenatal administration of ATRA resulted in nitrofen-exposed fetuses in a significantly increased SP-B count compared to placebo-treated lungs (***$P < 0.0001$, *vs.* Nitrofen+Placebo; $^{\#}P < 0.0001$, *vs.* Control+Placebo).

5.3 Scientometric analysis of the global research activity (V)

5.3.1 Global publication volume (V)

A total of 3,669 publications on CDH were identified, originating from 76 countries (**Figure 17**). North America and Europe constituted the two scientific centers in the field of CDH-related research. In contrast, the vast majority of African countries had an extremely low or no scientific output on CDH. Globally, the largest number of scientific articles relating to CDH was published by the USA [*n* = 1,250; (34.1%)], the United Kingdom [*n* = 279; (7.6%)] and Canada [*n* = 215; (5.9%)]. Most CDH papers were written in English [*n* = 3,432; (93.5%)], followed by French [*n* = 87; (2.4%)] and German [*n* = 81; (2.2%)].

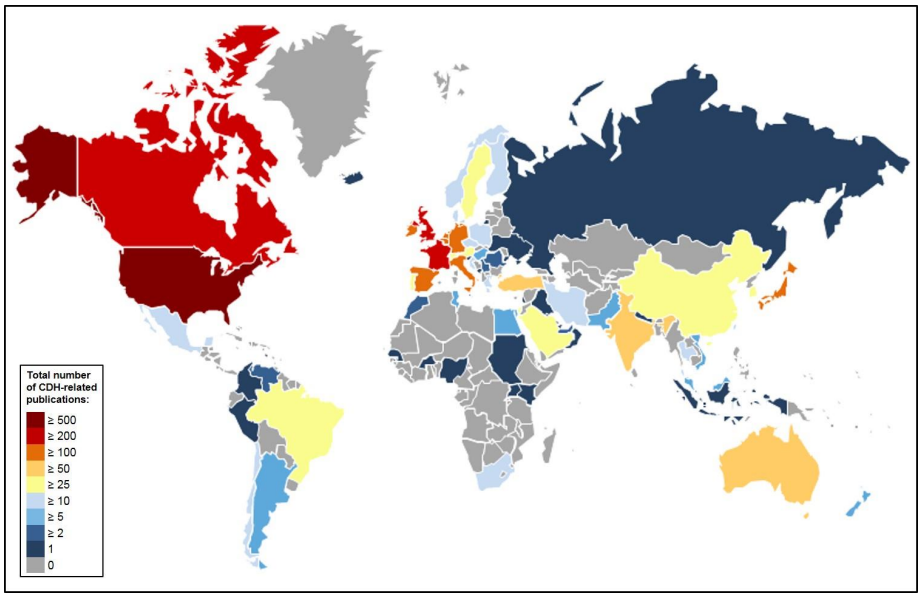

Figure 17 Choropleth mapping visualizing the global publication volume in the field of CDH research.

5.3.2 International research collaborations (V)

Clinicians and basic scientists in 53 (69.7%) of the identified 76 countries that published CDH-related work were involved in international research collaborations (**Figure 18**). The USA combined the highest number of cooperation articles on CDH (*n* = 152), followed by Belgium (*n* = 115) and the Netherlands (*n* = 93). The most productive collaborative network in the field of CDH research was established between the United Kingdom/Belgium (*n* = 53), followed by Belgium/Spain (*n* = 47) and the United Kingdom/Spain (*n* = 34). Luxembourg (*n* = 3), Venezuela (*n* = 2), Dominica, Iceland, Indonesia, Malta, Peru, St. Kitts & Nevis, Sudan and Ukraine (*n* = 1/each) only had joint CDH papers, whereas Turkey had with 3/92 (3.3%) the smallest percentage of cooperative items in relation to its total output. CDH researchers in 23 (30.3%) countries were not involved in any international collaborations. Of those, South Korean investigators released the largest number of CDH publications (*n* = 28), followed by authors from Iran (*n* = 11) and Tunisia (*n* = 8).

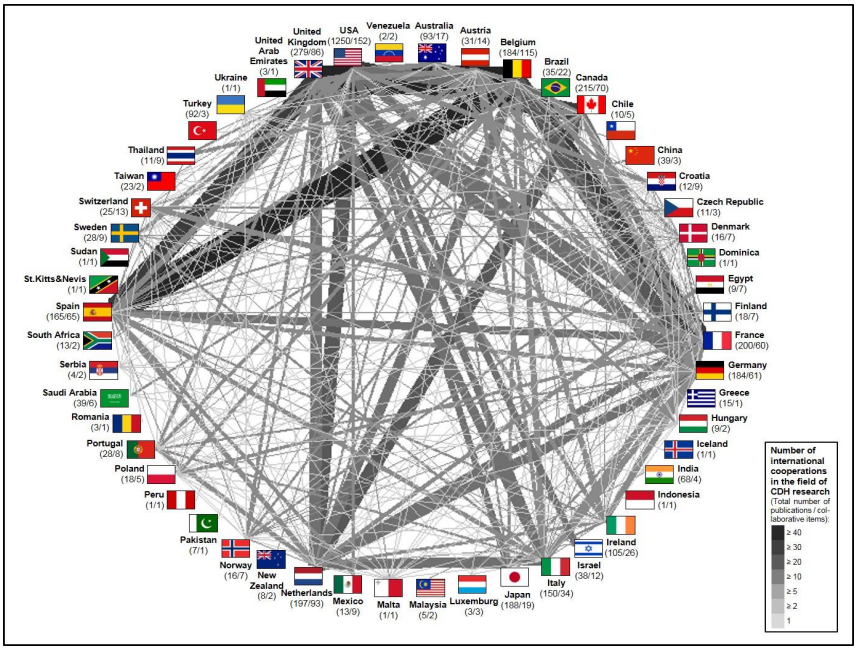

Figure 18 Network diagram of international collaborations and cooperation publications relating to CDH.

5.3.3 Citation rate and country-specific *h*-index (V)

The 215 identified CDH publications from Canada had the highest average citation rate per published item (22.8), followed by articles from the Netherlands (20.7) and USA (20.2). The USA had the highest country-specific *h*-index in the field of CDH-related research (72), followed by Canada (40) and the United Kingdom (38). In contrast, many scientific papers from African, Middle Eastern and Eastern European countries received extremely few citations and thus these countries frequently had a *h*-index of 1 or 0.

5.3.4 Most productive research institutions and authors (V)

All 3,669 scientific publications on CDH were evaluated in relation to their institutions of origin and contributing authors. The identified CDH articles were affiliated with a total of 2,187 institutions and 10,210 authors. The ten most productive CDH research institutions in the world were located in the USA, the Netherlands, Belgium, France, Ireland, the United Kingdom and Canada (**Figure 19a**). The ten most productive authors (appearing anywhere in the author list) in the field of CDH-related research came from the USA, Belgium, the Netherlands, Ireland, Spain and Germany (**Figure 19b**).

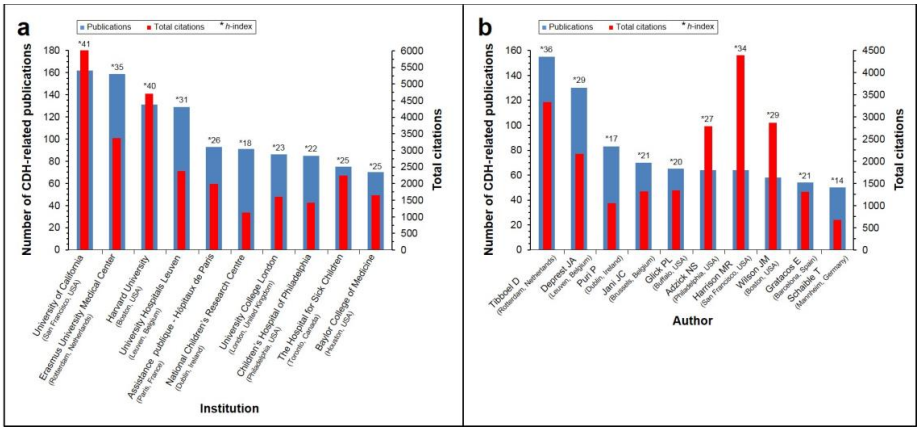

Figure 19 Ten most productive institutions (**a**) and authors (**b**) in the field of CDH-related research.

5.3.5 Scientific subject categories and document types (V)

Subject categories are defined standard categories in the Web of Science™ database, which represent general areas of science. These categories were distributed by the Journal Citation Reports™ for each scientific journal and its publications. Most articles relating to CDH research were assigned to the subject category "PEDIATRICS" (*n* = 1,723), followed by "SURGERY" (*n* = 1,474) and "OBSTETRICS/GYNECOLOGY" (*n* = 449). Other common categories were "GENERAL INTERNAL MEDICINE" (*n* = 370), "RADIOLOGY/NUCLEAR MEDICINE/MEDICAL IMAGING" (*n* = 259), "RESPIRATORY SYSTEM" (*n* = 212), "GENETICS" (*n* = 188), "CARDIOVASCULAR SYSTEM/CARDIOLOGY" (*n* = 96), "RESEARCH/EXPERIMENTAL MEDICINE" (*n* = 74) and "GASTRO-ENTEROLOGY/HEPATOLOGY" (*n* = 66).

Document types of the 3,669 identified CDH publications were classified as 2,576 original articles (70.2%), 494 meeting abstracts and proceedings (13.5%), 332 editorials and letters (9.0%), 149 reviews (4.1%) and 118 others (3.2%).

5.3.6 Publication and citation trend (V)

The first CDH-related paper was published in 1910 and the number of subsequent scientific publications increased almost annually, associated with a steady increase in citations (**Figure 20**). Until 1970, there was low publication activity, comprising of 161 articles. From 1970 onwards, the number of published items increased constantly with a steep rise in the early-mid 1990s, interrupted by a brief drop in the late 1990s/early 2000s, comprising a total of 3,508 articles (i.e. 95.6% of all scientific publications on CDH were published after 1970). Overall, authors published 19-fold more articles relating to CDH in 2016 than in 1970. Between 1922 and 2016, the 3,669 identified CDH publications received a total of 51,253 citations and an average of 533.9 citations per year (range, 0-3,215).

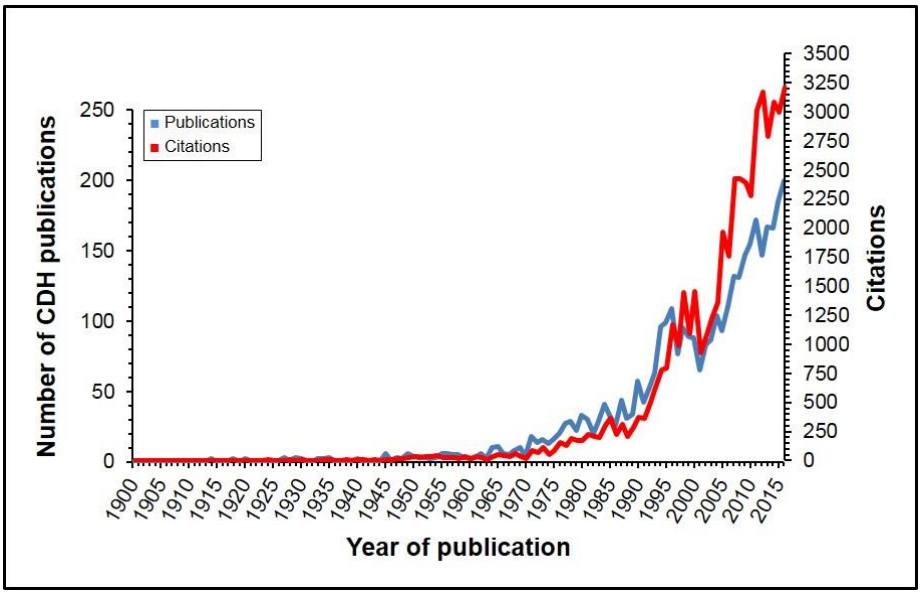

Figure 20 Overall number of CDH publications and received citations in the time span 1900 to 2016.

5.3.7 Notable scientific journals and publications (V)

All scientific journals listed in the Web of Science™ database were examined in regard to their individual output relating to CDH research and citation rates of relevant items were determined. The 3,669 CDH-related articles were published in 573 different journals with an average citation rate of 14.0 (range, 0-414) per publication (*h*-index: 85). The "*Journal of Pediatric Surgery*" was identified as the most productive journal (*n* = 649), whereas "*The Journal of Pediatrics*" had with 33.1 the highest average citation rate per published CDH paper (**Figure 21**).

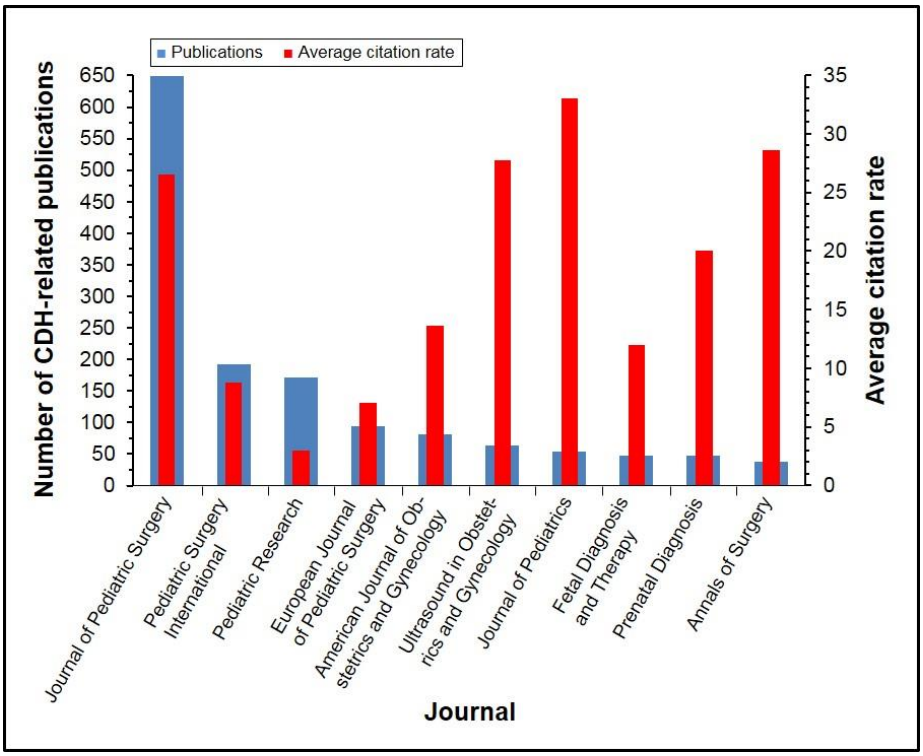

Figure 21 Ten most productive journals with regard to CDH publications and average citation rate per published article.

Table 2 lists the ten most-cited articles in the field of CDH research between 1910 and 2016.

Table 2 Ten most-cited publications in the field of CDH research.

Rank	Publication	Total citations	Citations per year
1	Cantrell JR, Haller JA, Ravitch MM. A syndrome of congenital defects involving the abdominal wall, sternum, diaphragm, pericardium, and heart. *Surg Gyn Obstet* 1958;107:602-614.	414	6.90
2	Metkus AP, Filly RA, Stringer MD, et al. Sonographic predictors of survival in fetal diaphragmatic hernia. *J Pediatr Surg* 1996;31:148-152.	351	15.95
3	Harrison MR, Keller RL, Hawgood SB, et al. A randomized trial of fetal endoscopic tracheal occlusion for severe fetal congenital diaphragmatic hernia. *N Engl J Med* 2003;349:1916-1924.	300	20.00
4	Stege G, Fenton A, Jaffray B. Nihilism in the 1990s: the true mortality of congenital diaphragmatic hernia. *Pediatrics* 2003;112:532-535.	269	17.93
5	Kitagawa M, Hislop A, Boyden EA, et al. Lung hypoplasia in congenital diaphragmatic hernia. A quantitative study of airway, artery, and alveolar development. *Br J Surg* 1971;58:342-346.	244	5.19
6	Boloker J, Bateman DA, Wung JT, et al. Congenital diaphragmatic hernia in 120 infants treated consecutively with permissive hypercapnea/spontaneous respiration/elective repair. *J Pediatr Surg* 2002;37:357-365.	224	14.00
7	Harrison MR, Jester JA, Ross NA. Correction of congenital diaphragmatic hernia in utero - 1. The model: intrathoracic balloon produces fatal pulmonary hypoplasia. *Surgery* 1980;88:174-182.	210	5.53
8	Difiore JW, Fauza DO, Slavin R, et al. Experimental fetal tracheal ligation reverses the structural and physiological-effects of pulmonary hypoplasia in congenital diaphragmatic hernia. *J Pediatr Surg* 1994;29:248-257.	209	8.71
9	Lipshutz GS, Albanese CT, Fekdstein VA, et al. Prospective analysis of lung-to-head ratio predicts survival for patients with prenatally diagnosed congenital diaphragmatic hernia. *J Pediatr Surg* 1997;32:1634-1636.	204	9.71
10	Deprest J, Gratacos E, Nicolaides KH, et al. Fetal tracheal occlusion (FETO) for severe congenital diaphragmatic hernia: evolution of a technique and preliminary results. *Ultrasound Obstet Gynecol* 2004;24:121-126.	201	14.36

6 Discussion

PH is considered to be one of the main reasons of neonatal mortality and long-term morbidity in infants with CDH [Keijzer and Puri, 2010; Rottier and Tibboel, 2005]. Decades of research have focused on attempts to improve lung maturation in these patients, which has led to the widespread use of exogenous surfactant, inhaled nitric oxide, high-frequency oscillation and ECMO [Garriboli et al., 2012]. However, more recently it became clear that these therapeutic options do not significantly reduce CDH-associated mortality rates nor provide considerable outcome benefits [Guner et al., 2018; Snoek et al., 2016; Putnam et al., 2016; Lally et al., 2004]. Due to the absence of sufficient lung-protective strategies, most of these newer treatment modalities have therefore merely replaced mortality with a higher rate of chronic lung disease and impaired neurodevelopment [Madderom et al., 2013; Wynn et al., 2013; Rocha et al., 2012]. Despite the necessity to find an optimal treatment for lung immaturity in CDH, extensive research in the field has not succeeded yet.

6.1 Methodology

The exact pathogenesis of CDH-associated PH remains incompletely understood because of the myriad of involved spatiotemporal processes comprising multiple, complex molecular and cellular interactions. Based on findings from experimental animal studies, it has been demonstrated that decreased alveolar formation and reduced synthesis of pulmonary surfactant contributes to the development of PH in CDH [Utsuki et al., 2001; Alfonso et al., 1996]. Alveolarization and maturation of distal airspaces is an essential phase in developing fetal lungs that requires well-coordinated expression of many regulatory factors, which in turn stimulate alveolar growth and surfactant production [Herriges and Morrisey, 2014; Morrisey and Hogan, 2010; Roth-Kleiner and Post, 2003]. Most of our current understanding about this important stage during pulmonary growth and associated changes in hypoplastic lungs with diaphragmatic defects has originated from different animal models [Chiu, 2014; van Loenhout et al., 2009; Mortell et al., 2006].

In the present work, the well established nitrofen model was used to investigate CDH-associated disturbances during alveolar formation, as the timing of the diaphragmatic insult and bilateral PH are remarkably similar to the human situation [van Loenhout et al., 2009; Beurskens et al., 2007]. However, although maternal exposure of the herbicide nitrofen to pregnant rodents during midgestation has been found to result in CDH in approximately 70% and PH in 100% of the offspring [Noble et al., 2007], a major shortcoming of this model is the fact that the potential teratogenic effects of nitrofen have not been proven to induce diaphragmatic defects and lung hypoplasia in humans so far [Keijzer and Puri, 2010]. Therefore, collaborative research studies utilizing genome-wide arrays and next generation sequencing technics may be more useful to identify potential new CDH-related genes in humans.

6.2 Role of LIFs as a potential target to enhance fetal alveolar development and lung maturation in the nitrofen CDH model

Pulmonary LIFs, which are mainly expressed in the alveolar interstitium and have been shown to account for approximately 50% of all resident alveolar wall cells in immature lungs [Kaplan et al., 1985], playing a crucial role in alveolar development by promoting alveolar epithelial cell differentiation and *de novo* production of surfactant phospholipids [Rehan et al., 2006; Torday et al., 2003; McGowan and Torday, 1997]. This specific subset of lung fibroblasts normally arises in fetal rat lungs during the late canalicular stage of pulmonary development with a significant increase over the last few days of gestation [Tordet et al., 1981]. It has been indicated that pulmonary LIFs contain large, cytoplasmatic lipid droplets, which enable their histological detection within the walls of developing fetal alveoli [Torday and Rehan, 2011; Brasaemle et al., 1997]. During the onset of alveolarization, these lipid inclusions were found to be associated with the expression of Thy-1, a 25-37 kDA heavily N-glycosylated, glycophosphtidylinositol-anchored cell surface protein that has recently been revealed as a regulator of alveolar LIF differentiation and lipid homeostasis in developing lungs [Varisco et al., 2012]. Furthermore, LIFs are characterized by a relatively high expression of Adrp shortly before birth [Schultz et al., 2002], which is a known downstream target of Thy-1 and most pronouncedly expressed in fetal and newborn lungs [Londos et al., 1999]. Adrp is a functional lipogenic marker of fully differentiated pulmonary LIFs that controls the intracellular uptake of neutral lipids in this specific subset of lung fibroblasts and their subsequent transport to AECII [Schultz et al., 2002]. Consequently, Adrp reflects the content of lipid droplets in alveolar LIFs [Magra et al., 2006] and is also a physiological determinant for the synthesis of surfactant phospholipids in AECII [Torday and Rehan, 2011]. Treatment strategies aiming to increase the expression of pulmonary LIFs therefore represent a promising therapeutic approach to enhance fetal alveolar development and thus lung maturation in CDH-associated PH.

In the present study, αSMA and ORO staining was combined to evaluate the distribution of LIFs in fetal rat lungs in detail. Given the fact that this specific subset of lung fibroblasts is characterized by an absence of αSMA expression [Torday et al., 2003], it is not surprising that confocal laser scanning microscopy showed absent αSMA immunofluorescence as well as markedly reduced lipid inclusions in the alveolar interstitium of CDH-associated PH compared to controls on E21.5. At first glance, this finding may appear in contrast to results from previous studies [Santos et al., 2007; Okazaki et al., 1997], which reported no significant differences in αSMA staining between hypoplastic lungs and control group in the rat model of nitrofen-induced CDH. However, the current experiments focused merely on the identification and localization of LIFs in the alveolar walls of fetal rat lungs, while other investigators used αSMA staining mainly to analyze changes in vascular smooth muscle cells of pulmonary arteries that eventually cause PPHN [Jesudason et al., 2006; Taira et al., 1998; Yamataka and Puri, 1997].

6.3 Disruption of *Thy-1* signaling and Adrp expression in hypoplastic rat lungs with nitrofen-induced CDH is associated with an overall reduction of pulmonary LIFs

The present study demonstrated a significant downregulation of pulmonary *Thy-1* transcripts in fetal rat lungs with CDH-associated PH compared to controls, which in turn suggests a disruption of *Thy-1* signaling in the nitrofen model. Immunohistochemistry confirmed this finding by showing a markedly decreased Thy-1 protein expression in alveolar LIFs, which was associated with an overall reduction of cytoplasmatic lipid droplets in this specific subset of lung fibroblasts. Two recent studies [Gosemann et al., 2012; Doi et al., 2010] have provided additional evidence for this intriguing theory by proving that further downstream components of the *Thy-1* signaling pathway [parathyroid hormone-related protein (PTHrP), PTHrP receptor and peroxisome proliferator-acivated receptor gamma] were also significantly downregulated in nitrofen-induced hypoplastic lungs during late gestation, while their relative mRNA expression peaked normally in control lungs. Hence, these discoveries suggest that upregulation of *Thy-1* signaling during this important stage of fetal lung development may be essential for alveolarization and associated distal airway maturation in rats with CDH-associated PH. Moreover, qRT-PCR revealed a significant reduction of pulmonary *Adrp* transcripts, which was further validated by specific immunohistochemical evaluation illustrating strikingly diminished Adrp immunoreactivity in alveolar LIFs. Taken together, these results verified that the quantitative decrease in pulmonary mRNA expression of *Thy-1* and *Adrp* in the nitrofen rat model was for the most part certainly also translated to the protein level. Immunofluorescence double staining ultimately confirmed an overall reduction of LIFs in the alveolar interstitium of CDH-associated PH with markedly reduced lipid inclusions, thus suggesting an impaired alveolar lipid homeostasis and abnormal LIF functioning in nitrofen-exposed rat lungs during the critical time period of fetal alveolarization and alveolar maturation.

6.4 Molecular and cellular effects of prenatally administered ATRA on pulmonary hypoplasia in the nitrofen CDH model

Because most newborn infants with CDH die primarily of respiratory failure secondary to severe PH, any therapeutic approach should be focused on reducing PH and promoting lung growth. Retinoids are known to play a key role during lung morphogenesis [Maden, 2004], including formation of primordial alveoli and maturation of distal airspaces [Simon and Mariani, 2007; Maden and Hind, 2004; McGowan et al., 2000; Massaro and Massaro, 1996]. Although it is widely accepted that disruption of retinoid signaling contributes to the pathogenesis of hypoplastic lungs and diaphragmatic defects [Coste et al., 2015; Clugston et al., 2010b; Montedonico et al., 2008b; Nakazawa et al., 2007; Mendelsohn et al., 1994], most studies have only focused on the severity of CDH along with possible reductions in the incidence by administration of vitamin A and its derivates [Babiuk et al., 2004; Thébaud et al., 1999]. Prenatal application of ATRA, which is one of the most biologically active metabolites of vitamin A within the retinoid signaling pathway, has been demonstrated to accelerate the proliferation of alveolar cells and thus having the potential to attenuate PH in nitrofen-induced CDH [Sugimoto et al., 2008]. However, the exact molecular and cellular effects of ATRA treatment on fetal alveolar growth remain poorly understood.

6.4.1 Effects of maternal ATRA application on fetal lung-to body weight ratio, *Adrp* signaling and LIF expression in hypoplastic rat lungs with nitrofen-induced CDH

We observed in this study that *in vivo* administration of ATRA resulted in a significantly increased lung-to-body weight ratio compared to placebo treatment. Furthermore, mRNA transcripts of *Adrp*, a known downstream target of *Thy-1* signaling and functional lipogenic marker characterizing pulmonary LIFs [Schultz et al., 2002], were significantly increased in hypoplastic lungs of nitrofen-exposed fetuses after prenatal application of

ATRA, which again was associated with markedly increased Adrp immunoreactivity mainly in the distal alveolar interstitium. In addition, the occurrence of cytoplasmatic lipid droplets in alveolar interstitial cells was notably increased in nitrofen-induced hypoplastic lungs after prenatal administration of ATRA compared to placebo treatment, which was accompanied by a marked increase of LIFs in the mesenchymal and interstitial compartments of distal alveolar walls.

6.4.2 Effects of maternal ATRA application on fetal alveolarization and maturation in hypoplastic rat lungs with nitrofen-induced CDH

In the alveolar region of immature lungs, ATRA has been shown to be stored in LIFs [Dirami et al., 2004; Shenai and Chytil, 1990; Okabe et al., 1984] and is utilized to regulate the expression of many retinoid-responsive genes, which in turn initiates the formation of alveolar septa, proliferation of AECII and associated synthesis of pulmonary surfactant proteins [Massaro and Massaro, 2010; Chytil, 1996; Mangelsdorf and Evans, 1995]. As normal functioning LIFs are able to synthesize ATRA [McGowan et al., 1995], it was not surprising that we did not find any structural or morphological differences between Control+ATRA and Control+Placebo. Stereo- and morphometric analysis of fetal lungs, a well established technique that allows accurate study of forming alveoli [Bolender et al., 1993], has revealed a significant progression in alveolar development after prenatal administration of ATRA. Nitrofen-exposed fetuses that received ATRA application shortly before birth showed enhanced radial alveolar count and decreased mean linear intercept compared to placebo treatment. These findings are consistent with previous studies, demonstrating that prenatal administration of ATRA stimulates alveolarization in nitrofen-induced hypoplastic lungs [Montedonico et al., 2008a; Montedonico et al., 2006]. Taken together, this highlights that ATRA is not only essential for alveolar growth, but also has the potential to rescue failed alveolar formation [Massaro and Massaro, 2003].

As there were no significant differences between nitrofen-exposed fetuses that had CDH compared to those who did not have CDH, our results confirmed that the therapeutic effects of prenatal treatment with ATRA occur independently of the diaphragmatic defect. However, it remains unclear whether ATRA has a direct effect on AECII or may function indirectly through a mechanism involving epithelial-mesenchymal interactions [Schuger et al., 1993].

6.4.3 Effects of maternal ATRA application on fetal *Lep* signaling and synthesis of surfactant phospholipids in hypoplastic rat lungs with nitrofen-induced CDH

Lep has been found to be critically involved in the regulation of distal airway development [Huang et al., 2008]. The lungs are one of the few organs in the fetus that express Lep and its functional receptor Lep-R [Bergen et al., 2002], which are mutually expressed by pulmonary LIFs and AECII [Torday et al., 2002]. The expression of Lep and Lep-R has been shown to arise just before the onset of AECII maturation, beginning during the late canalicular stage of fetal lung development with a significant increase over the last few days of gestation [Henson et al., 2004; Torday et al., 2002]. In a recent study, it has been demonstrated that *Lep* upregulates the intracellular expression and extracellular secretion of surfactant proteins in AECII [Chen et al., 2013]. Additionally, *Lep*-deficient mice exhibit decreased alveolarization with reduced pulmonary surfactant phospholipid synthesis [Tankersley et al., 1996]. Lep has been reported to increase the maturation of AECII and expression of surfactant protein B [Kirwin et al., 2006], which is accompanied by an increase in fetal lung weight. These findings highlight the important role of Lep and Lep-R during prenatal lung growth by promoting alveolar epithelial cell differentiation and *de novo* surfactant production, suggesting them as potential physiological markers of fetal lung maturity [Chen et al., 2013; Kirwin et al., 2006].

Previous studies have indicated that Lep and Lep-R expression in developing lungs is regulated by retinoid signaling [McGowan et al., 1995]. In this way, ATRA may promote alveolar development by accelerating the differentiation and subsequent proliferation of AECII [Belloni et al., 2000]. In the present study, it was found that mRNA transcripts of *Lep* and *Lep-R* were significantly increased in fetuses with nitrofen-induded PH after prenatal administration of ATRA compared to placebo treatment. This work also demonstrated that Lep and Lep-R immunoreactivity were markedly increased in interstitial and alveolar epithelial cells of nitrofen-exposed fetuses after maternal ATRA application compared to placebo treatment, which was accompanied by a notably increased SP-B expression in AECII. These results confirmed that the quantitative increases in *Lep* and *Lep-R* mRNA transcripts were translated to the protein level, thus indicating that ATRA upregulates *Lep* signaling in nitrofen-induced hypoplastic rat lungs, which in turn stimulates the synthesis of pulmonary surfactant phospholipids.

6.5 Use of retinoids during pregnancy

The use of retinoids during pregnancy is controversial and currently restricted by the Food and Drug Administration because of its teratogenic side effects on various developmental aspects of the embryo [Desai et al., 2007]. For instance, birth defects induced by ATRA embryopathy may include central nervous system abnormalities (e.g. facial nerve palsy, microencephaly, hydrocephalus), external ear abnormalities (e.g. microtia), cardiovascular abnormalities (e.g. transposition of the great vessels, hypoplastic left heart syndrome, ventricular septal defects, tetralogy of Fallot), craniofacial dysmorphia (e.g. midface hypoplasia, cleft palate and lip), eye abnormalities (e.g. ocular hypertelorism), thymus gland abnormalities and bone abnormalities (e.g. syndactyly) [Nau 2001; Lammer et al. 1985]. However, it has been reported that pregnant women with acute leukemia have been successfully treated with ATRA during the second and third trimester of pregnancy with no adverse effects on the newborn [Agarwal et al., 2015; Valappil et al., 2007]. This allows a possible time window for its use in pregnant women during late gestation when alveolarization of fetal lungs begins.

6.6 Scientometric analysis

Between 1910 and 2016, a total of 3,669 publications on CDH were indexed in Web of Science™ database, originating from 76 countries. The absolute number of CDH papers has increased nineteen-fold since the 1970s, associated with an equally steep increase of citations and replicating the same trend as shown in previous scientometric studies on other pediatric conditions [Friedmacher et al., 2019; Schöffel et al., 2017]. Advances in postnatal resuscitation and introduction of new therapeutic strategies in the 1990s and 2000s, respectively, most likely contributed to the steep increase of CDH research in these two decades. Not surprisingly, only a small number of North American and European countries were responsible for the majority of CDH-related research, which not only generated most of the scientific articles, but also papers high in quality. Of these, the USA, Canada, the United Kingdom, France and the Netherlands were the five leading countries with regard to the total number of CDH publications, average citation rate and *h*-index. This mirrors the worldwide trend for a greater volume of scientific articles to originate from high-income countries [Groneberg-Kloft et al., 2008; Braun et al., 1995], and further, for authors from these countries to dominate key roles in authorship. In comparison, the lack of publications from low- and middle-income countries reflects a pattern in all fields of medicine: that survival of infants with serious conditions such as CDH is often not feasible in countries with low resources or in healthcare systems, where medical professionals are too busy with clinical pressures to commit time to research. As significant progress cannot be made by a single researcher, there is currently a global movement in science towards strategically designed national or international collaborations in order to improve overall patient care [Greene, 2007]. This is particularly relevant for rare disorders like CDH as shown by the increasing number of cooperation papers and collaborative networks in this field.

The most productive collaborative network on CDH was established between the United Kingdom and Belgium. These findings can partly be explained by the efficient and well-funded academic structure in both countries, allowing leading experts more frequently to cooperate with their international colleagues [Wagner and Jonkers, 2017; Adams, 2013]. All of the most productive institutions and authors were either based in North America or Europe.

What have been the topics of the most-cited CDH work so far? Four out of the ten most-cited articles were directly linked with the intriguing concept of in-utero intervention for fetuses with CDH, reporting pioneering work from its experimental beginnings, subsequent evolution of this technique and a randomized controlled trial. Although there is currently insufficient evidence to recommend FETO as a part of routine clinical practice [Grivell et al., 2015], a few specialized fetal medicine centers in Europe, North and South America successfully perform this procedure [Persico et al., 2017; Belfort et al., 2017; Ruano et al., 2012; Dekoninck et al., 2011]. Recently, it has been reported that FETO improves neonatal survival in CDH fetuses with severe PH compared with standard perinatal management [Araujo Júnior et al., 2017; Al-Maary et al., 2016]. Today, FETO results in a survival rate of 50% to 60% [Deprest et al., 2014a]. Further results from ongoing international randomized trials are anticipated in the near future [Deprest et al., 2014b]. Two further papers dealt with prenatal predictors for postnatal CDH survival. With the advent of routine maternal ultrasound scanning, CDH can now be diagnosed prenatally in up to 60% of cases [Benachi et al., 2014]. Nowadays, the observed-to-expected lung area-to-head circumference ratio measured on 2D ultrasonography is routinely used by fetal medicine centers around the world as a good indicator of neonatal prognosis and chronic lung disease in survivors with CDH [Senat et al., 2018; Snoek et al., 2017]. Other valuable prognostic parameters are extent of liver herniation and observed-to-expected fetal lung volume on magnetic resonance imaging [Russo et al., 2017; Kastenholz et al., 2016]. However, gestational age at diagnosis should be taken into account when estimating postnatal morbidity and mortality [Bouchghoul et al., 2015].

Other highly cited themes were: CDH-associated mortality, pulmonary hypoplasia and lung-protective therapies. During the last two decades, CDH survival rates have slightly but significantly improved [Morini et al., 2017]. Whereas some specialized centers have reported survival rates of close to 90%, pooled results from the CDH Study Group indicated that today's overall survival rate is approximately 70% [Harting and Lally, 2014]. Defective lung alveolarization appears to be a common and potentially actionable phenotype in both patients and animal models of CDH [Donahoe et al., 2016]. These findings have revealed opportunities for the development of novel targeted treatment options, particularly in the pre- and postnatal stages, when therapeutic drugs combined with appropriate ventilation strategies and ECMO can have maximum clinical impact on surviving patients.

6.7 Limitations

One of the main limitations of this study was the use of a toxicologically introduced animal model due to lacking access to postmortem tissue from a human biobank. Although the nitrofen model is a well-established part of modern CDH research that has provided important insights into the underlying pathogenesis and associated pathophysiological alterations in pulmonary development, the potential teratogenic effects of this herbicide have never been proven to play a role in human cases. As some strains of rodents appear to have a higher susceptibility to nitrofen than others, which for instance makes its application difficult to investigate in genetically modified mice [Beurskens et al., 2007], pathogen-free Sprague-Dawley rats were used to achieve the highest possible rate of diaphragmatic defects and bilateral PH in the offspring. Even though the fundamental mechanisms by which these changes are induced in this CDH model are not fully understood, disturbances of the retinoid signalling pathway appear to be very likely [Montedonico et al., 2008b; Greer et al., 2003].

Another noteworthy limitation of this study was the fact that differences in pulmonary Thy-1, Adrp, Lep, Lep-R and SP-B protein expression between the experimental groups were only quantified by immunohistochemical staining of representative tissue sections. A major problem with Western blot analysis is not the method itself, but the availability of high-quality antibodies. Despite all being affinity-purified, none of the tested commercial antibodies provided accurate results with regard to detection of specific protein levels. This is a known issue with this technique [Gilda et al., 2015], why the present study mainly focused on the precise evaluation of the cellular protein expression.

The main limitations of the scientometric analysis was most likely related to the used search engine. Although the Web of Science™ database is a well-established platform in citation analysis and one of the most comprehensive, accurate and unbiased resources for literature searching, not all journals, institutions or individual authors that published CDH-related research are necessarily listed. The use of other search engines such as PubMed/MEDLINE would likely have resulted in marginally different figures. Additionally, the choice of database may have caused a potential language bias towards scientific articles from English-speaking countries [Van Leeuwen et al., 2001] and it is also known that authors and reviewers tend to be biased towards their native language in their citation practice [Link, 1998; Campbell, 1990]. As the applied search strategy was based on a title rather than a topic search to identify all papers which focused primarily on CDH research, a few relevant research items may not have been recognized by the automated computer search. Another possible bias may be the analysis of citation frequency and *h*-index as measures of scientific quality rather than using journal impact factors as a surrogate [Garfield, 2001]. In turn, it must be considered that self-citation by authors can considerably manipulate the *h*-index. Unfortunately, the Web of Science™ database does not provide a separate function for this type of large dataset, which would allow calculation of the *h*-index excluding self-citations. Nevertheless, this metric is a proven tool to compare different countries, institutions and authors working in one specific field [Bartneck and Kokkelmans, 2011].

6.8 Future considerations

A sound understanding of the etiology and pathogenesis of CDH together with PH and PPHN is fundamental in order to prevent these children from the devastating sequelae of this congenital malformation. Recent advances in prenatal intervention by minimally invasive techniques such as FETO and regenerative tissue engineering combined with stem cell therapy offer encouraging potential for future treatment strategies [De Coppi and Deprest, 2017; Shieh et al., 2017; Al-Maary et al., 2016; DeKoninck et al., 2015; Jeanty et al., 2014; Di Bernardo et al., 2014; Deprest et al., 2014a; Pederiva et al., 2013; De Coppi and Deprest., 2012; Deprest and De Coppi, 2012]. Translational research therefore represents an essential element in our quest for new treatment options for CDH-associated PH, which will mainly depend on multi-institutional and international collaborations [Lally and Skarsgard, 2017]. However, investigations of novel medical therapies and pharmaceutical compounds that have the ability to arrest or reverse PH in animal models of CDH require the application of standardized research methodologies [Eastwood et al., 2015]. The experiments performed in this work have allowed us to identify pulmonary LIFs as a potential target for prenatal treatment with ATRA to improve alveolar development in hypoplastic rat lungs with nitrofen-induced diaphragmatic defects. Further functional studies will need to be carried out in order to get a more comprehensive scientific knowledge of the underlying molecular and cellular effects of ATRA application during fetal alveolarization as well as the impact on the involved epithelial-mesenchymal interactions. Additional points for future research studies could be isolation, *in vitro* co-culture with ATRA and subsequent transplantation of “retinoic acid-enriched” pulmonary LIFs. To test the impact of this modified subset LIFs on immature lungs, one could create fluorescent-labeled cells and administer them to nitrofen-exposed rat fetuses prenatally. Probably the best way of administration with direct delivery of these cells to the hypoplastic lungs would be endoscopical microinjection into the trachea followed by FETO. Afterwards, pulmonary

tissue could be isolated and microscopically investigated for morphology and localization of the labeled LIFs. Moreover, isolated lung cells could be sorted by fluorescence-activated cell sorting to determine their differentiation pattern. Another option could be the injection of ATRA-treated and fluorescent-labeled LIFs into the trachea of nitrofen-exposed lung explants. It may be possible to follow these cells for a certain amount of time using live imaging combined with confocal laser scanning microscopy and study their individual behavior *in vitro* over several hours.

International multicenter consortiums and national research networks have addressed many critical knowledge gaps pertaining to CDH care. Most importantly, they have identified variability in both CDH practice and outcome among participating centers. Using combined data from these groups, national or international consensus guidelines for multidisciplinary CDH treatment may be produced to standardize best practices for patients with CDH, from prenatal diagnosis to hospital discharge, based on the best available clinical evidence. In addition, collaborations with global initiatives such as *CDH International* may help to foster further research activities and strengthen support groups.

7 Conclusions

PH, characterized by a significantly decreased number of terminal airway generations and alveolar immaturity, is one of the main reasons for the potential life-threatening respiratory insufficiency in newborns with congenital diaphragmatic defects, leading to high neonatal mortality and long-term morbidity. The objectives of this dissertation were to investigate the underlying molecular and cellular alterations in pulmonary LIFs and AECII at the end of gestation in the nitrofen-induced rat model of CDH, which form the basis for a therapeutic approach with ATRA. Alterations in *Thy-1* signaling and its downstream target *Adrp* in hypoplastic rat lungs with toxicologically induced diaphragmatic defects were systematically evaluated in two observational studies, and resulting changes in the amount of cytoplasmatic lipid droplets in LIFs and overall expression of this specific subset of lung fibroblasts were examined. The effects of prenatally administered ATRA on fetal alveolarization and surfactant phospholipid synthesis in nitrofen-exposed rat fetuses with CDH-associated PH were assessed in two *in vivo* treatment studies using molecular genetic, immunohistochemical/-fluorescence and stereo-/morphometric analysis techniques. Based on the results of these studies, the following conclusions can be drawn:

1. Disruption of *Thy-1* signaling in hypoplastic rat lungs leads, through a disturbed uptake of neutral lipids into pulmonary LIFs at the end of fetal gestation, to markedly reduced cytoplasmatic lipid content in this specific subset of lung fibroblasts, which is associated with an impaired alveolar development and PH in the nitrofen-induced CDH model. (I)

2. Decreased pulmonary Adrp expression during late gestation is accompanied by an overall reduction of LIFs in hypoplastic rat lungs, thereby suggesting that disruption of Adrp-regulated alveolar mesenchymal cell differentiation and lipid homeostasis in nitrofen-exposed fetuses with diaphragmatic defects may cause impaired alveolar formation and eventually PH through disturbed LIF functioning. (II)

3. Maternal application of ATRA shortly before birth increases the fetal expression of LIFs and alveolarization in hypoplastic rat lungs with nitrofen-induced CDH, suggesting that ATRA may have a therapeutic potential in attenuating CDH-associated PH by stimulating alveolar formation and distal airway maturation through increased expression of pulmonary LIFs. (III)

4. Prenatally administered ATRA upregulates *Lep* signaling in hypoplastic rat lungs, which in turn increases *de novo* production of pulmonary surfactant in nitrofen-exposed fetuses with diaphragmatic defects and thus may reduce experimentally induced PH by increased synthesis of surfactant phospholipids at the end of gestation. (IV)

In addition, this study draws the first detailed map of the global CDH research architecture based on an in-depth analysis of the scientific output from 1910 to 2016:

5. During this time span, CDH-related research has progressed from simple empirical observations to accumulation of best clinical evidence, becoming much more multidisciplinary with main research endeavors concentrating in a few high-income countries. Great strides in basic science and biomedical technology have contributed to a number of revolutionary new discoveries in the pathogenesis and pathophysiological mechanisms of CDH.

Collaborative research has led to substantial progress in prenatal diagnostics and interventions, implementation of standardized neonatal treatment protocols and most recently regenerative medicine therapy. All these advances hold now the promise of improving CDH patient care and outcome in the 21st century. International collaborations should therefore be strengthened to allow further evolution in this field. (V)

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