CLINICAL AND EXPERIMENTAL ASPECTS OF LUNG DEVELOPMENT AND INJURY IN CONGENITAL DIAPHRAGMATIC HERNIA

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CLINICAL AND EXPERIMENTAL ASPECTS OF LUNG DEVELOPMENT AND INJURY IN CONGENITAL DIAPHRAGMATIC HERNIA

Klinische en experimentele aspecten van longontwikkeling en longbeschadiging bij congenitale hernia diaphragmatica

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Part I

Introduction

Chapter 1

Introduction and review of the literature

1.1 Introduction

Congenital anomalies are expressions of abnormal growth and development. They presently form the second most frequent cause of death, after immaturity, in the perinatal period.[1] Congenital diaphragmatic hernia (CDH) is a congenital anomaly manifesting itself in about one in 3,000 total births[2] or one in 2,700 live births.[3] More than 95% of the diaphragmatic defects are posterolateral ones.[2] For a long time CDH was considered as a purely anatomical defect of the diaphragm.[4] But it has become clear that in many cases (ranging from 39 to 47%) it is associated with other anomalies, especially cardiac defects.[2,3,5] Despite improved neonatal intensive care, the overall survival rate does still not exceed 55 to 58%[2,3], and is even less in patients with associated anomalies.[2,3,5] However, higher survival rates have been reported: Wung and coworkers reported a survival rate of 94% in a group of CDH patients who were treated with very delayed surgery and a respiratory care strategy that avoids pulmonary overdistension.[6]

Inherent to CDH are hypoplastic lungs as judged by the criteria of Askenazi and Perlman[7]: decreased lung-body weight ratios and radial alveolar counts (RAC), a measure of the complexity of the respiratory acinus[8], have been reported by several authors.[9-11] Also inherent is a lower number of airway and vascular generations[12,13] and greater muscularity of the peripheral arteries.[12,14] These abnormalities may lead to respiratory insufficiency shortly after birth. Artificial ventilation with high peak inspiratory pressures and high O_2 fraction is often needed. This is associated with a high incidence of bronchopulmonary dysplasia in surviving CDH patients.[4]

Lung hypoplasia is also found in relation to other clinical conditions[15,16], for instance: oligohydramnios caused by renal malformations[11,17-19] or leakage of amniotic fluid[18,20], and anencephaly.[11] Lung hypoplasia in these cases is usually due to less and smaller alveoli, although a diminished number of airway generations has been described as well.[19] The vascular abnormalities found in CDH, however, are uncommon in these other cases.[19]

This review focuses on lung development in CDH. Fundamental knowledge of normal growth and development is essential to understand the mechanisms of

abnormal growth and development. Therefore, the fundamentals of normal lung development are described first, followed by the characteristic features of abnormal lung development in CDH. The rationale of different treatment modalities is briefly discussed. It is concluded by the aims of the investigations presented in this thesis.

1.2 Normal lung growth and development

1.2.1 Developmental anatomy

Reid has formulated three laws of human lung development:[21]

1. The bronchial tree is fully developed by 16 weeks of gestation;
2. Alveoli develop after birth, increasing in number until the age of eight years. Size increases until growth of the chest wall finishes with adulthood;
3. The preacinar vessels (arteries and veins) follow the development of the airways, the intraacinar vessels that of the alveoli. Muscularization of the intraacinar arteries does not keep pace with the appearance of new arteries.

Originally, lung development was thought to occur in five consecutive stages: the embryonic, pseudoglandular, canalicular, saccular, and alveolar period.[16,22] Later it became clear that the first two periods are histologically similar and should therefore be referred to as the pseudoglandular period, thus leaving four stages.[23,24] The histological characteristics of these four stages have been extensively reviewed by Merkus and coworkers.[23] During the *pseudoglandular* period the lung primordial system develops in humans until week 10-12 of gestation, followed by the differentiation of airways from weeks 12 to 16. Vascularization and further development of the acinus occurs in the *canalicular* stage from gestational week 16 to 28. During the *saccular* stage from weeks 26 to 36 the major event is the subdivision of sacculi. The *alveolar* stage is from term until about 18 years of age, although the onset of this stage is still debated.[23] The alveolar stage is the stage of alveolar acquisition and enlargement.

Several studies aimed at quantifying the number of alveoli at birth and thereafter; a wide range of data has been reported.[25-27] Most observations reveal that up to 85% of alveoli develop postnatally and that the most rapid alveolar multiplication occurs during the first three years of life[25,26] with a gradual slowing of multiplication in childhood.[24] Postnatal lung growth and the consequences of this process for the development of lung function in childhood and adolescence have

been reviewed by Merkus and coworkers.[23] The same lung developmental stages as in humans are recognized in other mammals, but between species these show important differences regarding the time limits of the various developmental stages.[22] In the rat, an animal species that is widely being used to study different aspects of lung development, the pseudoglandular stage occupies 86% of gestation, the saccular stage begins at 95% of gestation, and alveoli start to develop at postnatal day 4.[22]

The preacinar arteries that accompany the airways are all present by the end of 16 weeks gestation.[21] The intraacinar arteries supply the capillary bed and their multiplication is most rapid after birth to follow the alveolar multiplication. Muscularization is slow, however, and in the fetus and newborn muscular arteries are only found alongside airways and proximal to the alveolar surface.[21]

1.2.2 Factors controlling growth of airways and epithelium

Epithelial branching is one of the major events in lung morphogenesis. This process depends on interaction between epithelium and mesenchyme: epithelial cells are able to respond to inductive signals coming from the mesenchyme, and the mesenchyme is able to modulate patterns of epithelial branching.[28] The importance of epithelial-mesenchymal interactions was already described by Masters in 1976[29], and has been supported by studies in transgenic mice.[30] Several extracellular matrix factors have a role in the epithelial-mesenchymal interactions: laminin, fibronectin, proteoglycans such as syndecan, and collagens.[31,32] Massoud and coworkers observed that in the fetal rat lung the first generation of airways appear by a single new bud (monopodial branching), whereas later generations appear by dichotomous branching.[33]

1.2.2.1 Transcription factors

Several transcription factors are possibly involved in lung development. An extensive review regarding these factors has recently been published by Cardoso[28], and Glasser and coworkers reviewed the transgenic models that are available to study lung development.[30] Transcription factors are proteins with two important domains: one for transcription activation and one for DNA recognition and binding.[28] The homeodomain-containing (homeobox) proteins make up a large family of transcription factors. Examples of important genes during early lung development are Hox-genes, which are present at the early stages of development and are developmentally regulated. An important regulator of airway branching is

Hoxb-5.[28] Another relevant gene is TTF-1, which is expressed at the onset of thyroid and lung morphogenesis in the rat[34], and is thought to regulate distal epithelial cell phenotypes. In addition, TTF-1 is a major activator of lung-specific genes such as the surfactant protein B gene promotor.[35] Genes from the Hox family expressed in the lung mesenchyme interact, via signals, with TTF-1 in the epithelium to regulate lung morphogenesis.[28]

The steroid hormone receptor superfamily includes receptors for steroid and thyroid hormones, and for retinoids. Retinoid acid receptors (RARs) are regulators of cell growth and differentiation which exert a function during early and late stages of lung development. The roles of retinoids have been summarized by Zachman[36]: they include branching morphogenesis, alveolarization, and interactions with Hox gene expression and growth factors like epidermal growth factor (EGF) and transforming growth factor-ß (TGF-ß). Mendelsohn and coworkers reported lung hypoplasia and lung agenesis in RAR double mutant mice, indicating that RARs are essential for lung development.[37] Blommaart and coworkers showed that in the fetal rat lung two isoforms of the thyroid hormone receptor (THR) are expressed from day 13 onward: the α-isoform in the mesenchyme and the ß-isoform in the pulmonary epithelium, suggesting that thyroid hormones play a role in early lung development.[38]

Another transcription factor involved in early lung development is N-myc, a protein of the myc protooncogene family. Its expression is restricted to epithelial cells and in contrast to the Hox and RAR family it has little functional redundancy with other family members, allowing one member to compensate for the lack of others.[28] Targeted disruption of N-myc in transgenic mice resulted in lung hypoplasia.[30]

1.2.2.2 Growth factors

Important growth factors that are involved in early lung development are the insulin-like growth factors I and II (IGF-I and IGF-II). In developing mouse lung mRNA expression of IGF-I, IGF-II, the type I IGF receptor, and IGF-binding proteins — which modulate IGF activity — has been reported at early stages in mesenchymal and epithelial cells.[39] In human fetal lungs both epithelial and mesenchymal IGFs mRNA levels are predominant during the period of active epithelial growth.[40] IGF-I may stimulate the proliferation of fetal lung fibroblasts, which in their turn synthesize IGF-I to stimulate their own proliferation.[41] In fetal rat lungs platelet-derived growth factor (PDGF) is found in developing airway epithelial cells and mesenchymal cells at maximal levels at the late pseudoglandular

stage. Its expression declines at later stages, suggesting that this growth factor is important during fetal lung development.[42] Other growth factors, such as fibroblast pneumocyte factor (FPF), TGF-ß, and EGF are mainly involved in later stages of lung development during cell differentiation and maturation.[43,44] All growth factors mentioned above may regulate the extracellular matrix formation, which stimulates branching morphogenesis and cell differentiation.[31]

1.2.2.3 Neuropeptides

Pulmonary neuroendocrine cells (PNEC) are amine and peptide producing cells distributed throughout the airway mucosa. Several observations indicate that these cells are involved in lung development:[45]

1. they are the first cell type to differentiate in fetal lung;
2. they form elongated dendrite-like processes extending over long distances — an ideal arrangement for paracrine interactions with adjacent epithelial and/or mesenchymal cells;
3. they are preferentially located at airway bifurcations, mainly in clusters.

Gastrin-releasing peptide (GRP) is a major bombesin-like peptide produced by PNEC, which is involved in embryonic mouse lung branching morphogenesis.[46,47] Bombesin-like peptides released by PNEC are thought to bind to adjacent mesenchymal cells, subsequently activating the production of various fibroblast growth factors.[45] A recent study showed that the GRP-receptor mRNA expression in lungs of fetal rabbits was located in the distal airway epithelial tubes and the surrounding mesenchyme with maximal levels on gestational day 24. In the fetal rabbit and human lungs it was greatly reduced in the larger, better differentiated airways. These observations suggest that the GRP-receptor plays an important role during the canalicular stage of lung development.[48] Another peptide produced by PNEC is calcitonin gene-related peptide. In vitro, this peptide induces the proliferation of airway epithelial cells in guinea pigs[49] and human endothelial cells.[50]

1.2.2.4 Fetal lung liquid and respiratory movements

Another important factor controlling fetal lung growth, mainly studied in fetal sheep, is lung liquid. This is actively secreted by the pulmonary epithelium against the resistance of the upper airway; lung liquid volume and the intratracheal pressure (ITP) are maintained within precise ranges by the larynx[51]. Alcorn and coworkers showed that tracheal ligation performed in fetal sheep on day 105-110 (canalicular stage) resulted in increased lung growth, though lung maturation was

negatively influenced as assessed by the enhanced ratio of type 2 to type 1 pneumocytes.[52] On the other hand, lung liquid drainage resulted in growth retardation, but it had a positive effect on lung maturation.[52] Fetal lung liquid is also important in early stages of lung development: Souza and coworkers showed that reduction of the fluid secreted by lung epithelium in embryonic rats impairs lung growth, but not branching morphogenesis.[53]

Others showed that tracheal ligation, which results in increased ITP, is responsible for lung growth through cell proliferation. Lung maturation as assessed by measurement of surfactant phospholipids, or by quantitative morphometrics was not affected by tracheal ligation in these studies.[51] In lung hypoplasia models, fetal tracheal ligation did not only accelerate lung growth beyond normal limits, but could actually reverse the lung hypoplasia.[51]

In humans, breathing movements with a low amplitude and high frequency are observed from gestational week 12. Abolition of fetal breathing movements in fetal rabbits or lambs causes failure of lung growth. It has been suggested that fetal breathing movements control the volume of liquid that is retained in the lung.[15] Liu and coworkers showed that mechanical stretch of fetal rat lung cells, which simulates fetal breathing movements, can enhance cell proliferation by controlling PDGF-B and PDGF-ß-receptor gene expression.[54,55]

1.2.3 Factors controlling vascular growth

The normal structural development of the pulmonary vasculature has been extensively reviewed by Morin and Stenmark.[56] These authors describe two morphogenetic processes that contribute to the pulmonary vasculature: vasculogenesis and angiogenesis. Vasculogenesis begins with the differentiation and segregation of angioblasts, which are the precursors of endothelial cells. After differentiation into endothelium, angioblasts contribute to the expression of smooth muscle phenotype or they recruit smooth muscle cells (SMC) to the forming vessel. The forming of new vessels from preexisting primitive vascular channels is called angiogenesis.[56] Proliferation of endothelial cells, SMC, and fibroblasts are necessary for continued vascular growth.

The following growth factors have been put forward to be involved in vascular growth: fibroblast growth factors (FGFs), TGF-ß, PDGFs, IGF-I and II, and vascular endothelial growth factor (VEGF). Besides their mitogenic activity to vascular cells, some of these growth factors stimulate the production of other factors that are involved in vascular growth. For instance, TGF-ß increases the

production of collagens I and III and fibronectin by lung fibroblasts, and IGFs stimulate the synthesis of collagens and elastin.[40,56]

In the rat embryo the expression of VEGF receptors is one of the earliest events occurring in endothelial cell differentiation.[57] In human fetal lung at midgestation, VEGF is secreted from the pulmonary epithelium and vascular smooth muscle cells, and is thought to regulate growth of adjacent vascular endothelium.[58]

1.2.4 Differentiation and maturation of lung tissue

1.2.4.1 Development of the surfactant system

At the end of the pseudoglandular stage cuboidal epithelium is found in the developing lung tubules.[24] The transition from columnar cells to these cuboidal cells demarcates the pulmonary acinus, the respiratory portion of the lung. Using antibodies against surfactant-associated proteins, Otto-Verberne and Ten Have-Opbroek have shown that these cells are alveolar type II cells or their precursors. These cells have been detected in the mouse from day 14[24], in the rat from day 16[59], and in human lungs from week 11-12.[60] Further differentiation of the type II cells into flattened type I cells starts in the human from gestational week 16 onwards.[60]

Since 1959 has it been known that infants dying from respiratory distress syndrome were surfactant deficient.[61] Alveolar type II cells are the only cells in the lung responsible for the production, storage, and secretion of pulmonary surfactant. Surfactant is composed of 85-90% phospholipids, 10-15% proteins and small amounts of carbohydrate.[62,63] Surfactant phospholipids spread as a monolayer at the air/liquid interface, reducing the net contractile force of the alveolar surfaces, thus preventing the air spaces from collapsing at low lung volumes.[62] Phosphatidylcholine (PC) represents about 70-80% of the phospholipid fraction and 60-75% of PC is saturated.[62-64] The phospholipid composition of surfactant in different species has been reviewed by Rooney and coworkers; for the human, rat, and rabbit the proportion of PC is approximately within the above mentioned ranges.[64] There are two major pools of surfactant in the lung: the extracellular pool can be isolated from the lung by bronchoalveolar lavage, whereas the lamellar bodies, characteristic inclusion organelles in the type II cells, represent the intracellular surfactant pool.[62] A recent study of surfactant pool sizes in humans revealed that the extracellular pool size in human lung tissue is smaller than previously estimated in other species, which may make the human lung more susceptible to injuries that interfere with surfactant function.[65]

The production of surfactant increases at 85-90% of gestation: the volume density of lamellar bodies increases, whereas that of the glycogen stores decreases. Glycogen may provide both the energy and the substrate for the biosynthetic pathways of the surfactant phospholipids.[63] The pathway for PC synthesis has been extensively reviewed by Post and Van Golde.[62] The enzyme cholinephosphate cytidylyltransferase (CT) plays a key role in the regulation of PC synthesis and forms the rate-limiting step.[62,63,66,67] During lung maturation the amount of lecithin (phosphatidylcholine) increases, whereas the amount of another phospholipid, sphingomyelin, decreases. Thus, the ratio of these phospholipids increases towards the end of gestation and is therefore considered as a marker of lung maturity (l/s ratio). Another marker of lung maturity is the percentage of phosphatidylglycerol (PG), a phospholipid that increases at 35 weeks of gestation.[53] The process of lung maturation occurs at the cost of lung growth: in fetal rabbit, rat and chick lungs a rise in saturated PC levels concurred with a decline in DNA/protein ratios.[68]

Surfactant proteins are other components of pulmonary surfactant. Their functions and developmental regulation have been reviewed by Rooney and coworkers.[64] The major surfactant protein, SP-A, is detectable in amniotic fluid at 30 weeks of gestation and its amount increases with the rise of the l/s ratio. SP-A binds to phospholipids and acts together with two other proteins, SP-B and SP-C, to promote rapid formation of phospholipid surface films, reducing the alveolar surface tension. The fourth surfactant protein, SP-D, could have a function in pulmonary host defense mechanisms.[64]

Surfactant is secreted from the lamellar bodies in alveolar type II cells by exocytosis, and in newborn rabbits up to 95% of surfactant is reutilized by receptor-mediated endocytosis of the type II cells.[62,63] It has been suggested that lung maturation and surfactant production are initiated by endogenous factors in the lung, although the mechanism is not clear yet. These processes would then be accelerated and modulated by circulating hormones[62], which will be discussed later.

1.2.4.2 Development of the antioxidant enzyme system

The development of the surfactant system coincides with enhanced activity of antioxidant enzymes (AOE), such as catalase, glutathione peroxidase, and superoxide dismutase, as observed in lungs of fetal rats, rabbits, guinea pigs, hamsters, and lambs during the last 10-15% of gestation.[69-71] These AOE will scavenge or detoxify the highly reactive O_2 metabolites produced in the processes of normal

respiration in all cells[72], and together with other non-enzymatic antioxidants, such as vitamin A, C, and E, and glutathione, they form the host defenses against reactive O_2 species and O_2 free radicals.[73]

Exposure of neonatal rats to hyperoxia stimulates the AOE activities; this effect is regulated pretranslationally and preceded by an increase of mRNA levels of these enzymes.[74] Assumedly, in prematurely born neonates who need artificial ventilation with supplemental O_2 the antioxidant defenses, consisting of the AOE system and the non-enzymatic antioxidants, cannot adequately protect the immature lungs from damage by O_2 free radicals.[72,73]

1.2.4.3 The influence of hormones on lung maturation

Several hormones have been studied with respect to lung maturation, including glucocorticoids, thyroid hormones, estrogens, androgens, insulin, and prolactin[62,63]. This review pays attention to glucocorticoids and thyroid hormones only, because these two seem the most promising for clinical practice.

Glucocorticoid administration results in some anatomic changes in the lungs, notably larger alveoli and thinner interalveolar septae, and higher numbers of lamellar bodies and type II cells. In addition, lung compliance will improve, not only from increased surfactant production but perhaps also from other factors, e.g. increased elastin production.[63,75,76] The synthesis of phospholipids is stimulated by glucocorticoids, probably by stimulation of the activities of different enzymes such as CT and fatty acid synthase.[62,63] The fibroblasts in the fetal lung could well be the primary site of glucocorticoid action; fibroblasts produce FPF which in its turn acts on type II cells and stimulates surfactant synthesis.[62] Glucocorticoids also enhance the production of SP-A, SP-B, and SP-C.[75,77] Glucocorticoids stimulate the AOE activity together with the surfactant stimulation, which results in higher levels of AOE at birth in newborn rats and lambs.[78-81] The AOE response to hyperoxic exposure after birth has been reported to be increased[82] or unchanged[79]. The intensified catalase activity after prenatal dexamethasone is regulated at the level of gene transcription.[80]

The thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4), which show little placental passage, stimulate PC synthesis and CT activity, but not the synthesis of PG.[62,63] Thyroid-releasing hormone (TRH) readily crosses the placenta, thus raising the T_3-levels in the fetus. However, TRH has been shown to stimulate surfactant secretion, but not the synthesis.[62] Thyroid hormones have a negative effect on the activity of fatty acid synthase, and a negative or absent effect on the regulation of the surfactant proteins.[63,77]

Prenatal administration of T_3 or TRH to fetal rats was shown to depress AOE activity and to shorten survival during prolonged hyperoxia[83-85], whereas in fetal lambs no effect of TRH on AOE activity was reported.[81] Negative regulation of AOE gene expression at the level of gene transcription has been held responsible for the negative effect of TRH on AOE activity in fetal rats.[85]

In humans and several animal species the combined administration of glucocorticoids and thyroid hormones has a synergistic effect on surfactant synthesis.[62,63,77] Others, however, found no additive effects of TRH on the regulation of surfactant proteins or the amount of saturated PC.[86] Adding thyroid hormones to prenatal glucocorticoid treatment had no effect on lung compliance and AOE activity in lambs.[76,81] However, in rat pups the negative effects of TRH on the AOE activity and on survival persisted when TRH was given in combination with dexamethasone.[84,85] These findings make it likely that the possible synergistic effect of glucocorticoids and thyroid hormones on surfactant synthesis coincides with depressed AOE activity at birth and inadequate protection against O_2 free radicals.

In clinical trials glucocorticoids appeared to have a positive effect on the incidence of RDS and the mortality rate of prematurely born infants[75], though the addition of TRH to glucocorticoids was associated with maternal and perinatal risks.[87] The National Institutes of Health Consensus Development Conference on prenatal steroids is strongly supportive of the use of prenatal corticosteroids, even for 24 hours but, if possible, for 48 hours, in all fetuses between 24 and 34 weeks of gestation and at risk of preterm delivery.[88]

1.2.5 Differentiation and maturation of the pulmonary vasculature

Not only endothelial cell growth, but also the formation of a muscular coat, consisting of SMC and extracellular matrix, is essential for the development of vascular tone. Contractile, cytoskeletal, and extracellular matrix protein production undergo important changes during development. These changes may modulate vascular function and have been summarized by Morin and Stenmark.[56] The expression of the two major contractile proteins, actin and myosin, is developmentally regulated. Many contractile proteins that are important for SMC contraction are underexpressed in fetal and newborn vessels. In mature vessels the greater contractile capability is associated with a lessened potential for cell replication. Morin and Stenmark therefore suggest that the underexpression in neonatal SMC of many of the contractile and cytoskeletal proteins found in fully mature SMC, may

explain the rapid hyperplastic response observed in the neonatal vascular wall in response to stress.[56]

1.2.5.1 Regulation of pulmonary vascular tone

In utero, the pulmonary blood flow is low and the pulmonary vascular resistance is high.[56,89] Several factors modulating pulmonary vascular resistance in utero and during transition from intrauterine to extrauterine life have been summarized by Kinsella and Abman.[90] An important contributor is the low O_2 tension in utero (about 19 mm Hg) which results in vasoconstriction.[56,89,90] A possible role of prostaglandins, thromboxanes, and leukotrienes, all metabolites of arachidonic acid, in the maintenance of high pulmonary vascular resistance in utero is still debated but seems unlikely.[56,89]

Recently endothelin type 1 (ET-1) was put forward as a mediator of intrauterine pulmonary vasoconstriction.[90] Two different receptors have now been described in humans: the ET_A receptor, localized to vascular smooth muscle cells, which mediates vasoconstriction and bronchoconstriction, and the ET_B receptor, present on the vascular endothelial cell, which mediates vasodilatation by release of nitric oxide (NO) and vasodilating prostaglandins.[90,91]

Concomitant with the enhanced synthesis of surfactant and antioxidant enzymes towards the end of gestation, the resting pulmonary blood flow doubles from about 4% of combined ventricular output to about 8%.[89] At birth the pulmonary vascular resistance decreases more than 10-fold, leading to an 8 to 10-fold increase in pulmonary blood flow.[56] There is strong evidence that the decline of pulmonary vascular resistance is mediated by nitric oxide (NO).[56,89,90,92] The gene expression of NO synthase (NOS) of two different isoforms (neuronal NOS and endothelial NOS) is developmentally regulated in rat lungs with maximal levels towards term.[93] In addition, high NO levels may decrease the production of ET-1.[91]

Shortly after birth, with the onset of ventilation, the production of prostacyclin (PGI_2) is stimulated and this, together with the increased production of other pulmonary vasodilators such as other prostaglandins, bradykinin, and acetylcholine, sustains further pulmonary vasodilatation.[56,89] It has been suggested that NO, but not prostaglandins, is important as a mediator of the low resting pulmonary resistance in the newborn after the immediate postnatal state. The adult level of pulmonary vascular tone is reached in 2-6 weeks postnatally.[89]

In some clinical conditions, e.g. meconium aspiration syndrome, sepsis, and CDH, pulmonary vascular resistance does not decrease after birth. Persistent pulmonary hypertension of the newborn (PPHN) is a complex disorder characteri-

zed by pulmonary hypertension and altered vasoreactivity, with right-to-left shunting of blood across the patent ductus arteriosus or the foramen ovale, leading to severe hypoxemia. Morin and Stenmark extensively describe the PPHN-associated pathological changes of smooth muscle cells and extracellular matrix.[56] Management of PPHN focuses on correction of the alveolar hypoxia and hypercarbia, and dilatation of the pulmonary vasculature.

1.3 Abnormal lung growth and development in CDH

Two types of animal models have been developed to study the features of CDH: models in which CDH is surgically created and those in which CDH is induced by prenatal exposure of the animal to a teratogenic agent.[94-101]

Fetal lambs in the late pseudoglandular or canalicular stage of lung development were used for models of the surgical type. Early experiments involved an inflated balloon placed in the thoracic cavity to simulate compression of the lung by growing viscera. Deflation of the balloon could mimick correction of the diaphragmatic defect.[94] Another approach was to make a hole in the left diaphragm at midgestation.[94,95] A recent publication described the surgical creation of a diaphragmatic defect in fetal rabbits at the canalicular stage of lung development.[96] In both models lung hypoplasia was observed in the ipsilateral lung.[95,96] Mainly the lamb model has been used to study the neonatal pathophysiology of CDH, because the large size of the animals provides the opportunity to perform functional studies, and intrauterine therapeutic interventions such as drug therapy and fetal surgery. This type of models is generally criticized for its inherent shortcomings: the defect is created at a relatively late stage of lung development, so that information regarding the natural history of CDH is not obtained.

A different type of model was, therefore, developed, based on the effect of a teratogenic agent, the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen). When administered to the pregnant mother, Nitrofen is known to interfere in rats and mice with development of the lungs and the diaphragm in the offspring.[97-100] The intensity of its effect is dependent on the gestational age and the dosage.[98]

Nitrofen has a stereochemical configuration that strongly resembles that of thyroid hormone and it has been shown in vitro to decrease the binding of T_3 to the α_1 and β_1 form of the thyroid hormone receptor in a non-competitive way. However, it is questionable whether lung hypoplasia results from the decreased binding of T_3 to its receptor in the in vivo situation.[101,102] Lung hypoplasia is

observed in all Nitrofen-exposed fetuses, and is more severe when a diaphragmatic defect is present.[97,100,103]

Mainly rats have been used for the non-surgical model. This type of model has an important advantage because CDH is induced in an early stage of gestation, which provides the opportunity to study the natural history. Furthermore, the large litter size and the relatively short duration of gestation provide a relatively cheap animal model. Therapeutic interventions by antenatal administration of drugs are possible, but this model is less suitable to perform functional studies or to study surgical therapeutic interventions.

This review focuses on the findings with respect to the abnormal lung growth and development in CDH. In addition to observations in humans, results from experimental studies in the lamb and the rat are reported.

1.3.1 Developmental anatomy in CDH

In lungs of infants with CDH reduced numbers of airway and vascular generations have been observed.[12,13] Because the bronchial tree is fully developed at 16 weeks gestation, it is likely that lung growth must have been affected before this period.[20] Classically, it is believed that the pleuroperitoneal canal fails to close at 8-10 weeks gestation and that abdominal viscera herniate into the thoracic cavity, and reduce lung growth through competition for space.[94] Because competition persists during later gestational stages the development of the pulmonary acinus is impaired as well, thus explaining the decreased RAC observed in lungs of CDH patients.[9-12]

Lungs of CDH patients show abnormal morphology of the pulmonary arteries: muscle mass is increased, and muscle is found in arteries of a smaller diameter than normal.[12,14] Similar findings have been observed in rats[104] and lambs[94] with CDH.

After repair of the diaphragmatic defect improvement of the pulmonary abnormalities has been observed, especially in the contralateral lung. Beals and coworkers observed postnatal lung growth at the alveolar level and vascular remodelling resulting in larger and less muscular arteries within the first weeks.[105] A normal total lung volume, though with abnormal structure, was found in three cases of CDH at autopsy after 2.5 to 64 months.[106,107]

Long-term pulmonary sequelae revealed normal or restricted lung capacity together with a normal diffusion capacity under resting conditions.[108-111] Spirometric results were normal[112] or mild airflow obstruction was present.[108,109,111,113,114] Ventilation-perfusion lung scans showed diminished perfusion on the ipsilateral

side, suggesting residual vascular abnormalities in the most hypoplastic lung.[111,114-116] A recent study showed lower maximal O_2 consumption and exercise tolerance in CDH patients than in controls, but this merely reflects a lower degree of physical fitness rather than illness.[117] These studies make not clear whether the observed abnormalities result from the pulmonary abnormalities in CDH or that modes of treatment such as artificial ventilation contribute to the long-term sequelae.

Kluth and coworkers studied the natural history of CDH and lung hypoplasia in Nitrofen-exposed embryonic rats.[118] They noted a diaphragmatic defect on gestational day 14, which corresponds to 5 weeks gestation in humans. Lung hypoplasia was observed at 16 days gestation, corresponding to 8 weeks gestation. In controls with normal development, the pleuroperitoneal openings were too small to allow herniation of the bowels into the thoracic cavity. Protrusion of the liver through the diaphragmatic defect reduces the space available for normal lung growth, and in this concept the lung hypoplasia is therefore secondary to the diaphragmatic defect.[118] However, others have postulated that Nitrofen primarily inhibits growth of the lung bud itself which, therefore, does not grow sufficiently downwards in the pleuroperitoneal canal. Then the complete development of the posthepatic mesenchymal plate, which forms the main tissue of origin for the diaphragm, will be disturbed. Thus, primary hypoplasia of the lung bud may be responsible for the hypoplasia of the posthepatic mesenchymal plate which in turn allows the development of CDH.[99] It has also been suggested that both lung hypoplasia and CDH result from a common pathogenetic process.[100] In humans, it was not possible to show an association between CDH and any of a number of teratogenic agents, or between CDH and maternal thyroid dysfunction.[119] Therefore, the natural history in the rat model of CDH should only be extrapolated to the human situation with a fair amount of reserve.

1.3.2 Expression of growth controlling factors in CDH

The expression of extracellular matrix components, transcription factors, growth factors and neuropeptides has hardly been studied with respect to abnormal lung development in CDH. The abnormal number of airway generations in the lung in CDH suggests a disturbed pattern of branching morphogenesis. However, it is not clear whether insufficient epithelial-mesenchymal interaction or insufficient stimulation by transcription factors or neuropeptides, such as bombesin, contribute to this feature. Preliminary findings in Nitrofen-exposed rats indicate that the

expression pattern of fibronectin, laminin, and collagens III and IV is not different between CDH and controls (A.E. Brandsma, personal communication). In lungs of CDH rat pups, the expression of thyroid hormone receptors in both the ipsilateral and contralateral lung is similar to that of lungs in controls at all stages studied.[120]

The expression of neuropeptides in CDH has been studied in only a few cases. In three human cases of CDH the expression of GRP was lower than in gestational age-matched control lungs, which suggests a connection between normal lung development and levels of GRP.[121]

The role of fetal lung liquid with respect to the pathogenesis of lung hypoplasia in CDH has not been studied. But in the CDH lamb model, tracheal ligation resulted in accelerated lung growth achieved by cell proliferation. It has been assumed that mechanical alveolar distension may stimulate the activity of growth factors. Therefore, application of tracheal occlusion, which is called PLUG (Plug the Lung Until it Grows), may offer new perspectives for therapeutic intervention in CDH.[51,122]

Only one factor that modulates growth of the pulmonary vasculature has recently been studied in rats with CDH. In control lungs this factor, VEGF, was detected in the vessels at the hilum and in pulmonary parenchyma from gestational day 20 onwards, but it was absent in lungs from CDH rats studied from day 16 to day 22. This suggests that decreased VEGF expression in CDH accounts for altered endothelial cell growth resulting in changed pulmonary vascular reactivity.[123]

1.3.3 Differentiation and maturation of lung tissue in CDH

Abnormal differentiation of the lungs has been described in infants with CDH: they showed a retarded development of the pulmonary acinus, with resulting less alveoli in the ipsilateral and the contralateral lungs, though the ipsilateral lung was more affected in all cases studied.[9,10] The same was true for a subjective maturity score of the lungs in CDH.[10] However, a wide variability in morphological and biochemical maturation has been observed.[9] Pringle and coworkers found in lambs with CDH that both the ipsilateral and the contralateral lungs were morphologically immature, showing a solid aspect with an abundance of type II cells.[95] Nitrofen-exposed lungs in the rat had a lower RAC which was lowest in those cases that had developed CDH.[97] Brandsma and coworkers studied the differentiation of Nitrofen-exposed lungs using an antibody against SP-A. They concluded that Nitrofen-exposed lungs show retarded differentiation from type II into type I cells,

irrespective of the presence of CDH. In addition, lungs of rats with CDH showed retarded development of future airspaces.[103]

1.3.3.1 The surfactant system in CDH

The studies in humans, lambs, and rats conducted so far do not decisively answer the question whether morphologically retarded differentiation delays or alters the development of the surfactant system. The lamellar bodies in the type II cells that are responsible for the intracellular surfactant pool have been studied in human lungs and in the rat model. The ipsilateral lungs of infants with CDH had fewer lamellar bodies than the contralateral lung.[9] In rats with CDH there was no difference in numbers compared with controls, but an unusual appearance of the lamellar bodies has been observed.[124]

In amniotic fluid of CDH patients both normal and decreased l/s ratios have been reported.[125,126] The concentrations of saturated PC and SP-A were lower[127], whereas the concentration of PG was normal.[125] Normal l/s ratios and PG have also been found in amniotic fluid of lambs with CDH.[128] However, in the same lambs the total amounts of phospholipids and the percentage PC in bronchoalveolar lavage (BAL) fluid, representing the extracellular surfactant pool, were lower than those of controls, suggesting that the l/s ratio in amniotic fluid does not adequately reflect the lung maturity state.[128,129] In BAL fluid of rats with CDH normal phospholipid fractions and normal SP-A concentrations have been reported[124], but lower disaturated PC concentrations have been observed in lung homogenates.[130]

Recent findings in the rat model of CDH suggest that the smaller amount of FPF produced by lung fibroblasts in CDH is responsible for a lower CT activity in fetal type II cells. This enzyme forms the rate-limiting step in PC synthesis[66] and its decreased activity may therefore explain the lower levels of PC and disaturated PC that have been reported in CDH (L.J.I. Zimmermann, personal communication).

The expression of SP-A, SP-B, and SP-C mRNAs have been studied in lung homogenates of rats with CDH. On gestational day 18 a significantly lower level of SP-A mRNA was observed in CDH lungs than in control lungs, and the same was true for SP-B and SP-C mRNA on gestational day 20. However, on day 22 the mRNA levels of all three proteins were similar for CDH and control lungs, suggesting that delayed lung maturation in CDH, as far as the levels of surfactant protein mRNAs are concerned, is abolished towards term.[131]

1.3.3.2 The antioxidant enzyme system in CDH

The AOE activity has been studied in the rat model of CDH only. It appeared that catalase, superoxide dismutase, and glutathione peroxidase had normal activity during the last days of gestation and at birth. A short period of artificial ventilation with 21% O_2 declined the activity of glutathione peroxidase, which even became more pronouncedly after ventilation with 100% O_2. These findings make it likely that the development of the AOE system is not retarded in CDH, though that they may be prone to develop oxygen-induced lung damage.[132]

1.3.3.3 The influence of hormones on lung maturation in CDH

The effects of prenatal hormonal treatment on lung maturity have been studied in rats and lambs with CDH. Glucocorticoids had a positive effect on the abnormal lung morphology seen in CDH: the airspaces became larger and the septae became thinner.[133,134] After dexamethasone the concentration of disaturated PC increased and the glycogen levels decreased in lungs of rats with CDH, whereas the mRNA levels of SP-A, SP-B, and SP-C remained unchanged.[133,135] Prenatal administration of cortisol in lambs resulted in a significant decrease of glycogen in the contralateral, but not the ipsilateral lung, whereas the concentration of disaturated PC did not change.[134] Lung compliance and postductal pO_2 were significantly higher in cortisol treated lambs compared to saline-treated controls.[134]

In rats, the administration of TRH alone increased the disaturated PC concentration in CDH lungs, but had no effect on glycogen. A synergistic effect of dexamethasone and TRH on the increase of disaturated PC level and the decrease of glycogen was observed in lungs of CDH pups.[135] The effects of hormones on the AOE system in CDH have not been studied yet.

1.3.4 Differentiation and maturation of the pulmonary vasculature in CDH

The expression of myosin heavy chain isoforms has been studied in the rat model of CDH to evaluate the smooth muscle cell differentiation. The lungs of controls and those of rats with CDH showed similar patterns in the expression of α-actin, SMemb, SM1, SM2, and PDGF.[123,136] This suggests that differences in smooth muscle cell differentiation do not account for the vascular abnormalities in CDH. However, in rat lungs with CDH increased intracellular levels of calcitonin gene-related peptide were found towards the end of gestation. This neuropeptide acts as a pulmonary vasodilator, and may be involved in the development of pulmonary hypertension in CDH.[136]

1.3.4.1 Regulation of the pulmonary vascular tone in CDH

Persistent pulmonary hypertension contributes to the high mortality and morbidity rate in CDH.[4] Apart from the morphological abnormalities in the pulmonary vasculature observed in CDH patients, it may well be that an altered expression of factors that are known to be involved in the regulation of the vascular tone at birth determine the pathogenesis of PPHN in these patients.

One of the factors studied recently is endothelin. Increased plasma levels of endothelin have been reported in infants with and without CDH who suffered from PPHN.[137,138] In addition, lungs of CDH patients showed increased ET-1 immunoreactivity as compared with lungs of control patients at autopsy.[137] In lungs of rats with CDH the mRNA levels of ET-1 did not differ from those of controls, and in both groups the mRNA levels rose significantly after prenatal treatment with dexamethasone.[133]

It has been suggested that NO is an important mediator to reduce the pulmonary vascular resistance at birth.[56,89,90,92] A failure to release NO may contribute to the pathogenesis of PPHN in CDH. Therefore, several studies in the lamb model and the rat model have been performed to evaluate the expression of endothelial NO synthase (eNOS) at the end of gestation or shortly after birth.[133,139-141] In lambs with CDH a qualitative analysis showed the presence of eNOS in the main pulmonary artery trunks, and likewise in controls.[139] Quantitative studies of the lung parenchyma in rats with CDH, however, showed that the eNOS activity and the corresponding mRNA expression were lower than in control lungs.[140,141] Suen and coworkers found normal eNOS mRNA levels that were not changed after prenatal treatment with dexamethasone in lungs of CDH pups.[133] These findings suggest that a deficiency of endogenous NO production may contribute to PPHN associated with CDH.

The role of prostaglandins, which may reduce the pulmonary vascular tone postnatally after the onset of ventilation[56,89], has not been studied in CDH during the transition from intrauterine to extrauterine life. Increased plasma levels of the stable metabolites of the pulmonary broncho-, and vasoconstrictor thromboxane A_2 (TxA_2) and the pulmonary vasodilator prostacyclin (PGI_2), TxB_2 and 6-keto-$PGF_{1\alpha}$, respectively, have been observed in CDH patients during episodes of hypoxemia[142], and in the immediate postoperative period.[143-145] This may be a reflection of PPHN and not a specific feature of CDH, however, because increased levels of eicosanoids have also been reported in plasma and in BAL fluid of infants with PPHN without CDH who were treated with conventional ventilation or with extracorporeal membrane oxygenation (ECMO).[146-149]

1.4 Concluding remarks and aims of the studies

Many aspects of abnormal lung growth and development in CDH have already been studied, and the most important findings are summarized in this overview. However, many other aspects still remain to be studied: The etiology of CDH in humans is still unknown. Though the abnormal developmental anatomy has been extensively described, the role of lung growth controlling factors, such as growth factors and neuropeptides, has not been elucidated yet. Many studies indicate that lung differentiation is delayed in CDH; it is still debated, however, whether the morphologically immature lungs are surfactant deficient. Positive effects of prenatal hormonal therapy on lung morphology, lung compliance, and surfactant content in animal models of CDH have been described, but the lung morphology following artificial ventilation and the effects of prenatal hormones on antioxidant enzyme activity in CDH have not been studied. Furthermore, it is still unknown what structural and functional factors contribute to PPHN in CDH patients. Assumedly, altered concentrations of vasoactive agents, such as eicosanoids, may be involved in abnormal regulation of the pulmonary vascular tone in lungs of CDH patients in the perinatal period. It is unclear why such high incidence of bronchopulmonary dysplasia has been observed in CDH patients; an imbalance in factors that should protect the lungs from barotrauma and oxygen toxicity on the one hand, and inflammatory mediators on the other hand, may contribute to this phenomenon. In addition, eicosanoids involved in the pathogenesis of PPHN in CDH may also induce inflammatory processes, and thus contribute to the above mentioned imbalance. From the few studies that have addressed the long-term pulmonary sequelae in CDH it appeared that the structure of the lungs remains abnormal, and that only mild lung function abnormalities were present. It is not clear, however, whether the observed abnormalities result from the pulmonary abnormalities in CDH or from modes of treatment such as artificial ventilation.

Studies regarding the etiology of CDH in humans are not within the scope of this thesis. The studies reported in this thesis were conducted to investigate various, related aspects of lung development and lung injury in CDH. Figure 1 shows a concept with respect to the abnormal lung development in CDH and the contribution of different factors. Factors that may contribute to lung injury following artificial ventilation in CDH are shown in Figure 2. In this figure, we also suggest two possibilities for intervention to prevent ungoing lung injury.

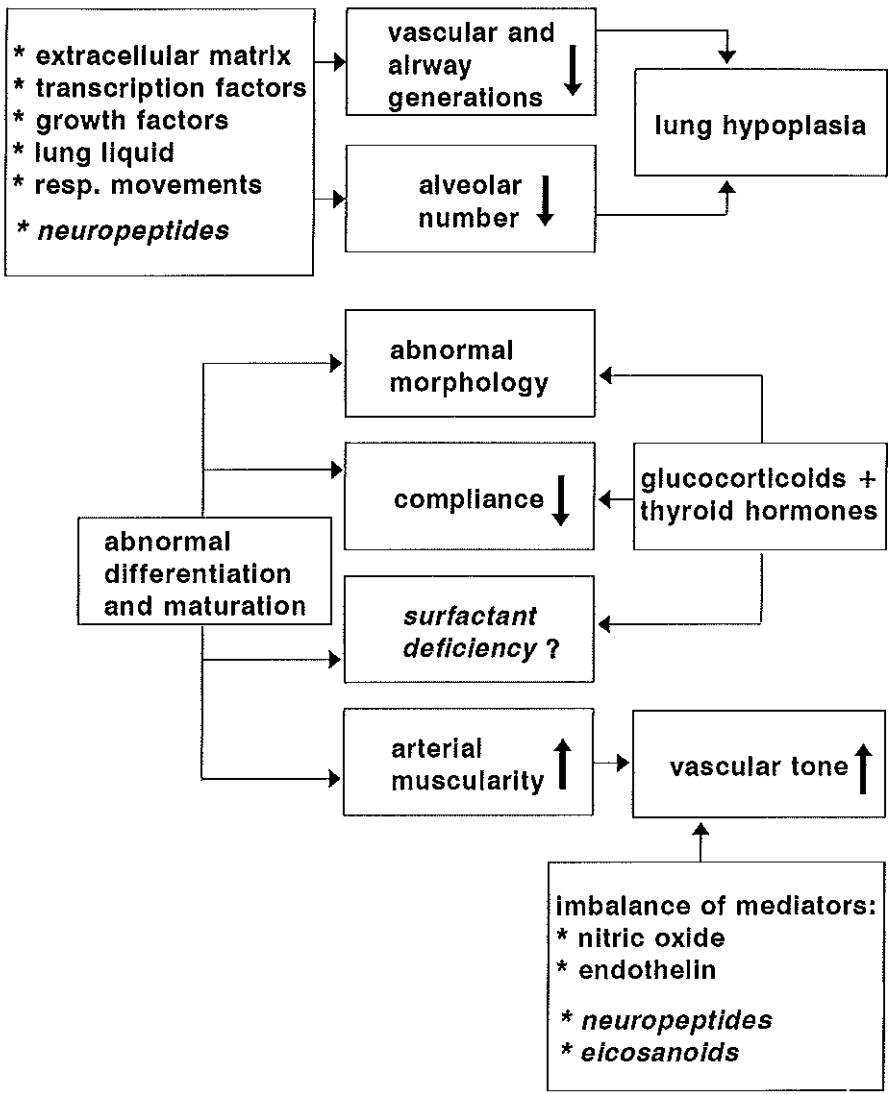

Figure 1: Concept of factors involved in abnormal lung development in CDH. Aspects that will be described in this thesis are shown in italics.

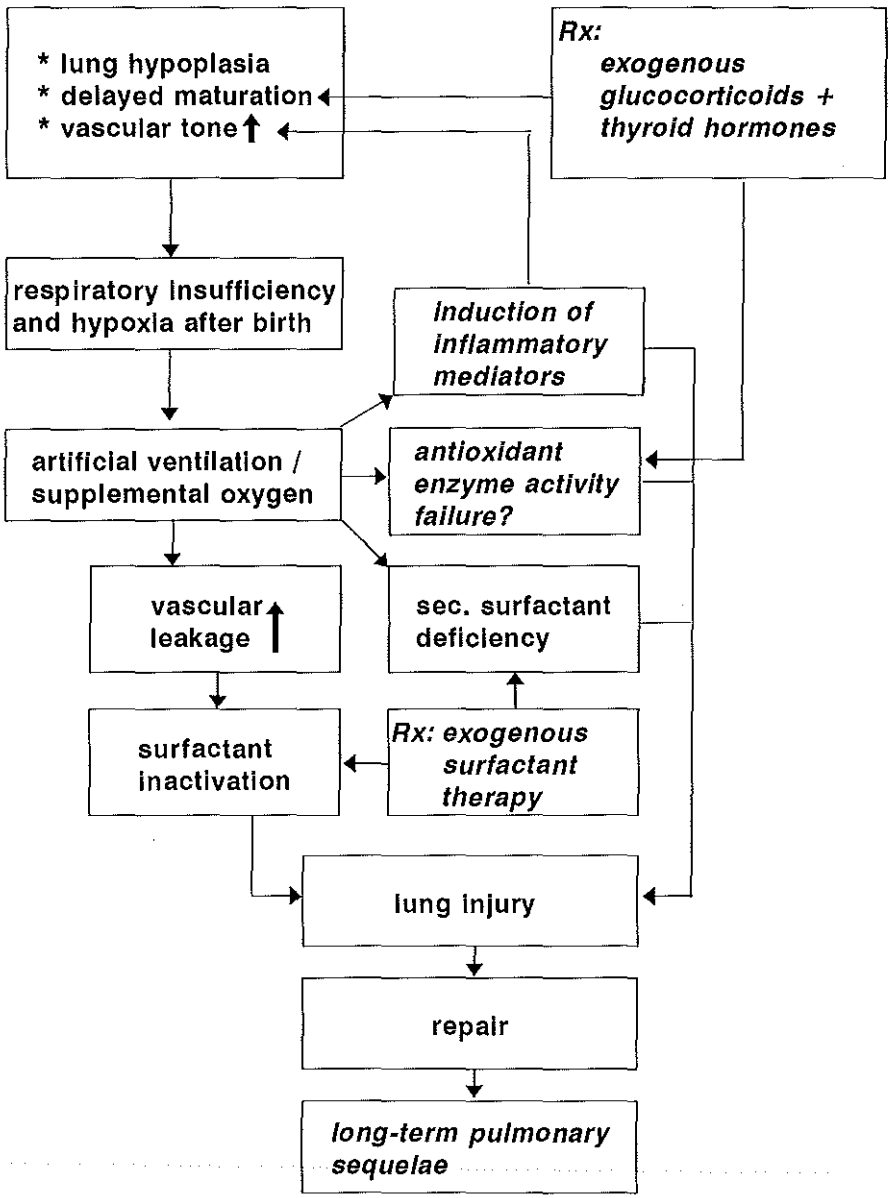

Figure 2: Concept of factors contributing to lung injury caused by artificial ventilation in CDH. Aspects that will be described in this thesis are shown in italics.

The studies presented in this thesis which aim at answering the following questions are indicated *in italics* in Figures 1 and 2:

1. Does retarded differentiation of the lungs in CDH result in primary surfactant deficiency?
2. Is the expression of pulmonary neuroendocrine cells during lung development altered in CDH?
3. Are eicosanoids involved in the pathogenesis of PPHN in CDH?
4. What are the long-term pulmonary sequelae in CDH patients and do they differ from the pulmonary sequelae in matched controls who also underwent artificial ventilation in the neonatal period?
5. Are the lungs in CDH prone to damage by oxygen free radicals on account of a failing antioxidant enzyme system?
6. Does an uneven distribution of exogenous surfactant explain why this therapy not always yields positive results in CDH?
7. What are the effects of prenatal hormonal modulation on antioxidant enzyme activity and lung morphology in CDH?

First, studies of the aspects of lung development will be presented (Part II of this thesis), followed by studies of the aspects of lung injury (Part III). Each part consists of clinical and experimental studies, which will be described in that order.

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Part II

Aspects of lung development in CDH

Chapter 2

Prospective evaluation of surfactant composition in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age-matched controls*

2.1 Summary

It has been suggested that infants with congenital diaphragmatic hernia (CDH) have morphologically and biochemically immature lungs. However, normal lecithin/sphingomyelin ratios (l/s ratios) and phosphatidylglycerol (PG) concentrations have been reported in amniotic fluid of CDH patients. We hypothesized that surfactant deficiency in lungs of CDH patients would be reflected in an altered surfactant composition in BALF compared to that of age-matched controls. Therefore, we measured the concentrations of different surfactant phospholipids and the fatty acid composition of phosphatidylcholine (PC) in BALF of conventionally ventilated CDH patients, ECMO-treated CDH patients, age-matched conventionally ventilated controls without pulmonary abnormalities, and ECMO-treated infants without CDH. No significant differences between the concentrations of PC and PG, and the l/s ratios were found between the four groups. The fatty acid composition of PC in conventionally ventilated patients showed a median percentage of palmitic acid of 68% in CDH patients and 73% in controls ($p < 0.001$). Our findings indicate that the concentrations of different phospholipids are similar in CDH patients and controls without CDH, but that the surfactant composition of PC is slightly altered. A primary surfactant deficiency which determines their clinical course seems unlikely in infants with CDH, but secondary surfactant deficiency following respiratory failure may be involved.

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2.2 Introduction

Infants with congenital diaphragmatic hernia (CDH) have abnormal morphological development of lungs and intrapulmonary blood vessels.[1-4] Artificial ventilation with high peak inspiratory pressures and a high inspired oxygen fraction is often required in the neonatal period. This treatment may result in bronchopulmonary dysplasia, a chronic lung disease which occurs mainly in prematurely born infants with respiratory distress syndrome and surfactant deficiency.[5,6] Bronchopulmonary dysplasia has been described in 33% of CDH survivors, despite a mean birth weight of nearly 3000 grams.[7] Several publications suggest that in infants with CDH the lungs are biochemically immature[1,3,8,9], but this is contradicted by others.[10] Contradictory results regarding lung maturity in CDH have also been reported in animal models.[11-13] In premature neonates with respiratory distress syndrome or chronic lung disease the composition of surfactant has been studied in tracheal aspirates or in bronchoalveolar lavage fluid (BALF).[14,15] In CDH patients surfactant levels in lung tissue have only been measured in non-survivors[3] and the surfactant composition only in amniotic fluid.[8-10] However, it has been suggested that in CDH the surfactant composition in amniotic fluid does not adequately predict the actual surfactant status.[16]

During normal lung development the production of surfactant increases towards the end of gestation, and the composition of different phospholipids and phospholipid fatty acids changes.[17-20] We hypothesized that surfactant deficiency in lungs of CDH patients would be reflected in an altered surfactant composition in BALF compared to that of age-matched controls. Therefore, we measured the concentrations of different surfactant phospholipids and the fatty acid composition of phosphatidylcholine (PC) in BALF of ventilated CDH patients and of age-matched, ventilated controls without pulmonary abnormalities.

2.3 Patients and methods

2.3.1 Patients

The study was performed in our Pediatric Surgical Intensive Care Unit between December 1993 and January 1996. Four different groups were studied: two groups of CDH patients and two control groups. Thirteen CDH patients underwent conventional ventilation (referred to as the CDH-CV group), and five infants with

CDH were treated with venoarterial extracorporeal membrane oxygenation (ECMO; referred to as the CDH-ECMO group) using standardized treatment protocols.[21-23] In four patients CDH had been diagnosed prenatally. Ten patients in the CDH-CV group and all five ECMO patients suffered from respiratory insufficiency within six hours after birth, and were, therefore, considered to be high-risk patients[7]; the other three patients developed respiratory failure after 10 hours, 36 hours, and 28 days, respectively. Operative repair by an abdominal approach was performed in 11 conventionally ventilated children and in three ECMO patients after preoperative clinical stabilization[21]; four of the five patients who died had not been operated on.

Fourteen other children without pulmonary abnormalities, who were mainly ventilated perioperatively, served as age-matched controls (referred to as the C-CV group). They were selected for the best possible match for gestational age and birth weight. In addition, six term born infants without CDH who were treated with ECMO were included in the study (referred to as the C-ECMO group).

For all patients in our institution the entry criteria for ECMO were: gestational age at least 34 weeks, birth weight at least 2000 grams, artificial ventilation for less than 7 days, alveolar-arterial oxygen difference ($A\text{-}aDO_2$) > 600 torr.[22,23] A maximal PaO_2 of at least 10 kPa was an additional entry criterium for CDH patients. During ECMO ventilatory settings were usually reduced to PIP 12-16 cm H_2O, PEEP 5-6 cm H_2O, rate 10-15/min, and FIO_2 0.25-0.3.

In all 38 patients sputum cultures were routinely performed every three days. All patients received antimicrobial therapy. Informed consent was obtained according to the guidelines of the Institutional Review Board of our hospital.

2.3.2 *Study design*

Of each patient the following data were recorded: gestational age, birth weight, diagnosis, survival, age at admission and at discharge or death, age at start of respiratory insufficiency, and, if applicable, age at start ECMO, duration of ECMO and/or artificial ventilation, and duration of supplemental O_2 therapy.

Bronchoalveolar lavage was performed on day 1, day 3, and day 7 after onset of ventilation or ECMO, and once every week thereafter as long as endotracheal intubation was continued and the child remained in our Intensive Care Unit. An unstable clinical condition during the first days was a reason to modify this schedule.

The following data were recorded at each BAL procedure: medication, clinical events such as surgery, mean airway pressure (MAP), oxygenation index (OI), and

$AaDO_2$.[21] Arterial blood gases and serum urea concentration, to correct BAL parameters for dilution, were obtained within six hours from the time of BAL.

2.3.3 BAL procedure

The procedure was performed directly after routine endotracheal suctioning by the nursing staff using a technique described by Grigg and coworkers.[24] The patient's head turned left, a 5 Fr open-ended catheter (outer diameter 1.7 mm; Sherwood Medical, Petit Rechain, Belgium) was passed down the endotracheal tube and placed in wedge position. Warmed NaCl 0.9% was instilled in 2 aliquots of 1 ml/kg each. Gentle manual suctioning with a 20 ml-syringe was directly performed after each aliquot. In most cases the ventilatory circuit was not broken. The whole procedure took always less than one minute. The volume of the recovered fluid was measured and then the fluid was immediately centrifuged at 900 g for 5 minutes. The supernatant was frozen at -80°C.

2.3.4 Measurements in BAL fluid

A total of 78 samples was obtained (CDH-CV n=33; CDH-ECMO n=16; C-CV n=19; C-ECMO n=10). In 300 µl of the supernatant the lipids were extracted according to Bligh and Dyer.[25] The phospholipids were then separated by thin-layer chromatography[26] using Kieselgel 60 TLC plates (Merck, Darmstadt, Germany) and a Canag Linomat IV (Merck, Darmstadt, Germany). The concentrations of PC, phosphatidylglycerol (PG), and sphingomyelin (S) were determined by a phosphorus assay according to Bartlett.[27]

In 22 samples (CDH-CV n=8; CDH-ECMO n=4; C-CV n=10), sufficient fluid was recovered to perform analysis of the PC fatty acid composition by gaschromatography (Hewlett Packard 5890 series II, Amstelveen, The Netherlands). For this experiment lipids were extracted in 300 µl of BAL fluid and surfactant phospholipid isolation was performed as described above. The PC was derivatised[28], and the fatty acid methyl esters were extracted. One µl of fatty acid extract was injected splitlessly on a Omega wax 250 column (10 m, 0.25 mm ID, 0.25 µm film thickness; Supelco, Zwijndrecht, The Netherlands). At injection (280°C) the column was at 80°C for 2 min and increased with 25°C/min to 180°C, and then with 10°C/min to 220°C, held for 1.5 min. Subsequently, the temperature was ramped with 25°C/min to 240°C, for 2 min.

To correct for dilution, urea was determined in serum with a routinely used in-house urease-based assay. Urea in BAL fluid was measured using a commercially available urease-based kit (Merck 3334; Merck, Darmstadt, Germany) that was more sensitive than the assay for measurement of plasma concentrations. The volume of epithelial lining fluid (ELF) was calculated from the formula: ELF = (BAL fluid urea/serum urea) x BAL fluid volume.[24]

2.3.5 Data analysis

Data were expressed as median (range) unless stated otherwise. Comparisons of patient characteristics and BALF recovery between the groups were performed using one-way analysis of variance with the Student-Newman-Keuls test, or the non-parametric Kruskall-Wallis test when appropriate. To compare the concentrations of surfactant phospholipids in BALF, the data were divided in four categories of duration of ventilation or ECMO treatment: Day 1-2, Day 3-5, Day 6-14, and Day 15 or more. In nine cases more than one measurement of phospholipids of an individual patient was performed within a single time category, in which cases the average of the measurements per time category was used. Phospholipid concentrations in BALF were compared between the two conventionally ventilated groups, or between both ECMO-treated groups using the non-parametric Mann-Whitney test. To obtain a homogeneous group, two CDH-CV patients were excluded from this part of statistical analysis: one prematurely born patient (gestation 34 wks, birth weight 1550 gr) and one infant in whom CDH was diagnosed at the age of 28 days. To determine the reproducibility of our methods Wilcoxon's signed rank test was used to compare duplicate measurements or to compare two consecutive samples of the same patient.

For the analysis of the PC fatty acid composition the relative contribution of different fatty acids was calculated. Because of the small number of samples for this part of the study data from conventionally ventilated patients were only statistically analyzed on Day 1-2 using Student's t-test or the non-parametric Mann-Whitney test. In four cases two measurements of fatty acid composition of an individual patient were performed on Day 1 and 2 or on Day 5-7; the average of both measurements was then calculated. One sample from the one-month old CDH patient was again excluded from statistical analysis. Spearman's rank correlation coefficient was used to study the relationship between different parameters in BALF and ventilatory parameters. Two-tailed statistical significance was assumed at a 5% level.

Table 1: *Patient characteristics of controls and CDH patients*

	CDH-CV; n=13 (11)[a]	C-CV; n=13 (13)[a]	CDH-ECMO; n=5 (2)[a]	C-ECMO; n=6 (6)[a]
gestational age (wks)	38 (34-41)	38 (36-41)	39 (34-42)	40 (36-41)
birth weight (gr)	3140 (1550-4340)	2705 (2350-3910)	3000 (2380-3630)	3800 (2610-4325)
ventilation (d)[b]	10 (3-33)[d]	3 (1-8)	42.5 (34-41)[e]	8 (6-14)
supplemental O_2 (d)[b]	15 (3-47)[d]	4 (1-12)	58 (35-81)[e,f]	14 (7-25)
age at start ECMO (h)	----	----	16 (6-42)	24 (10-55)
duration of ECMO (d)	----	----	14 (6-25)	5 (3-7)
MAP[c] (Day 1-2)	20 (4.6-50)	10 (6.5-25)	----	----
(Day 3-5)	22 (9.7-44)[d]	8.6 (5.1-11)	----	----
OI[c] (day 1-2)	5.4 (1-22)	3.6 (2.6-13)	----	----
(day 3-5)	5.5 (3-13)[d]	1.6 (1.1-2.8)	----	----
A-aDO_2[c] (day 1-2)	121 (17-211)	61 (18-230)	----	----
(day 3-5)	64 (33-235)[d]	22 (18-35)	----	----

The median (range) values are shown for different patient characteristics. C-CV = conventionally ventilated controls; CDH-CV = conventionally ventilated CDH patients; C-ECMO = ECMO-treated controls; CDH-ECMO = ECMO-treated CDH patients.

[a] the total number of patients per group are shown, the number of survivors are shown in brackets; [b] only data from survivors are shown; [c] parameters calculated only at the time that BAL was performed, day 1-2: controls n=9, CDH-CV n=6; day 3-5: n=5 for both groups; [d] significantly different from C-CV, $p < 0.05$; [e] significantly different from all three other groups, $p < 0.05$; [f] one child again received O_2-therapy one month later and is still O_2-dependent at 27 months of age.

2.4 Results

2.4.1 Patient characteristics

The characteristics of all patients are summarized in Table 1. Ventilated controls had the following diagnoses: meconium peritonitis (n = 4), oesophageal atresia (n = 4), M. Hirschsprung, necrotizing enterocolitis, volvulus, vesical exstrophy, hiatus hernia with Ehler-Danlos syndrome, wet lung (each n = 1). Controls treated with ECMO had the following diagnoses: meconium aspiration with persistent pulmonary hypertension of the newborn (PPHN; n = 3), neonatal sepsis with pulmonary hemorrhage and PPHN, blood aspiration and PPHN, pneumonia (each n = 1).

Ten CDH-CV patients had left-sided CDH and three had a right-sided defect. Two of them, who never met the ECMO entry criteria (low birth weight or pO_2 < 10 kPa), died during the first day of life. Four CDH-CV patients were O_2-dependent at the age of 28 days. One of the CDH-ECMO patients had a bilateral diaphragmatic defect, the other four had left-sided CDH. Two survivors were operated on while undergoing ECMO, and they were both O_2-dependent at 28 days. The other three CDH-ECMO patients died within 5 to 11 days after decanulation because of recurrent therapy-resistant PPHN. Gestational age and birth weight were not significantly different between the four groups (Table 1).

2.4.2 Correction for dilution

To correct for dilution, the urea concentrations in BAL fluid and in serum were used to calculate the volume of epithelial lining fluid (ELF). In all groups the concentrations of PC and PG in BALF showed a strong positive correlation with those in ELF ($p < 0.001$). We therefore decided to present the uncorrected data, i.e. per ml BAL fluid. The proportions of recovered fluid did not significantly differ between the groups: C-CV 39 (13-75)%, CDH-CV 25 (14-68)%, C-ECMO 26 (17-56)%, and CDH-ECMO 37 (11-64)%, respectively.

2.4.3 Surfactant phospholipids in BAL fluid

To determine the reproducibility of the measurements four different samples were measured in duplicate or triplicate; there were no significant differences between the measurements. In addition, in four patients who were in a clinically stable

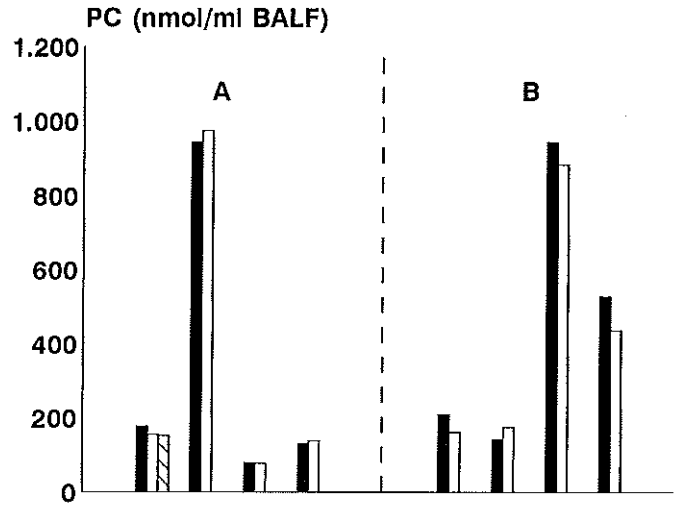

Figure 1: The reproducibility of the measurements was determined in four different samples in which the PC concentration in BALF was measured in triplicate or duplicate (panel A). In four patients who were in a clinically stable condition, as reflected by the values of MAP and OI, the PC concentrations in two samples taken on consecutive days were compared (panel B). No significant differences were observed.

condition, as reflected by the values of MAP and OI, the phospholipid concentrations in two samples taken on consecutive days were compared, and no significant differences were observed. The results for PC are shown in Figure 1. Differences between the groups were not statistically significant.

The concentrations of PC within the different time categories are shown in Figure 2. The concentrations of PG and the lecithin (PC)/sphingomyelin ratios (l/s ratios) are listed in Table 2. In nine samples PG could not be detected: in four samples of CDH-CV patients (including one of a prematurely born infant), in four samples of three CDH-ECMO patients, and in one C-ECMO patient. None of the parameters studied showed significant differences between controls and CDH patients. The median concentrations of PC and PG, and the l/s ratio did not change significantly during the course of treatment in any of the groups. Data from three CDH-CV patients with right-sided CDH did not differ from the others with left-sided CDH (not shown). The same was true for the infants who were still O_2-dependent at the age of 28 days. In the conventionally ventilated groups, there were no significant correlations between ventilatory parameters and the concentrations of PC and PG or the l/s ratio.

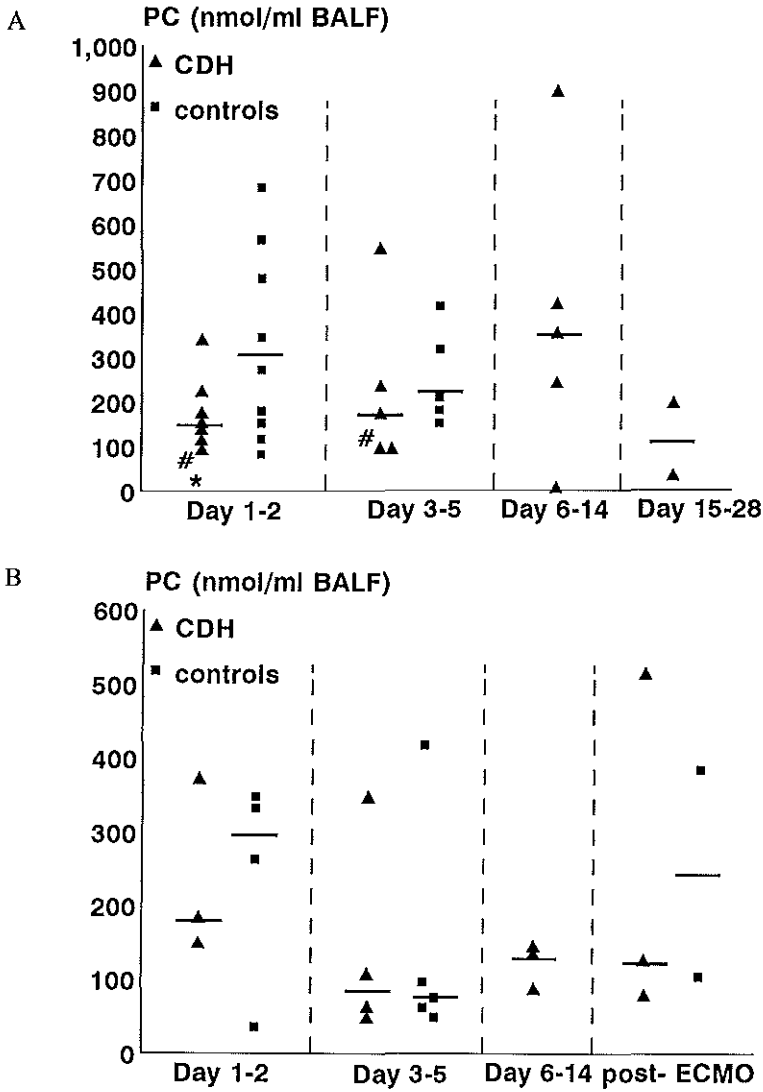

*Figure 2: The PC concentrations in BALF at different time points are shown for conventionally ventilated CDH patients and controls (2A) and for ECMO-treated patients (2B). After 5 days of ventilation or ECMO no samples from controls were obtained. For ECMO-treated patients measurements within the first week after decanulation are shown. The median values are indicated for each time category. No significant differences were observed. #: data from a patient in whom CDH was diagnosed at the age of 28 days; *: value from a CDH patient born after 34 weeks of gestation who died during the first day of life. Both patients were excluded from statistical analysis to obtain a homogeneous group.*

***Table 2:** Surfactant phospholipids in BALF of CDH patients and controls*

		PG (nmol/ml BALF)	l/s ratio
CDH-CV	Day 1-2 (6)[a]	22.5 (2.5-56.4)	18 (13-24)
	Day 3-5 (5)	12.4 (0.7-61)	27 (5-84)
	Day 6-14 (6)	5.7 (0-106)	68 (0.4-228)
	Day 15 > (2)	0.5 (0-1)	36 (4-69)
C-CV	Day 1-2 (10)	51.2 (1.5-89.2)	29 (2-95)
	Day 3-5 (5)	22.5 (13-25.6)	47 (10-137)
CDH-ECMO	Day 1-2 (3)	4.6 (3.9-38.3)	24 (15-33)
	Day 3-5 (4)	16.8 (0-30.4)	10 (5-180)
	Day 6-14 (3)	10 (2.5-10.1)	18 (17-96)
	post-ECMO (3)	16 (0-59)	28 (4.4-180)
C-ECMO	Day 1-2 (4)	21.7 (10.5-47.5)	23 (3.2-34)
	Day 3-5 (5)	4.5 (0-20)	8.8 (4.4-22)

The median (range) values are shown for phosphatidylglycerol (PG) and l/s ratio in BALF. C-CV = conventionally ventilated controls; CDH-CV = conventionally ventilated CDH patients; C-ECMO = ECMO-treated controls; CDH-ECMO = ECMO-treated CDH patients.
[a] *The numbers of patients per group are shown in brackets.*

2.4.4 Fatty acid composition of PC

The relative contributions of the most important fatty acids are shown in Table 3. The percentage of palmitic acid (16:0) was significantly higher in C-CV patients than in CDH-CV patients during the first two days of ventilation ($p = 0.001$), whereas the proportion of arachidonic acid (20:4w6) was higher in CDH-CV patients compared with C-CV ($p=0.02$). The total proportion of saturated fatty acids was 82 (80-82)% in C-CV and 78 (77-80)% in CDH-CV on Day 1-2 ($p = 0.001$); the proportion of unsaturated fatty acids (mono-unsaturated and poly-unsaturated fatty acids) was thus 18 and 22%, respectively. In three C-CV patients the proportion of palmitic acid on Day 3 was 70 (69-71)%, in two CDH-CV patients the proportions were 60 and 61% on Days 5-7. In two CDH-ECMO

patients the fatty acid composition was determined: the percentages palmitic acid in one patient consecutive measurements on Day 1, 4, and 12 of ECMO were 64, 62, and 63%, respectively; in another CDH-ECMO patient this percentage was 70% on Day 1. Both of these patients died a few days after decanulation from ECMO. In one patient in whom CDH was diagnosed at the age of 28 days the proportion of palmitic acid was 74%.

Table 3: *Fatty acid composition of phosphatidylcholine in conventionally ventilated CDH patients and controls*

	CDH-CV		C-CV	
	Day 1-2 (n=4)	Day 3-7 (n=2)	Day 1-2 (n=6)	Day 3 (n=3)
14:0	4.7 (3.3-6.3)	3.2 (2.6-3.8)	4.4 (3.2-6.4)	5.7 (4.5-6.8)
16:0	68 (67-70)[a]	60.5 (60-61)	73 (71-75)	70 (69-71)
16:1w7	4.6 (2.8-7.5)	3.8 (3.6-4)	4 (0.8-4.8)	4.9 (0.3-5.2)
18:0	4.3 (3.6-6)	4.8 (3.1-6.4)	3.7 (2.9-4.6)	3.5 (3.4-4.4)
18:1w9	6.3 (5.7-7.2)	9.9 (9-10.9)	5.5 (5.4-6.4)	7.1 (6.9-7.3)
18:2w6	2.9 (2.2-3.2)	5.9 (5.5-6.4)	2.3 (1.7-2.7)	2.1 (1.8-2.6)
20:4w6	2.9 (1.8-3.5)[a]	3.1 (2.9-3.3)	1.5 (1.1-2.2)	2.3 (1.5-2.5)

The median (range) percentages of fatty acids in PC are shown for conventionally ventilated CDH patients (CDH-CV) and controls (C-CV). Statistical comparison between groups was only performed during the first two days. Other fatty acids studied are 14:1, 15:0, 16:1w9, 18:1w7, 18:3w6, 18:3w3, 20:2w6, and 20:3w6. These fatty acids contributed each less than 1.5% and were, therefore, not shown. [a] *significantly different from control group on Day 1-2;* $p < 0.05$.

2.5 Discussion

In the present study, which is to our knowledge the first study of surfactant composition in BAL fluid of CDH patients, we found similar concentrations of PC and PG, and l/s ratios in BALF of infants with CDH and of age-matched controls without CDH. The fatty acid composition of PC showed a slightly, but significantly lower proportion of palmitic acid in samples of conventionally ventilated CDH patients compared with controls, and the same was true for the total proportion of saturated fatty acids.

The composition of surfactant in tracheal aspirates, BALF, and amniotic fluid has mainly been studied in premature infants with respiratory distress syndrome, and several indicators of lung immaturity have been put forward. Towards the end of gestation the proportion of phosphatidylcholine increases, whereas that of sphingomyelin decreases; this results in an increase in the l/s ratio.[17] L/s ratios of at least 2 to 3 reflect lung maturity.[19,29] Phosphatidylglycerol can be detected from gestational week 35 onward, and the presence of this phospholipid is highly predictive (up to 100%) that respiratory distress syndrome will not occur.[19,30]

Several studies indicate that in CDH the lungs are not only morphologically, but also biochemically immature.[3,8,9] In lung tissue of infants with CDH the concentration of disaturated PC (DSPC) in ipsilateral lungs was significantly lower than that in contralateral lungs and that in lungs of age-matched controls.[3] The contralateral lungs of CDH patients had similar DSPC levels as control lungs.[3] In amniotic fluid of infants with CDH decreased l/s ratios, saturated PC levels, and surfactant protein-A levels have been reported[8,9], whereas others found normal l/s ratios and PG concentrations.[10] In amniotic fluid of lambs with CDH normal l/s ratios and PG concentrations were present, whereas in BALF of the same lambs the total amount of phospholipids and the percentage PC were significantly lower compared with controls, thus suggesting that the l/s ratio in amniotic fluid does not reflect lung maturity in CDH.[16] Decreased DSPC levels have also been observed in lungs of rat pups with CDH.[12] In BALF of rat pups with CDH the composition of surfactant and the concentrations of surfactant protein-A did not differ from those of control pups.[13] Preliminary results from Batenburg and coworkers show that the mRNA levels for the surfactant proteins A, B, and C are lower in rat pups with CDH compared with controls on gestational day 18 or 20, but that similar levels are present on day 22.[31]

Our observations support findings from Sullivan and coworkers to the effect that the lungs in CDH are not surfactant deficient.[10] We found that the concentrations of PC and PG, and the l/s ratios in BAL fluid of term born CDH patients were not significantly lower than those of age-matched controls, but there was a trend towards decreased levels for all three parameters. This may indicate that an initial delay in lung maturation has largely been regained towards the end of gestation.[31] Eight of the nine samples with negative PG were obtained from CDH patients. One of the PG-negative samples was from an infant of 34 weeks gestation who had a very low concentration of PC in BAL fluid, which may indicate that the lungs of this CDH patient were immature. From the other seven CDH patients earlier samples were obtained with positive PG. Assumedly, the negative values in these

samples are indicative of a secondary surfactant deficiency caused by respiratory failure.[32]

The fatty acid composition of phosphatidylcholine has been studied in tracheal or gastric aspirates of preterm infants with and without respiratory distress syndrome (RDS) and of fullterm infants, and in BAL fluid of adults.[32-34] During the first days of life the percentages of palmitic acid in PC ranged from 58% and 71% in tracheal aspirates of preterms with and without RDS, respectively[32], and it was 59% in fullterm, healthy infants.[34] In healthy adults the proportion of palmitic acid in PC was 68%.[32] The percentage palmitic acid in CDH patients in our study was slightly, but significantly lower than in BAL fluid of control patients, but data from both groups were within the ranges that have been published for humans without lung disease.[32-34]

For the BAL procedure, we used a method that has been validated by Grigg and coworkers.[24] They showed that with their technique the catheter is introduced into the right main bronchus and wedged in one of the basal segments of the lower lobe. It is likely, therefore, that we also performed BAL in the right lungs, which is the contralateral lung in 14 of the CDH patients that we studied. According to the findings of Nakamura and coworkers[3] this means that we studied the composition of surfactant in the most mature lung. However, in three cases with a unilateral right-sided diaphragmatic defect the data were not different from the other cases with a left-sided CDH. Moreover, Batenburg and coworkers did not observe any differences in mRNA levels of surfactant proteins between the ipsilateral and contralateral lungs in rat pups with CDH (personal communication, J.J. Batenburg).

We compared the data of the CDH patients with those of controls who were selected for the best possible match for gestational age and birth weight. Our conventionally ventilated control patients did not suffer from pulmonary diseases, and the ventilatory requirements were of a shorter duration and were milder compared to those of the CDH patients. We found no significant differences in concentrations of surfactant phospholipids between ventilated CDH patients and controls without CDH, and no correlation between surfactant components in BAL fluid and ventilatory parameters. Furthermore, the results in ECMO-treated CDH patients were not different from those of the other CDH patients, who had a milder course of the disease. This indicates that it is unlikely that surfactant deficiency contributes to the clinical course in CDH.

The ECMO-treated controls without CDH suffered from primary pulmonary diseases and it can be argued whether they could be considered as true controls.

Their concentrations of PC and PG, and l/s ratios were, however, not different from those in the other groups, especially the non-ECMO controls without lung disease. In a newborn piglet model of meconium aspiration syndrome normal phospholipid concentrations in BALF have been found, but the endogenous surfactant activity was significantly decreased compared with controls.[35] We did not study the surfactant activity, and functional impairment of surfactant by protein leakage and fibrogen degradation products[36,37] cannot be excluded by our data.

In conclusion, our findings suggest that primary surfactant deficiency is unlikely in infants with CDH and that this possibly does not determine the clinical course in these patients. The altered composition of fatty acids in phosphatidylcholine, and the absence of PG in some samples of CDH patients may be caused by their respiratory failure.[32] More studies are needed to confirm our findings.

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Chapter 3

Abnormal expression of pulmonary bombesin-immunoreactive cells in infants with congenital diaphragmatic hernia*

3.1 Summary

Infants with congenital diaphragmatic hernia (CDH) have a high neonatal mortality and morbidity owing to lung hypoplasia and persistent pulmonary hypertension. Pulmonary neuroendocrine cells produce bombesin, a peptide with growth factor-like properties involved in lung development. We examined the expression of bombesin-immunoreactive pulmonary neuroendocrine cells (PNEC), and clusters of these cells called neuroepithelial bodies (NEB), in the lungs of three groups of infants: patients with CDH, newborns with lung hypoplasia due to other causes, and controls without lung abnormalities. Morphometric analysis included: 1) percentage immunostained airways; 2) percentage immunostained epithelium (i.e. frequency of PNEC and NEB); and 3) NEB size. Controls and infants with lung hypoplasia did not differ with respect to bombesin-immunoreactivity. The ipsilateral and the contralateral lungs in CDH had a similar bombesin-immunostaining pattern of PNEC and NEB. The bombesin-immunoreactivity varied between CDH cases, possibly due to the differences in clinical presentation. The mean NEB size was significantly increased in infants with CDH compared to the other two groups ($p = 0.02$). Some CDH cases with large NEBs also showed a high percentage immunostained epithelium. Lung-body weight ratio correlated positively with the percentage immunostained airways, and negatively with the NEB size. We conclude that in lungs of CDH patients bombesin-immunoreactivity in PNEC and NEB differs from that of infants with lung hypoplasia due to other causes and controls. The increased bombesin-immunoreactivity observed in some cases of CDH may reflect a compensatory mechanism related to impaired lung development and/or failure of neuropeptide secretion during neonatal adaptation.

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3.2 Introduction

Neuroepithelial bodies (NEB) are clusters of innervated pulmonary neuroendocrine cells (PNEC) that produce amines and peptides.[1,2] Distributed throughout the airway mucosa, PNEC and NEB may play an important role in lung development[3-7] and during neonatal adaptation[3,4] as NEB are transducers of the hypoxic stimulus and could, therefore, act as airway chemoreceptors in the regulation of respiration.[8]

The principal peptide produced by PNEC is gastrin-releasing peptide (GRP), the mammalian counterpart of bombesin.[4] Antibodies against bombesin or GRP are most widely used as marker of PNEC in human lungs, since bombesin-immunoreactive cells have been identified in fetal lungs from 7-10 weeks of gestation onward.[2,9,10] Bombesin-immunoreactive PNEC differentiate in a craniocaudal direction and the highest number of cells is found in the small peripheral airways towards the end of gestation.[3,10] Experimental studies revealed that bombesin regulates lung branching morphogenesis[11] and stimulates lung growth and maturation.[6] A recent study revealed that in mammalian lung the expression of the GRP-receptor is developmentally regulated and that the GRP-receptor plays an important role especially during the canalicular stage of lung development.[12]

Infants with congenital diaphragmatic hernia (CDH) have abnormal morphological development of lungs and intrapulmonary blood vessels.[13,14] The high neonatal mortality and morbidity in these children is ascribed to the extent of lung hypoplasia and persistent pulmonary hypertension.[15] We have previously reported the expression of calcitonin gene-related peptide (CGRP) positive PNEC in a rat model of CDH:[16] Lungs of fullterm rat pups with CDH contained increased numbers of CGRP-immunostained PNEC compared to the lungs of controls. In the rat, CGRP-immunoreactivity has been studied during normal lung development and has been proven to be a reliable marker of PNEC[17], whereas no bombesinlike-immunoreactivity can be detected in this animal species.[18] In the human lung the reverse is true: CGRP-immunoreactivity has been reported from gestational week 20 onwards[19], but inconsistently and only in a limited number of cells.[18,19]

In this study, using morphometric methods, we investigated the expression of bombesin-immunoreactivity in PNEC of lungs from patients with CDH and compared them to lungs from infants with lung hypoplasia due to other causes, and to lungs from control infants without lung abnormalities who died during the perinatal period.

3.3 Patients and methods

3.3.1 Patients

Cases of CDH and of lung hypoplasia due to other causes were selected from the autopsy files of the Departments of Pathology in a large Pediatric Center in Canada (The Hospital for Sick Children in Toronto), and in 10 different hospitals in the Netherlands and spanning the period from 1967 to 1995. Age-matched controls without lung abnormalities were selected from the files of the Hospital for Sick Children in Toronto. The cases for this study were selected on the basis of the clinical diagnosis, of the fixative that had been used (only formalin-fixed tissues were examined), and of good histological preservation of lung tissue with the presence of intact airway epithelium to identify PNEC and NEB. The use of artificial ventilation for a longer period with high inspiratory peak pressures, especially in cases of CDH, leads to diffuse epithelial damage and hence precludes examination of this lung cell population. Consequently, cases with severe epithelial damage were excluded. Lung hypoplasia was established according to the lung body weight ratio, using the criteria of Askenazi and Perlman.[20] Most CDH patients were born at term and cases from both other groups were therefore selected to obtain the best possible match for gestational age. Control cases were further selected to obtain the best possible match for age at death. Thus, ten CDH patients were included, as well as seven children with lung hypoplasia and four controls (see Table 1, 2, and 3).

3.3.2 Histological examination

Routine 4-µm sections of formalin-fixed, paraffin-embedded lung tissue were immunostained for bombesin using the indirect Avidin Biotin Complex staining procedure as previously described.[21] All sections were digested with 0.5% pepsin (Sigma) and incubated overnight with the primary monoclonal antibody (dilution 1:800) against bombesin (Boehringer Mannheim, Germany). Counterstaining was performed with hematoxylin.

With the aid of a projecting microscope (magnification x700), the total area of airway epithelium of 20 non-cartilaginous airways per section and the bombesin-immunostained epithelium of these airways were traced on paper.[22] The same procedure was done for the 20 largest NEB, mainly located in the medium sized airways, in each section. All drawings were scanned at similar brightness and

contrast level, using a Hewlett Packard Scanjet connected to an Apple Macintosh computer. Morphometric analysis included measurements of the total epithelial surface area of 20 airways containing bombesin-immunostained cells (referred to as immunostained airways), the bombesin-positive areas of airway epithelium in these 20 airways, and the surface area (size) of 20 bronchial NEBs, using the Apple Macintosh National Institutes of Health (NIH) Image 1.53 program. The bombesin-immunostained area in relation to the total epithelial area, referred to as the percentage of immunostained epithelium (%IMS-epithelium)[22] was calculated from the resulting data. In addition, the percentage of immunostained airways (%IMS-airways) was determined for all sections by counting.[23] The average %IMS-epithelium, NEB size, and %IMS-airways were determined per section.

All available sections per case (range 2-5, 62 in total) were studied; 15 sections contained less than 20 non-cartilaginous airways to determine the %IMS-epithelium. The median number of airways studied in these sections was 15 (range 6-19). The %IMS-epithelium per case was measured in a median of 40 airways in the CDH-group (range 35-80); of 60 airways for the lung hypoplasia group (range 40-85), and of 60 airways (range 52-60) for the controls. Twenty bronchial NEBs could be obtained in all sections. The mean values from the different sections of each case were calculated and compared with other cases.

3.3.3 Data analysis

All data presented are median (range). Differences between groups were tested by one-way analysis of variance with the Student-Newman-Keuls test for multiple comparisons or by the non-parametric Kruskal-Wallis test if appropriate. The relation between clinical data and morphometric results was studied by least square regression. For statistical analysis of the morphometric data, two prematurely born infants (one with CDH and with one lung hypoplasia; cases 10 and 17, respectively), and two other patients with CDH (one with multiple congenital anomalies, and one with prolonged artificial ventilation; cases 8 and 9, respectively) were excluded to obtain homogeneous groups. Statistical significance was assumed at two tailed 5% level.

3.4 Results

The clinical data are shown in Tables 1-3. Gestational age, birth weight, and age at death were not significantly different between the three groups; the lung-body weight ratio was significantly higher in controls than in both other groups ($p < 0.001$), whereas the lung hypoplasia cases had a higher lung-body weight ratio than CDH patients ($p < 0.05$).

Bombesin-immunostaining was positive in all sections studied. Qualitative analysis of immunostaining revealed variable intensity of positive immunostaining. The presence of intensely immunostained NEB was observed in 6 CDH cases (cases 2, 3, and 4-7), in 2 cases of lung hypoplasia (cases 11 and 17), and in one control case (case 19). Brown, moderately immunostained NEB were found in one CDH case (case 8), 2 cases of lung hypoplasia (case 15 and 16) and in 3 controls (case 18, 20, and 21). Pale stained NEB were found in 3 CDH cases (case 1, 9, and 10), and in 3 lung hypoplasia cases (case 12-14). In 3 cases of CDH (case 2, 3, and 7) large NEB, sometimes located "beneath" the epithelium, were found in the large airways and at the bronchoalveolar junctions (Figures 1 and 2).

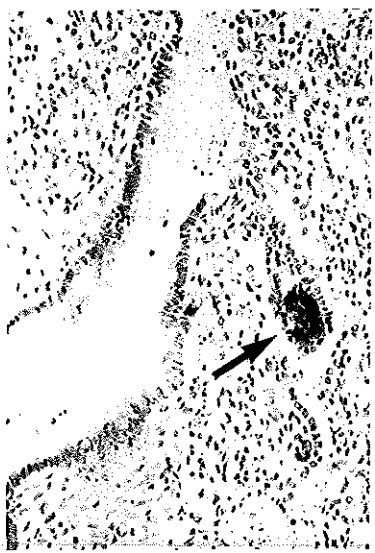

Figure 1: Contralateral lung from a CDH patient (case 3) with severe lung hypoplasia and a large NEB located at bronchoalveolar junction (arrow).

Figure 2: Ipsilateral lung from a CDH patient (case 2) showing some large NEBs that seem to be located "beneath" the airway epithelium (arrows).

Table 1: *Congenital diaphragmatic hernia: Clinical data and results*

Case	Gestation (weeks)	Birth weight (grams)	Lung-body weight ratio	Age (hours)	Diagnosis	%IMS-epithelium	NEB size (μm^2)	%IMS-airways
1	40	3140	0.003	2	CDH left	4.05	293	66.5
2	39	2890	0.003	0.2	CDH left	9.67	542	82.3
3	42	3355	0.002	0.5	CDH right	12.96	642	80
4	40	3040	0.001	1.7	CDH left	8.22	395	80.8
5	42	3585	0.001	1.5	bilat. CDH	7.46	467	82
6	41	3850	0.003	2.5	CDH right	7.54	400	74.8
7	39	2570	nr	2	bilat. CDH	8.61	656	85
8	38	1200	0.002	sb	bilat. CDH, Fallot's Tetralogy, polycystic kidneys	2.99	403	81.5
9	40	nr	nr	72	CDH left	4.02	231	69
10	28	1600	0.011	17	CDH left, HMD	1.83	122	38
median	40	3040	0.0025	1.8		7.5	401	80.4

nr = not recorded; sb = stillborn; bilat. CDH = bilateral CDH; HMD = hyaline membrane disease

Table 2: *Lung hypoplasia: Clinical data and results*

Case	Gestation (weeks)	Birth weight (grams)	Lung-body weight ratio	Age (hours)	Diagnosis	%IMS-epithelium	NEB size (μm^2)	%IMS-airways
11	36.5	2070	0.006	2	Potter syndrome	6.9	324	84
12	36	1970	0.010	2	Potter syndrome	4.76	252	55.8
13	40	2790	0.006	2	obstructive uropathy	7.32	330	53.3
14	40	3600	0.010	0	Potter syndrome	4.83	263	87.7
15	40	2600	0.009	2	oligohydramnios syndrome	7.95	384	91
16	37	2680	0.007	4.5	Prune Belly syndrome	6.68	472	89.5
17	28	1036	0.010	1	PROM	5.58	263	85.3
median	37	2600	0.009	2		6.68	324	85.3

PROM = premature rupture of membranes

Table 3: *Control cases: Clinical data and results*

Case	Gestation (weeks)	Birth weight (grams)	Lung-body weight ratio	Age (hours)	Diagnosis	%IMS-epithelium	NEB size (μm^2)	%IMS-airways
18	40	3760	0.015	sb	asphyxia	7.36	339	94.7
19	38	3380	0.026	sb	abruptio placentae	6.72	289	96.7
20	40	3340	0.018	2	asphyxia	5.88	215	85.5
21	39	2600	0.022	1.5	asphyxia	7.43	335	95.3
median	39.5	3360	0.020	0.75		7.04	312	95

sb = stillborn

This phenomenon was not observed in lung hypoplasia cases (Figure 3) or in controls (Figure 4).

Figure 3: *Lung hypoplasia case (case 16) showing NEBs within the epithelium of a peripheral airway and bronchoalveolar junction (arrows).*

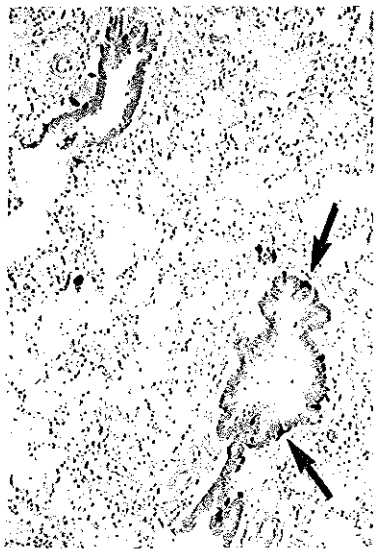

Figure 4: *Lung from control patient (case 20) showing PNEC and NEBs within the epithelium of a peripheral airway (arrows).*

Morphometric data in CDH cases were similar for the ipsilateral and contralateral lungs. Therefore, data from all lung sections of each case were averaged. Statistical analysis of 17 cases (7 CDH cases, 6 lung hypoplasia cases, and 4 controls) showed a higher %IMS-airways in controls than in CDH patients (95 (86-97)% versus 81 (67-85)%, respectively; $p = 0.02$). The NEB size was significantly larger in lungs of infants with CDH compared to the other two groups (467 (293-656) μm^2 in CDH versus 327 (252-472) μm^2 in lung hypoplasia, and 312 (215-339) μm^2 in controls; $p = 0.02$). The lung-body weight ratio correlated positively with the %IMS-airways ($p = 0.05$) and negatively with the NEB size ($p = 0.02$). The %IMS-epithelium was not significantly different between the groups. However, some cases of CDH with large NEB (Table 1, cases 2, 3, 5, and 7) had also a high value for %IMS-epithelium (Figure 5).

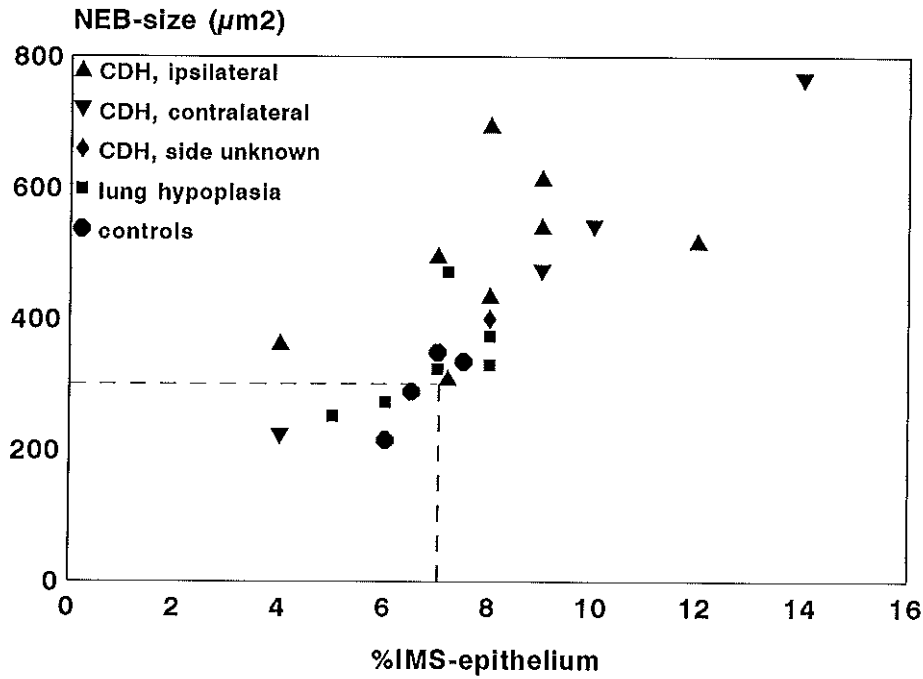

Figure 5: The mean NEB size and the %IMS-epithelium of the ipsilateral and contralateral lungs are shown for CDH patients included in statistical analysis (n=7); the three CDH patients who were excluded from statistical analysis include cases with multiple congenital anomalies (case 8), prolonged exposure to hyperoxia (case 9), and prematurity, prolonged artificial ventilation, and hyaline membrane disease (case 10). Lung hypoplasia cases (n=6); one prematurely born infant was excluded (case 17), and controls (n=4). For CDH patients the data from the ipsilateral and contralateral lungs are shown separately; from some slides it was not clear whether they represented the ipsilateral or the contralateral lung. These are indicated as separate symbols (CDH, side unknown). For both other groups the mean value of both lungs is shown. The dashed lines indicate the median NEB-size and median %IMS-epithelium of the control cases.

3.5 Discussion

We found that the expression of bombesin-immunoreactivity in pulmonary neuroendocrine cells in lungs of patients with CDH was different from that in lungs of infants with lung hypoplasia due to another cause, and also differed from

those without pulmonary abnormalities. Controls had a higher percentage of immunostained airways, which was also reflected in the positive correlation between the %IMS-airways and the lung-body weight ratio. In some CDH cases very large NEBs were found concomitant with a high percentage of immunostained epithelium.

The bombesin-immunoreactivity in PNEC and NEB of lungs of infants and children has been investigated for several different pulmonary diseases.[2] Only few data on bombesin-expression in lungs of patients with hypoplastic lungs are available. Absent or very low bombesin-immunoreactivity has been reported in earlier studies in infants with lung hypoplasia or CDH.[4,24] Jaramillo and coworkers reported that the density of GRP-immunoreactive cells in lungs of children with anencephaly and lung hypoplasia was similar to that of anencephalic patients without lung hypoplasia and that of normal controls, but more GRP-positive cells were located in the airways in anencephalic patients with lung hypoplasia than in those of patients without lung hypoplasia.[25] It is not clear whether differences in tissue processing, methodology, or the gestational ages may explain the differences with our observations.

An important prerequisite for our study was the preservation of airway epithelium. Therefore, patients with extensive epithelial damage who had been ventilated for more than a few hours had to be excluded. This resulted in a selection of CDH patients with severe lung hypoplasia resulting in death shortly after birth. Most children had died within the first two hours after birth, and only two children had lived for more than 2.5 hours. The number of cases studied was the maximal number that could be retrieved from files of the Pathology Departments of 10 institutions in the Netherlands and in The Hospital for Sick Children in Toronto between 1967 and 1996 (prior to this period, Zenker's fixative has been used, precluding immunostaining for bombesin). This is, to our knowledge, the first study of bombesin-immunoreactivity in infants with CDH with such a cohort of cases studied.

The variation in bombesin-immunoreactivity observed in CDH cases may be partly explained by the differences in clinical history. The lungs of the patients who survived longest (cases 9 and 10) and of a dysmaturely, stillborn patient with multiple congenital anomalies (case 8) showed pale immunostaining, relatively small NEB, and the lowest %IMS-epithelium. Patient number 10 was prematurely born and had hyaline membrane disease, a condition which is known to decrease bombesin-immunoreactivity.[4,9,26] It can be assumed that the prolonged treatment with artificial ventilation and oxygen therapy in cases 9 and 10, and the renal

abnormalities in case 8 may have influenced the bombesin-immunoreactivity in the lungs. These cases were not included in the statistical analysis, in order to maintain uniform clinical parameters.

The largest NEB and the highest %IMS-epithelium were found in lungs from CDH patients with severe hypoplasia who could not be ventilated adequately and died shortly after birth. Whether the function of these large NEB is abnormal is speculative. It is known that NEB are transducers of the hypoxic stimulus[8], and that increased exocytosis with secretion of neuropeptides occurs during hypoxia.[27] We speculate that large NEB may indicate failure of neuropeptide release i.e. that the hypoxic secretory response has not occurred. Such a phenomenon has been reported in infants with asphyxia and loss of brainstem function.[28] On the other hand, it can be assumed that during resuscitation of the CDH patients using artificial ventilation and a high inspired oxygen fraction the local concentration of oxygen in the airways was high, which may have inhibited exocytosis. This is supported by the findings of Lauweryns and coworkers who reported that a low oxygen concentration of the inhaled air, but not hypoxemia stimulates secretion of serotonin by NEB.[29]

The lungs of children with lung hypoplasia secondary to renal- or genitourinary abnormalities, showed variation in bombesin-immunoreactivity. The pattern of bombesin-immunoreactivity was, however, different from that in CDH patients. Half of the cases with lung hypoplasia showed pale immunostaining, and the NEB size and the %IMS-epithelium were within the same range as that found in controls. The differences in bombesin-immunostaining between the lung hypoplasia cases could not be explained by differences in gestational age or in clinical presentation.

In conclusion, we found that the bombesin-immunoreactivity in lungs of CDH patients differs from that of age-matched controls and from that of infants with lung hypoplasia due to other causes. Infants with the most severe lung hypoplasia and persistent pulmonary hypertension, as reflected by their very early age at death, have increased bombesin-immunoreactivity. Because bombesin stimulates lung growth and lung maturation[6] it may be assumed that the increased bombesin-expression reflects a maximal response of the lung to compensate for the abnormal growth in CDH. In an experimental setting using a rat model of CDH enlarged NEB and increased expression of CGRP have been reported.[16,30] In the developing rat lung, CGRP-immunoreactivity reaches its maximum near term[17]; a developmental pattern similar to that of bombesin in the human lung.[3] We therefore propose that the rat model of CDH is suitable to perform further studies

of the altered expression of neuroendocrine cells and their peptides in the lungs in CDH.

It can also be assumed that the increased bombesin-immunoreactivity observed in some CDH patients, reflects the extent of persistent pulmonary hypertension. This assumption is supported by a study that reported increased bombesin-immunoreactivity in older patients with primary pulmonary hypertension[31], although bombesin itself exerts no effect on the pulmonary vascular tone.[32,33] The large bombesin-positive NEBs in lungs of CDH patients may, however, contain other peptides such as leu-enkephalin[21], endothelin[2], or serotonin[2], which are known to induce pulmonary vasoconstriction.[2,32] However, our present data do not allow for conclusions regarding the role of NEB in abnormal lung development in CDH.

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Chapter 4

Pulmonary neuroendocrine cells during lung development in CDH

4.1 Pulmonary neuroendocrine cells in neonatal rats with congenital diaphragmatic hernia*

4.1.1 Summary

Lung hypoplasia and persistent pulmonary hypertension are the principal causes of high mortality and morbidity in infants with congenital diaphragmatic hernia (CDH). Amine and peptide producing pulmonary neuroendocrine cells (PNEC), widely distributed throughout the airway mucosa, are thought to play an important role in both pulmonary development and in regulation of pulmonary vascular tone. Furthermore, recent studies show increased levels of calcitonin gene-related peptide (CGRP), a pulmonary vasodilator produced by PNEC, during chronic hypoxia. The article reports data on morphometric analysis of CGRP immunoreactive PNEC clusters (neuroepithelial bodies, NEB) in a rat model of CDH. CDH was induced in neonatal Sprague Dawley rats by oral administration of 2,4-dichloro-p-nitrophenyl-ether (Nitrofen) to the mother at 10 days of gestation. Sections of lungs from term neonatal rats with and without CDH and controls were immunostained for CGRP (marker of NEB) with specific antibody against rat CGRP. NEB size and number of NEB/area of lung were assessed using a semiautomatic image analysis system. In lungs of neonatal rats with CDH the number of NEB per surface area of lung parenchyma was significantly increased compared to the age-matched controls. Although the mean size of NEB was larger in CDH, the differences were not significant. This is the first study of PNEC in CDH. Whether the phenomenon observed in this study results in altered NEB function including imbalance in vasoactive mediators requires further studies, especially in the human being.

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* **IJsselstijn H, Perrin D, de Jongste JC, Cutz E, Tibboel D**
J Pediatr Surg. 1995; 30:413-415

4.1.2 Introduction

Congenital diaphragmatic hernia (CDH) is a serious malformation with a high mortality and morbidity due to pulmonary hypoplasia and pulmonary hypertension.[1-3] The mortality rate of 40 to 50 % has not decreased during the past few years despite changing concepts in treatment including delayed surgery and extracorporeal membrane oxygenation (ECMO).[4] Factors that may contribute to pulmonary hypertension in general as well as in CDH have been studied intensively. Pulmonary neuroendocrine cells (PNEC), a known source of a variety of biological active compounds, have only been studied recently. These amine and peptide producing cells are widely distributed throughout the airway mucosa and are found as solitary cells or as clusters that are called neuroepithelial bodies (NEB).[5] PNEC are thought to play an important role during lung development[5,6] and neonatal adaptation[5,7], particularly in the regulation of pulmonary vascular tone.[8] One of the peptides produced by PNEC is calcitonin gene-related peptide (CGRP). In human beings, CGRP immunoreactive cells are found from 22 weeks of gestation[9] mostly within the epithelium of distal conducting airways and around blood vessels.[10] Both in rats and in humans CGRP is known to exhibit a potent vasodilatory[8,10,11] and a bronchoconstricting activity. Recent studies showed increased levels of intracellular CGRP in chronically hypoxic rats[12] and in lungs of children with bronchopulmonary dysplasia.[13] Because PNEC were not previously studied in CDH, we investigated the distribution and frequency of CGRP immunoreactive PNEC in a rat model of CDH and pulmonary hypoplasia.[14] The aim of this study was to determine whether these cells and their mediators may play a role in problems associated with CDH in newborns.

4.1.3 Materials and methods

Female Sprague-Dawley rats (Harlan Olac, England) were mated overnight (day 0 of gestation). To induce CDH, a subgroup of pregnant rats received orally 100 mg of 2,4-dichloro-p-nitrophenylether (Nitrofen: Rohm Haas Company, Philadelphia, PA), dissolved in 1 mL of olive oil, on day 10 of gestation. Nitrofen administration induces a left-sided or bilateral diaphragmatic defect in 70-90% of the offsprings using this regimen. The offsprings of the rats without Nitrofen administration served as normal controls. Food and water were supplied ad libitum during the whole period of pregnancy. At gestational day 22 (term) the mother was anesthesized by inhalation of ether and a cesarean section was performed. The fetuses

were removed and killed before any breathing occurred. The presence of a diaphragmatic defect was assessed and the lungs, with trachea attached, were removed for histological examination. Three study groups were included: normal controls (n=7), rats that developed CDH (n=9) and rats without CDH (non-CDH) (n=3) in the Nitrofen group.

The lungs were fixed by immersion in Davidson solution (40 vol% ethanol 100%, 5 vol% acetic acid 96%, 10 vol% formaldehyde 37%, 45 vol% saline; pH 7.3) and embedded in paraffin. Immunostaining for CGRP was performed with specific rabbit polyclonal antibody against rat CGRP (CA-08-220, Cambridge Research Biochemicals Incorporated, Wilmington, DE) using a well established protocol.[15,16]

Morphometric analysis including measurements of NEB size, number of NEB per section, surface area of lung sections and frequency of NEB (the number of NEB per mm^2 of lung) were performed using Apple McIntosh National Institutes of Health (NIH) Image 1.49 programme. All values were expressed as mean ± SEM. Group means were compared by Student's t-test and significance was accepted at 5% level.

4.1.4 Results

All except two in the CDH group had major left-sided or bilateral diaphragmatic defects. Two animals with a small right-sided defect were not included in further analysis. In all groups a positive staining for CGRP was detected. More prominent and numerous NEBs were found in lungs of CDH rats (Figure 1a) compared with normal controls (Figure 1b). The findings of the morphometric analysis are summarized in Table 1.

Table 1: *Morphometric analysis of lungs of neonatal rats with CDH immunostained for CGRP*

	CDH (n=7)	non-CDH (n=3)	control (n=7)
NEB size (μm^2)	332 ± 27	302 ± 37	290 ± 16
number of NEB	20.9 ± 2.2	16.3 ± 1.2*	30.0 ± 4.3
area of lung (mm^2)	22.2 ± 1.1*†	29.8 ± 3.8*	51.4 ± 1.2
number of NEB per mm^2 lung	0.95 ± 0.11*†	0.52 ± 0.04	0.58 ± 0.07

*Values are expressed as mean ± SEM. * significantly different from control; $p < 0.05$; † significantly different from non-CDH; $p < 0.05$.*

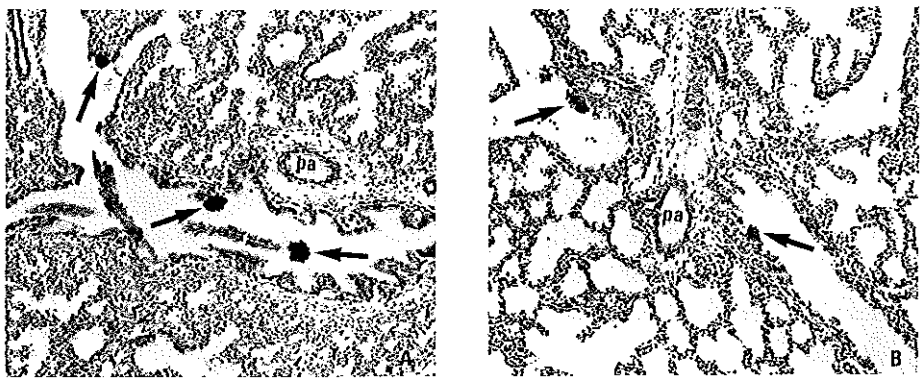

Figure 1: Representative pictures showing some large NEB (arrows) in a lung from a rat pup with CDH (A), and from a control lung (B). pa = pulmonary artery

The pulmonary area in rats exposed to Nitrofen was significantly smaller compared with that of controls ($p < 0.001$), and in CDH it was significantly smaller than in non-CDH rats ($p < 0.01$). The number of NEB per mm^2 of lung area in rats with CDH was significantly greater compared with that of both other groups ($p < 0.01$ compared with controls, $p < 0.05$ compared with non-CDH). Furthermore, the mean size of NEB was greater in CDH rats, but this was not statistically significant.

4.1.5 Discussion

Our findings suggest that in rats with CDH and pulmonary hypoplasia there is a proportionally higher number of NEB immunostained for CGRP. Nitrofen-exposed animals without CDH had a smaller lung area compared with normal controls. However, the relative frequency of NEB per mm^2 of lung was comparable to the frequency of NEB seen in controls. This suggests that in CDH there may be an additional factor apart from the pulmonary hypoplasia with a resulting delayed maturation of lung parenchyma.

With respect to the role of PNEC in lung development, different hypotheses are put forward. Cutz et al[6] demonstrated that in the developing human lung the differentiation of PNEC proceeds in a craniocaudal direction with more prominent serotonin-immunoreactive cells during the early stages. In contrast, bombesin-immunoreactive cells reach their maximum number at birth. Spindel et al[17] found that levels of GRP, the mammalian homologue of bombesin, increase during

gestation and remain elevated until several months after birth, whereas GRP mRNAs reach their maximum levels from 16 to 30 weeks of gestation and then decline by 34 weeks of gestation. Wada et al[7] studied the developmental changes in the expression of the CGRP gene in rat lungs. CGRP-positive cells did not appear in lung tissue before the 18th day of gestation and declined within 1 week after birth. These studies suggest involvement of PNEC in normal lung development and a possible role for CGRP in pulmonary adaptation from late intrauterine stages to the early neonatal period.

Earlier studies from our group in rats showed that morphologically hypoplastic lungs are less mature near term.[18] It can be assumed that in CDH the immaturity of the lungs is also reflected in the number of NEB. The findings of Wada et al[7] do not support this assumption. Further studies are required to show whether our findings reflect immaturity of the lung in CDH or reflect that a new characteristic of the lung in CDH has now been discovered.

Stahlman et al[19] performed a study of colocalization of peptide hormones in PNEC of human fetuses and newborns. They demonstrated that in normal fetuses the percentage of granules labeled for CGRP was consistently lower compared with abnormal fetuses and children dying from pulmonary disease. This percentage increased with the severity of pathological changes, being highest in hyaline membrane disease and bronchopulmonary dysplasia.

Springall et al[12,16] described an increase in intracellular levels of CGRP in PNEC of hypoxic rats. This could have important implications in the vasoconstrictor response to hypoxia. Furthermore, Youngson et al[20] demonstrated that NEB are transducers of the hypoxic stimulus and therefore may function as airway chemoreceptors in the regulation of respiration. In our experiment the neonatal rats were killed immediately after a cesarean section before severe hypoxia after birth could occur. The adaptation from intrauterine to extrauterine life is unlikely to explain our findings for the same reason. A process already existing in utero may
result in the higher number of NEB seen in CDH. The immunoreactivity of PNEC in CDH in humans is presently being investigated.

Whether an altered NEB function, including imbalance in vasoactive mediators, is involved in the continuing high mortality and morbidity of CDH is still unclear. Further studies in rats and in humans with other mediators such as serotonin are now being performed in our department.

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4.2 Calcitonin gene-related peptide expression is altered in pulmonary neuroendocrine cells in developing lungs of rats with congenital diaphragmatic hernia*

4.2.1 Summary

Congenital diaphragmatic hernia (CDH) is associated with high neonatal mortality due to lung hypoplasia and persistent pulmonary hypertension. Pulmonary neuroendocrine cells (PNEC), scattered throughout airway epithelium, produce calcitonin gene-related peptide (CGRP), a potent vasodilator. We previously reported altered distribution of CGRP-positive PNEC in fullterm rats with CDH, suggesting that in CDH abnormal development of PNEC leads to an imbalance in vasoactive mediators. In the present study we examined the expression of CGRP-positive PNEC at different stages of lung development in rats with CDH, induced by administration of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) on gestational Day 10. Cesarean sections were performed on Days 16, 18, 20, or 22, and the lungs were examined by immunocytochemistry. On Day 16 CGRP-immunostaining was negative; on Day 18 CGRP was expressed in lungs of all controls (not exposed to Nitrofen) but only in some CDH pups. On Day 20 CGRP-immunoreactivity was similar in CDH pups and controls. On Day 22 (term) a higher proportion of CGRP-positive cells was found in CDH lungs. The proportion of immunostained epithelium and the size of the neuroendocrine cell clusters (neuroepithelial bodies; NEB) were not significantly different from controls. Supraoptimal dilution immunocytochemistry, applied to quantify the intracellular CGRP level on Day 22, yielded similar results in CDH and controls. We conclude that CGRP-expression in PNEC and NEB is delayed in CDH during the early stages of lung development. Since CGRP also exhibits growth factor-like properties on endothelium and epithelial cells, the lack of this factor during a crucial developmental stage (canalicular period) may be causally related to lung hypoplasia. Whether the higher

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proportion of CGRP-positive cells in CDH observed at term reflects an abnormal function of these cells remains to be determined.

4.2.2 Introduction

Amine and peptide producing pulmonary neuroendocrine cells (PNEC) are distributed throughout the airway mucosa as solitary cells and as innervated clusters, called neuroepithelial bodies (NEB).[1,2] They have an important role during lung development[2,3] and neonatal adaptation[2,4], particularly in the regulation of pulmonary vascular tone.[5] Furthermore, NEB are transducers of the hypoxic stimulus and could, therefore, be functioning as airway chemoreceptors in the regulation of respiration.[6]

One of the peptides produced by PNEC is calcitonin gene-related peptide (CGRP).[7,8] In humans, CGRP-immunoreactive cells have been identified from 22 weeks of gestation onward[9], mostly within the epithelium of distal conducting airways.[10] In rat lungs they have been identified from gestational day 18, with the highest expression near term.[4] Several studies have indicated that CGRP has potent vasodilatory and bronchoconstricting activity.[5,10,11] Other biological activities include stimulation of growth with effects on endothelial cells and airway epithelium.[12,13] Increased levels of intracellular CGRP have been found in lungs of chronically hypoxic rats[14] and in children with bronchopulmonary dysplasia.[15]

Infants with congenital diaphragmatic hernia (CDH) have abnormal morphological development of lungs and intrapulmonary blood vessels.[16,17] The high neonatal mortality and morbidity in these infants is ascribed to the severity of lung hypoplasia and persistent pulmonary hypertension.[18] Our previous study in a rat model of CDH revealed that lungs of fullterm rat pups contained relatively more CGRP-immunostained NEB than lungs of age-matched controls.[19] This finding suggests that NEB may play a role in the pathogenesis of lung hypoplasia, or lead to an imbalance of vasoactive mediators in CDH. The aim of the present study was to investigate the developmental pattern of pulmonary CGRP-positive cells in lungs of fetal rats with CDH during different stages of lung development.

4.2.3 Materials and methods

4.2.3.1 Animal model

Female Sprague-Dawley rats (Harlan Olac, England) were mated during one hour (day 0 of gestation). Eleven of 19 pregnant rats received 100 mg of 2,4-dichloro-

phenyl-p-nitrophenylether (Nitrofen; Rohm Haas Company, Philadelphia, PA) in 1 ml of olive oil orogastrically under light ether anesthesia on Day 10 of gestation.[20,21] The remaining eight rats provided control pups. Nitrofen induces a large left-sided diaphragmatic defect with severe lung hypoplasia, sometimes in combination with a small right-sided defect, in up to 80% of the offspring using this regimen. Food and water were supplied ad libitum during the whole period of pregnancy. The pregnant dams were anesthetized by inhalation of diethylether and a cesarean section was performed on Day 16 (2 litters each group), Day 18 (3 Nitrofen-litters, 2 control litters), Day 20 (3 Nitrofen-litters, 2 control litters), or Day 22 (3 Nitrofen-litters, 2 control litters). The fetuses were removed and killed after cervical intersection with a needle before any breathing occurred. Autopsy revealed the presence of a diaphragmatic defect in the Nitrofen-exposed rat pups. The lungs, with trachea attached, were removed for histological examination. Three groups were studied: rat pups with CDH (Day 16: n = 8; Day 18: n = 6; Day 20: n = 10; Day 22: n = 7) and pups without CDH (non-CDH; Day 16: n = 4; Day 18: none; Day 20: n = 7; Day 22: n = 5) in the Nitrofen group, and control pups (Day 16: n = 5; Day 18: n = 8; Day 20: n = 4; Day 22: n = 4).

This experimental protocol was approved by the Animal Care and Use Committee of the Erasmus University Rotterdam.

4.2.3.2 Histological examination

The lung tissue was processed according to Springall et al.[14] The lungs were fixed by immersion in Bouin's fluid, dehydrated in graded ethanols, embedded in paraffin, and 4 µm sections were cut. Immunostaining for CGRP was performed with specific rabbit polyclonal antibody against rat CGRP (CA-08-220, Cambridge Research Biochemicals Incorporated, Wilmington, DE) by the peroxidase-antiperoxidase method.[22] The optimal dilution of the primary antibody of 1:400 was used according to the manufacturer's instructions. Counterstaining was performed with hematoxylin.

With the aid of a projecting microscope, the total area of the lungs and all CGRP-positive areas (both PNEC, i.e. solitary cells, and NEB, i.e. clusters of three or more cells) were traced on paper (magnification x700).[23] Morphometric analysis included measurements of the size of CGRP-positive areas, the number of CGRP-positive areas per lung, the total surface area of lung sections, the CGRP-positive areas of airway epithelium, and the total epithelial surface area of the immunopositive airways. These measurements were performed using the Apple Macintosh National Institutes of Health (NIH) Image 1.53 program. The resulting

data were used to calculate the number of CGRP-positive areas per mm^2 lung (frequency of CGRP-positive cells)[19], and the epithelial CGRP-positive area in relation to the total epithelial area, referred to as the percentage of immunostained epithelium (%IMS-epithelium).[23] The percentage of airways containing CGRP-positive cells, referred to as immunopositive airways (%IMS-airways) was determined by counting.[24] The size of CGRP-immunostained NEB, consisting of 3 or more cells, was determined by counting the number of nuclei. The median number of nuclei of the NEB in each case was used for data analysis.

Lung sections taken on gestational Days 16 and 18 were used to establish the presence of CGRP-positive cells. The median number of nuclei in NEB in lung sections from Day 18 rat pups was also determined. In lungs from rats of gestational Days 20 and 22 the number of CGRP-positive areas, and the CGRP-positive frequency were measured. In addition, the %IMS-epithelium, the %IMS-airways, and the median number of nuclei of NEB were determined in controls and in rat pups with CDH on gestational Day 22.

4.2.3.3 Supraoptimal dilution immunocytochemistry

Supraoptimal dilution immunocytochemistry was used to compare levels of anti-CGRP immunostaining in the CDH and the control groups lung sections from gestational days 20 and 22.[14,25] The lung sections used for this experiment were adjacent to the lung sections described above. They were stained with a supraoptimal concentration of primary antibody of 1:24,000, which is a 60-times dilution of the optimal dilution[25], or a with a concentration of 1:60,000, which is the actual dilution used by Ebina et al.[25] Coverslips were then mounted on wet sections which were immediately examined. The number of CGRP-positive areas per lung and the size of those areas were determined as described above. Thereafter these sections were reincubated with primary antibody at the optimal dilution concentration of 1:400. Finally, sections were washed, dehydrated, and mounted following counterstaining with hematoxylin. Then all sections were reexamined. The ratio of cells staining with supraoptimally diluted versus optimally diluted anti-CGRP, referred to as the cell count ratio, was considered an index of the level of intracellular CGRP.[25]

4.2.3.4 Data analysis

Values were expressed as means ± SEM, unless stated otherwise. Differences between groups were tested by one-way analysis of variance with the Student-Newman-Keuls test for multiple comparisons, or by the non-parametric Kruskal-

Wallis test if appropriate. Chi-square test was used for proportions. Paired t-tests were used to compare differences between left and right lungs in individual cases and to compare differences in size of CGRP-positive areas following supraoptimal dilution immunocytochemistry. Statistical significance was assumed at two tailed 5% level.

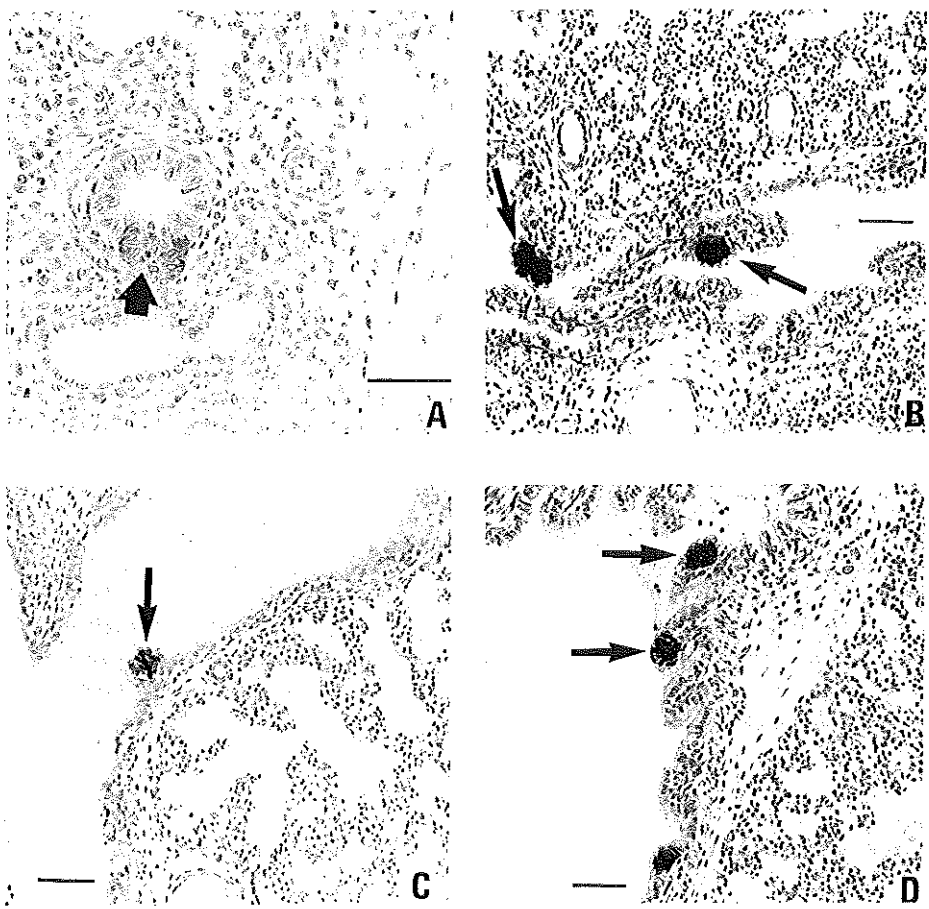


Figure 2: Weak CGRP-positive staining was present in lungs of CDH pups and controls on gestational Day 18, which is indicated here (arrow) for the left lung in a CDH rat (2A); representative pictures from left lungs on gestational Day 22 of a CDH rat (2B), of a Nitrofen-exposed pup without CDH (2C), and of a control pup (2D), showing a central airway with some large NEB (arrows). The NEB in CDH seemed bigger and more prominent than in other groups. Counterstaining with hematoxylin. Scalebars respresent 50 µm.

4.2.4 Results

On gestational Day 16, CGRP-immunostaining was negative in lungs of all three groups studied. On Day 18, weak staining was found in all but one lung of controls and in only some lungs of CDH rats (Table 2, and Figure 2a). Both the numbers of cases with positive CGRP-immunostaining in the left, ipsilateral, lungs, and the numbers of CGRP-positive areas (PNEC and NEB) in the right, contralateral, lungs tended to be lower in CDH than in controls, but the difference was not significant (Table 2).

Table 2: *CGRP-immunoreactivity in rat lungs on gestational day 18*

	Control		CDH	
	left	right	left	right
cases with CGRP-pos. PNEC	8/8	7/8	4/6	4/6
cases with CGRP-pos. NEB	7/8	5/8	4/6	3/6
# CGRP-pos. area	2.5 (1-6)	2.5 (0-6)	1.5 (0-5)	1 (0-2)
# CGRP-pos. NEB	2 (0-6)	2 (0-6)	1 (0-4)	0.5 (0-1)
NEB-size	4 (3-6)	6 (3-9)	3 (3-5.5)	5 (3-6)

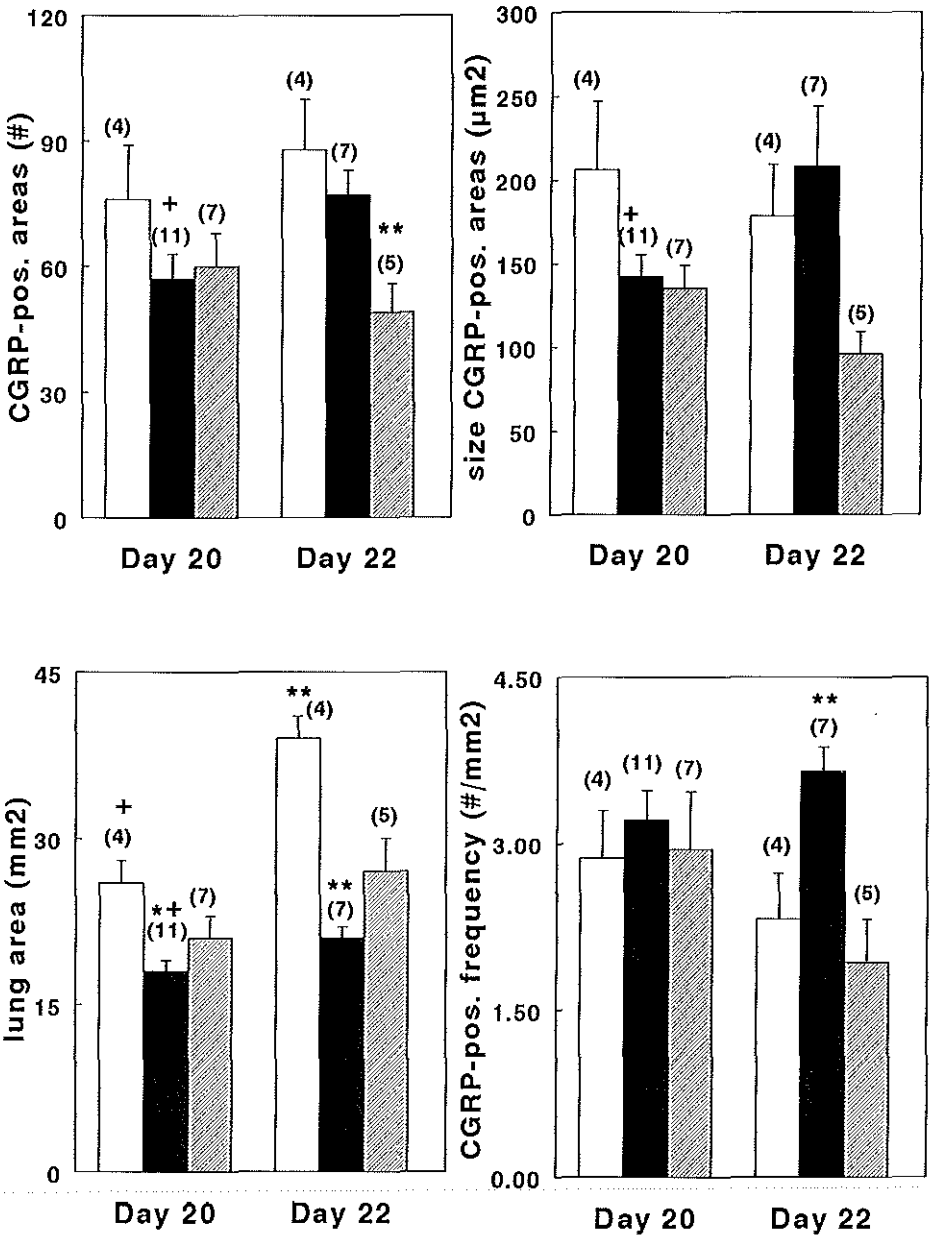
For controls and CDH rat pups the number of cases in which CGRP-positive PNEC and the number in which CGRP-positive NEB (consisting of three or more cells) were found, the number of CGRP-positive areas (consisting of PNEC and NEB), the number of CGRP-positive NEB, and the size of the NEB (determined by the median number of nuclei) are shown for each lung. For all parameters, except the number of CGRP-positive cases, the median and range values are indicated.

Figure 3: *For controls (open bar), CDH pups (black bar), and Nitrofen-exposed pups without CDH (striped bar) the means ± SEM values are shown on gestational Day 20 and Day 22 for: the number of CGRP-positive areas, consisting of PNEC and NEB (3A), the size of the CGRP-positive areas (3B), the total lung surface area (3C), and the numbers of PNEC and NEB per mm^2 lung area (CGRP-positive frequency; 3D). The numbers of animals per group are indicated in brackets.*

**: significantly different from controls on the same gestational day; $p < 0.05$.*

***: significantly different from both other groups on the same gestational day; $p < 0.05$.*

+: significantly different from the same group on Day 22; $p < 0.05$.


CGRP-pos. areas (#)
120
90
60
30
0
(4)
+
(11)
(7)
(4)
(7)
**
(5)
Day 20
Day 22
size CGRP-pos. areas (µm2)
300
250
200
150
100
50
0
(4)
+
(11)
(7)
(4)
(7)
(5)
Day 20
Day 22
lung area (mm2)
45
30
15
0
+
(4)
**+
(11)
(7)
**
(4)
**
(7)
(5)
Day 20
Day 22
CGRP-pos. frequency (#/mm2)
4.50
3.00
1.50
0.00
(4)
(11)
(7)
**
(7)
(4)
(5)
Day 20
Day 22

On days 20 and 22 there were no significant differences between the left lungs and the right lungs with respect to the number, the size, and the frequency of CGRP-positive areas in any of the three groups (data not shown). In pups with CDH the left lung was significantly smaller than the right lung at both time points (Day 20: 7.7 ± 0.6 versus 9.9 ± 0.5 mm^2; Day 22: 9 ± 0.5 versus 12.5 ± 1 mm^2).

The results of morphometric analysis from the rat pups (the left and right lungs combined) on Days 20 and 22 are shown in Figure 3. On Day 20 the mean total lung area was significantly smaller in CDH pups than in controls (Figure 3c; $p = 0.006$). The mean number of CGRP-positive areas, their mean size, and their frequency were not significantly different. Both on Day 20 and 22 there was a tendency towards a difference in size of CGRP-positive areas between the three groups, but this was not significant ($p = 0.08$ for both gestational ages). On Day 22 the mean frequency of CGRP-positive cells in CDH was significantly higher than in both other groups (Figure 3d; $p = 0.003$). Significant differences between Day 20 and Day 22 are shown in Figure 3 for each group.

Additional measurements in lung sections from Day 22 CDH pups and controls revealed that the percentage of immunopositive airways was higher in the left lung in CDH than in controls (Table 3; $p = 0.03$). Moreover, there was a tendency towards a higher %IMS-epithelium in the left lung in CDH compared with controls, but this was not significant. Such differences were not found in the right lungs. Both in CDH pups and controls none of the PNEC/NEB parameters showed significant differences between the left and right lungs (Table 3).

***Table 3:** CGRP-immunoreactivity in rat lungs on gestational day 22*

	Control		CDH	
	left	right	left	right
% IMS-epithelium	6.5 ± 0.7	7.5 ± 1.1	8.1 ± 0.7	7.5 ± 0.7
% IMS-airways	64 ± 2	63 ± 3	69 ± 1*	68 ± 3
CGRP-pos. NEB	11 ± 3	10 ± 2	12 ± 2	9 ± 1
NEB size	4.9 ± 0.4	6.1 ± 0.8	5.4 ± 0.6	4.7 ± 0.3

For controls and CDH pups the percentage immunostained airway epithelium, the percentage immunopositive airways, the total number of CGRP-positive NEB (consisting of 3 or more cells), and the size of the NEB (determined by the median number of nuclei) are shown for each lung. For all parameters the mean ± SEM values are indicated.

**: significantly different from left lung in controls; p = 0.03.*

Histological examination of lung slides from Day 22 suggested that more prominent, and larger NEB were present in CDH (Figure 2b) than in controls (Figure 2d). Therefore, we counted the number of NEB and determined the median number of nuclei in NEB for each lung. These parameters yielded no statistical differences between CDH and controls (Table 3), but in CDH several large NEB with up to 38 nuclei were found, whereas the largest NEB in controls contained not more than 20 nuclei.

Supraoptimal dilution immunocytochemistry with the primary antibody diluted 1:24,000 revealed that on gestational Day 20 the cell count ratio was 87 ± 3% in CDH and 61 ±8% in controls (Figure 4; p = 0.01). On Day 22 no such difference was found. The mean size of the CGRP-positive areas that stained with the supraoptimal dilution was not different from the mean size after restaining with the optimal dilution (data not shown). Experiments with a supraoptimal dilution of 1:60,000 showed a cell count ratio of 25 ± 3% in CDH and 29 ± 3% in controls on Day 20 (NS), this was 26 ± 3% and 23 ± 4% respectively on Day 22 (NS). However, the CGRP-positive areas were significantly larger in CDH on days 20 and 22, and in controls on Day 20 after staining with a dilution of 1:60,000 than after restaining with the optimal dilution (Figure 5). Representative pictures showing the CGRP-immunoreactivity in CDH using the supraoptimal dilution of 1:60,000 followed by optimal staining are shown in Figure 6.

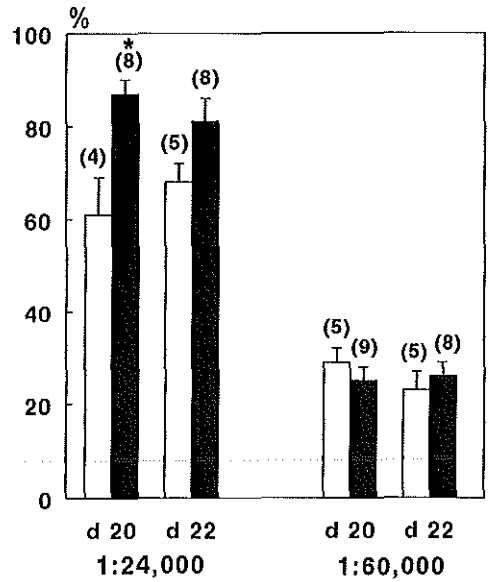


*Figure 4: The mean ± SEM percentage of cells that stained following immunostaining with a supraoptimal dilution of the primary antibody of CGRP compared with optimal staining (the cell count ratio) is shown for controls (open bar) and CDH (black bar) on Day 20 and Day 22. The left panel shows the cell count ratio using the supraoptimal dilution of 1:24,000, whereas the right panel shows the cell count ratio using a supraoptimal dilution of 1:60,000. The numbers of animals per group are shown in brackets. *: significantly different from controls; p = 0.01.*

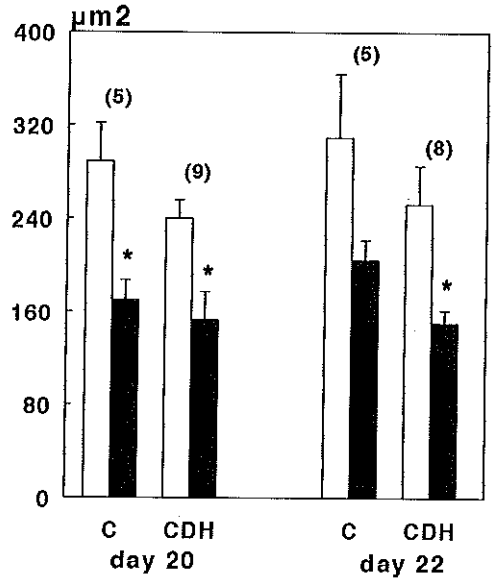


*Figure 5: The mean ± SEM size of the CGRP-positive areas are shown for CDH and controls on gestational Day 20 and Day 22 after immunostaining with a supraoptimal dilution of 1:60,000 (open bar) followed by staining with the optimal dilution of 1:400 (black bar). The numbers of animals per group are shown in brackets. *: significantly different from staining with the supraoptimal dilution in the same group on the same gestational age; $p < 0.01$.*

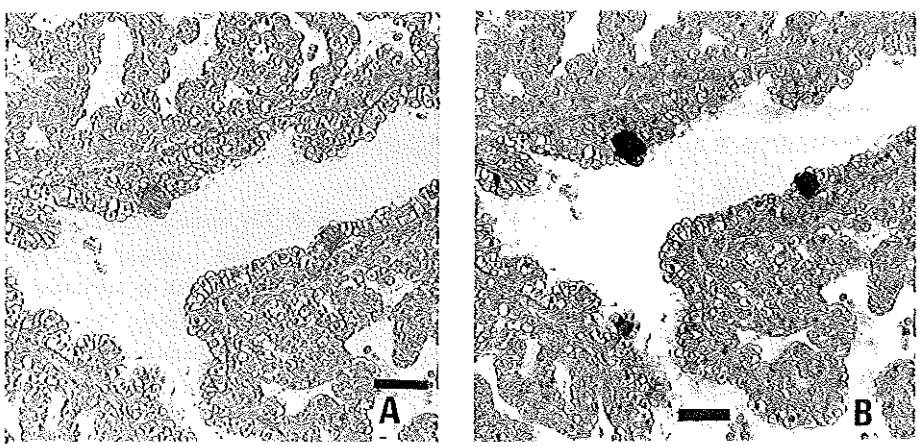


Figure 6: Representative pictures showing weak staining in the right lung in CDH on Day 22 after immunostaining with the supraoptimal dilution of 1:60,000 (6A), followed by staining with the optimal dilution of 1:400 (6B). No counterstaining. Scalebar represents 50 µm.

4.2.5 Discussion

In the present study we found some delay in the development of CGRP-positive PNEC in lungs of rats with CDH on gestational Day 18; i.e. CGRP-positive PNEC were observed in all controls, but not in all CDH cases. Our findings on days 20

and 22 could indicate that lung development in CDH accelerates towards the end of gestation.

In rats with normal lung development NEB have been identified from gestational day 15 onward using PAS-lead hematoxylin technique.[8,26] CGRP-immunoreactivity in fetal rats has been observed in cultures up from day 15+2[27], and in vivo up from day 18[4], which is in accordance with our findings in control fetuses. The number of NEB increases towards term and decreases rapidly during the first week after birth.[4,26] Our findings in controls and in Nitrofen-exposed rat pups without CDH support earlier observations that in normal fetal rat lungs the maximal CGRP-immunoreactivity is reached on gestational Day 20.[4]

In CDH pups, however, both the number and the size of the CGRP-positive areas increased significantly between Day 20 and Day 22. The number of CGRP-positive PNEC and NEB per mm^2 (frequency of CGRP-positive cells) was significantly higher in CDH compared with both other groups on Day 22. This previously described phenomenon suggests that the PNEC in CDH lungs contain increased levels of CGRP towards the end of gestation.[19,28] On the other hand, especially on Day 22, the lung tissue had a more compact appearance in CDH and less airspaces were apparent than in controls. The frequency of CGRP-immunopositive cells may, therefore, not adequately reflect their actual number. This assumption is confirmed by findings of Brandsma et al.[29] that on Day 22, but not on Day 19, the proportion of future airspaces was twice as high in lungs of controls and of Nitrofen-exposed pups without CDH compared with those of CDH pups. Hence a significantly higher proportion of lung tissue was present in CDH at that time.

Further morphometric analysis was concentrated on lung tissue samples from controls and CDH pups of gestational Day 22 to determine whether an increased level of CGRP is present in CDH lungs towards term. The similar %IMS-airways and %IMS-epithelium for the right lungs in both groups disagrees with this assumption. However, the left, more hypoplastic lung in CDH had a significantly higher %IMS-airways than controls. There was also a tendency towards higher %IMS-epithelium indicating that more CGRP-immunoreactive cells may be present in these lungs in CDH on Day 22. In both lungs of CDH pups larger and more prominent NEB were present compared with those in control lungs. While the mean size of the CGRP-positive areas and the mean number of nuclei per NEB were not significantly different between CDH and controls, several NEB in CDH lungs were found with a double number of nuclei compared to NEB in control

lungs. Interestingly, hyperplasia of bombesin-immunostained NEB has been observed in lungs of human cases of CDH (unpublished data).

The higher cell count ratio in CDH than in controls on gestational Day 20 found after immunostaining with a supraoptimal dilution of 1:24,000 suggests that more intracellular CGRP was present in the PNEC of CDH pups. To emphasize the differences in the intracellular CGRP-content we repeated the supraoptimal dilution experiment using a dilution of 1:60,000. Surprisingly, the cell count ratio decreased to the same level for CDH and controls, both on Day 20 and on Day 22. For both groups the mean size of the CGRP-positive areas decreased significantly when immunostaining with a dilution of 1:60,000 was followed by restaining with the optimal dilution, suggesting that only the largest NEB contained enough intracellular CGRP to stain with this supraoptimal dilution. The similar cell count ratios at a dilution of 1:60,000 in CDH and controls indicate the presence of similar numbers of large NEB containing similar levels of CGRP.

It remains speculative whether CGRP-immunoreactivity in CDH increases towards the end of gestation, as suggested previously.[19,28] In favour of this assumption are the higher frequency of CGRP-immunoreactive cells in CDH, and the increased number of CGRP-positive airways in the ipsilateral, most hypoplastic lungs in CDH. On the other hand, three observations indicate that shortly before birth CGRP-immunoreactivity is similar in CDH and controls: a) the similar total number and size of CGRP-positive areas, b) no significant differences were found in the proportion of immunostained epithelium, and c) supraoptimal dilution experiments revealed no significant differences between CDH and controls on Day 22.

Several findings support earlier reports that Nitrofen itself has a negative effect on lung development[21,29]: a) Nitrofen-exposed pups have a significantly smaller lung surface area than controls on Day 20 and Day 22; b) on Day 20 lungs of rat pups exposed to Nitrofen with and without CDH have a similar lung surface area; c) no significant differences in CGRP-immunoreactivity were found between the ipsilateral, most hypoplastic lungs and the contralateral lungs in CDH. On Day 20 the mean number and size of CGRP-positive areas were slightly, but not significantly smaller in CDH than in controls, and the mean CGRP-positive frequency was similar in both groups. This suggests that decreased CGRP-expression from Day 18, which probably reflects the effect of Nitrofen exposure, persists in CDH, but that subsequent expression of CGRP-immunoreactivity may be faster in CDH than in controls. It can be assumed that lung hypoplasia could be mediated via a growth factor, i.e. CGRP.

That in lungs of Nitrofen-exposed rat pups with CDH the frequency of CGRP-positive cells was increased compared to those without CDH, and that in lungs of rats with CDH prominent NEB were present, suggest that Nitrofen exposure alone is not sufficient to explain the retarded lung development in this CDH model. This assumption is supported by findings of Brandsma et al.[29] who reported delayed development of airspaces only if CDH was present.

Altered expression of neuropeptides may lead to an abnormal function of NEB as O_2 sensors in the perinatal period.[2,6] Under normal circumstances increased O_2 tension at birth would decrease the stimulation of NEB, whereas continued hypoxia (as in CDH cases) would increase stimulation by two possible scenarios[30,31]: Mediators with effects on the pulmonary circulation may be released locally, or signaling via innervation may occur. Further studies with O_2 sensor inhibitors or CGRP inhibitors are necessary to determine whether an abnormal function of NEB contributes to the pathophysiology of CDH.

In conclusion, the present study shows that the developmental pattern of CGRP-immunoreactivity in lungs of CDH rats differs from that of controls. The differences in CGRP-immunoreactivity observed on Day 18 suggest a delayed expression at the late pseudoglandular/early canalicular stage of lung development in CDH. During this crucial developmental period, with formation of capillaries and angiogenesis, lack of CGRP may result in decreased vascularization[12], a feature of pulmonary abnormalities in CDH.[16,17] This, and the fact that CGRP has a role in the regulation of airway epithelial cell proliferation[13], suggest that CGRP could at this stage act as a growth factor, similarly to the expression of bombesin-like peptides in the human lung.[8]

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Chapter 5

Lung eicosanoids in perinatal rats with congenital diaphragmatic hernia*

5.1 Summary

Abnormal levels of pulmonary eicosanoids have been reported in infants with persistent pulmonary hypertension (PPH) and congenital diaphragmatic hernia (CDH). We hypothesized that a dysbalance of vasoconstrictive and vasodilatory eicosanoids is involved in PPH in CDH patients. The levels of several eicosanoids in lung homogenates and in bronchoalveolar lavage fluid of controls and rats with CDH were measured after cesarean section or spontaneous birth. In controls the concentration of 6-keto-$PGF_{1\alpha}$, TxB_2, PGE_2, and LTB_4 decreased after spontaneous birth. CDH pups showed respiratory insufficiency directly after birth. Their lungs had higher levels of 6-keto-$PGF_{1\alpha}$, reflecting the pulmonary vasodilator prostacyclin (PGI_2), than those of controls. We conclude that in CDH abnormal lung eicosanoid levels are present perinatally. The elevated levels of 6-keto-$PGF_{1\alpha}$ in CDH may reflect a compensation mechanism for increased vascular resistance.

5.2 Introduction

Eicosanoids are arachidonic acid metabolites which are produced in different tissues in human and animal species.[1] They have been studied extensively in relation to the perinatal pulmonary circulation, and have been implicated in several physiologic and pathologic conditions such as persistent pulmonary hypertension (PPH).[2-7]

Prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), and thromboxane A_2 (TxA_2) are all generated via the cyclooxygenase pathway; the latter has a pulmonary vasoconstricting activity, whereas the other two are pulmonary vasodilators.[6]

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Increased circulating levels of PGE_2 may contribute to the pathogenesis of patent ductus arteriosus.[8] Leukotrienes, which are formed by the 5-lipoxygenase pathway, may have a key function in maintaining the elevated pulmonary vascular resistance in the fetus[6,9], although this could not be confirmed in other studies.[7,10]

Children with congenital diaphragmatic hernia (CDH) have abnormal morphological development of lungs and intrapulmonary blood vessels.[11,12] The high neonatal mortality and morbidity is ascribed to the extent of lung hypoplasia and PPH.[13] Increased levels of leukotrienes, and of metabolites of PGI_2 and TxA_2 have been reported in plasma and in bronchoalveolar lavage (BAL) fluid of both PPH patients without CDH and children with CDH.[14-21]

We hypothesized that the pulmonary vascular abnormalities in CDH cause abnormal transition of the pulmonary circulation at birth, associated with a dysbalance of vasoconstrictive and vasodilatory eicosanoids. Therefore, we studied the content of different eicosanoids — metabolites from the cyclooxygenase and one from the lipoxygenase pathway — in lung homogenates and in BAL fluid of perinatal rats with CDH.[22] The pulmonary vascular abnormalities in these rat pups strongly resemble those of children with CDH.[23]

5.3 Materials and methods

5.3.1 Animal model

Female Sprague-Dawley rats (Harlan Olac, England) were mated during one hour (day 0 of gestation). Nine of 18 pregnant rats received 100 mg of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen: Rohm Haas Company, Philadelphia, PA) in 1 ml of olive oil orogastrically under light ether anaesthesia on day 10 of gestation[22]; the remaining nine rats provided control pups. Nitrofen induces a large left-sided diaphragmatic defect with severe lung hypoplasia in up to 80% of the offspring using this regimen.[22] Food and water were supplied ad libitum during the whole period of pregnancy. Nine pregnant dams were anaesthetized by inhalation of diethylether and a cesarean section was performed on day 22 (Nitrofen-exposed litters n=5; control litters n=4). While they were kept in the membranes to prevent any breathing, the fetuses died after cervical intersection with a needle, and were weighed. Only rat pups that could be processed within the first 30 minutes of anaesthesia were included. In the remaining litters (Nitrofen-exposed n=4, and controls n=5) spontaneous birth on day 22-23 was awaited; within 5-10 minutes

after birth they were killed as described above, and weighed. The presence of a diaphragmatic defect in all Nitrofen-exposed rat pups was revealed by autopsy. To obtain a homogeneous group only Nitrofen-exposed rat pups with left-sided or bilateral diaphragmatic defects with concomitant severe lung hypoplasia were included, and Nitrofen-exposed pups with small right-sided defects or without CDH were excluded. Thus four different groups were studied: CDH rat pups after cesarean section or born spontaneously, and control pups after cesarean section or born spontaneously. Either BAL procedure or dissection of the lungs for preparation of homogenates was then performed.

5.3.2 Lung homogenates

The lungs were removed, stripped of non-pulmonary tissue, separated, weighed, frozen in liquid N_2, and stored at -70°C until further processed. Then they were homogenized in 1 ml of Krebs-solution, and centrifuged at 2500 g. The content of eicosanoids and protein was measured in the supernatant. Ten samples were obtained in CDH pups after cesarean section and four in spontaneously born pups. In controls the numbers were n = 11 and n = 23, respectively.

5.3.3 BAL procedure

After opening of the abdominal cavity and assessment of the diaphragmatic defect in the Nitrofen-exposed pups, the thorax was opened, and a tracheotomy was performed. A polyethylene catheter (Portex, England; outer diameter 0.61 mm or 1.0 mm, inner diameter 0.28 or 0.5 mm, for CDH pups and controls respectively) was inserted into the trachea and ligated. A 1 ml-syringe with NaCl 0.9%, heated to 37°C, was connected to the catheter, and the lungs were washed as described before.[24] In CDH pups the lungs were washed with seven to 10 times 0.05 to 0.1 ml. Lungs from control pups were washed four times with 0.25 to 0.45 ml, until 1 ml of fluid had been recovered. Samples that were visibly contaminated with blood were excluded. Ten samples were obtained in each CDH group, and 13 samples in each control group. The BAL fluid was directly frozen in liquid N_2 and stored at -70°C until assay.

5.3.4 *Measurement of eicosanoids and total protein*

The following eicosanoids were measured by radioimmunoassay: 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin), PGE_2, TxB_2 (the stable metabolite of TxA_2), all three generated by the cyclooxygenase pathway, and leukotriene B_4 (LTB_4), a lipoxygenase-derived metabolite of arachidonic acid. All assays were performed as described in detail previously.[25] Total protein was measured by ELISA at 595 nm using a commercially available protein reagent and protein standard (Instruchemie B.V., Hilversum, The Netherlands).

5.3.5 *Data analysis*

All eicosanoid levels are expressed as pg/μg protein (mean ± SEM), unless stated otherwise. Differences between groups were tested by Student's t-test or by the non-parametric Mann-Whitney test if appropriate. Statistical significance was assumed at 5% level.

5.4 Results

All spontaneously born control pups had a regular respiration rate and were pink within minutes after birth. Respiratory insufficiency with gasping and cyanosis was observed in rat pups with CDH, but not in controls, directly after birth.

The lung weights in spontaneously born control pups were significantly lower than those in controls delivered by cesarean section (Table 1; $p < 0.001$). This was not the case in the CDH pups: the lung weights were similar in both groups (Table 1). Control lungs were significantly heavier than lungs in CDH ($p < 0.001$).

5.4.1 *Results in lung homogenates*

First, data from the left and the right lungs in all groups were analyzed separately to determine whether there were consistent differences in eicosanoid levels between the ipsilateral and contralateral lungs in CDH (data not shown). This was not the case, however, and data from both lungs were therefore pooled.

In CDH pups protein per mg wet lung weight was higher than in controls: 28.8 ± 0.8 μg and 27.5 ± 1.1 μg after cesarean section and spontaneous delivery in CDH, respectively, and 13.5 ± 0.1 μg and 18.5 ± 0.6 μg in controls, respectively

Table 1: *Lung weights and total amount of eicosanoids in lung homogenates of controls and CDH pups after delivery by cesarean section or after spontaneous birth*

		Cesarean section	Spontaneous birth
lung weight (mg)	Control	149 ± 2	115 ± 4[b]
	CDH	62 ± 2[a]	62 ± 3[a]
6-keto-$PGF_{1\alpha}$ (pg)	Control	4340 ± 210	3260 ± 160[b]
	CDH	7830 ± 320[a]	7670 ± 270[a]
TxB_2 (pg)	Control	27620 ± 1600	21560 ± 850[b]
	CDH	21070 ± 1320[a]	19970 ± 880
PGE_2 (pg)	Control	42110 ± 2210	30410 ± 1450[b]
	CDH	27750 ± 2050[a]	24690 ± 1630[a]
LTB_4 (pg)	Control	3410 ± 180	2950 ± 130[b]
	CDH	2160 ± 150[a]	2610 ± 350

All data are expressed as mean ± SEM. The numbers per group are: n=11 and n=23 for the controls delivered by cesarean section and by spontaneous birth, respectively; n=10 and n=4 for the respective CDH groups. [a] *significantly different from control, same delivery mode,* $p < 0.05$; [b] *significantly different from cesarean section, same group,* $p < 0.05$.

($p < 0.001$). In controls the protein content per mg lung weight was significantly lower in pups who were delivered by cesarean section than in spontaneously born pups ($p < 0.001$), but this was not true for the total protein content in both lungs (2020 ± 9 µg after cesarean section and 2060 ± 24 µg after spontaneous birth). In all control pups the total amount of protein was higher than in CDH pups, whose lungs contained 1760 ± 12 µg and 1710 ± 8 µg protein in the respective groups ($p < 0.001$).

The eicosanoid concentrations per µg protein measured in the lung homogenates are shown in Figure 1. In controls the concentrations of all eicosanoids per µg protein (Figures 1A-D) and the total amount of eicosanoids (Table 1) were significantly lower in spontaneously born pups, compared to those in the cesarean section group. In CDH pups the eicosanoid levels were not affected by the delivery mode; this was also the case for the eicosanoid content per mg lung weight (data not shown).

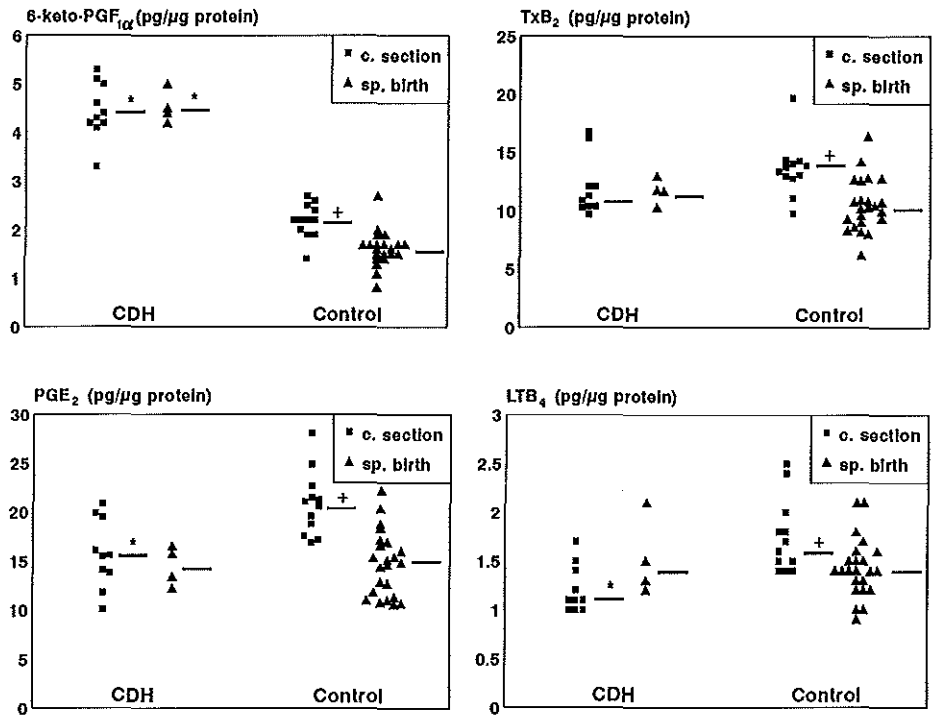

*Figure 1: Concentration of 6-keto-$PGF_{1\alpha}$, TxB_2, PGE_2, and LTB_4 per µg protein in lung homogenates of rat pups with CDH and controls delivered by cesarean section (squares) or spontaneously born pups (triangles). Median is indicated for each group. * significantly different from controls, similar mode of delivery, $p < 0.01$; + significantly different from controls after spontaneous birth, $p < 0.02$.*

The levels of 6-keto-$PGF_{1\alpha}$ per µg protein (Figure 1A; $p < 0.001$) and the total amount of 6-keto-$PGF_{1\alpha}$ (Table 1; $p < 0.001$) were significantly higher in CDH than in controls. In addition, the ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was calculated for each group; in the cesarean section group it was 0.38 ± 0.03 and 0.16 ± 0.01 for CDH and controls, respectively ($p < 0.001$), and for spontaneously born rat pups 0.39 ± 0.02 and 0.16 ± 0.01, respectively ($p < 0.001$).

Controls born by cesarean section had higher total TxB_2 than CDH pups (Table 1; $p = 0.006$) and a tendency towards higher TxB_2 per µg protein (Figure 1B; $p = 0.08$). No such differences for TxB_2 were observed in the spontaneously born rat pups. PGE_2 per µg protein was significantly higher in control pups delivered by cesarean section than in CDH pups (Figure 1C; $p = 0.003$). The total amounts of

PGE_2 were higher in controls than in CDH pups, irrespective of the delivery mode (Table 1). The concentration of LTB_4 per µg protein (Figure 1D) and the total amount of LTB_4 (Table 1) were significantly higher in controls than in CDH pups after cesarean section ($p < 0.001$), whereas both groups showed similar LTB_4 levels after spontaneous delivery.

5.4.2 Eicosanoids in BAL fluid

In BAL fluid a wide range of eicosanoid concentrations was observed. In controls the concentrations per ml BAL fluid of 6-keto-$PGF_{1\alpha}$, TxB_2, and LTB_4 were higher after spontaneous birth than after cesarean section (Table 2; $p = 0.01$, 0.06, and < 0.001, respectively). However, after correction for dilution, with total protein as marker, only LTB_4 was significantly higher after spontaneous birth (Table 2; $p = 0.02$). CDH pups showed higher uncorrected concentration levels of TxB_2 and LTB_4 in spontaneously born rats compared with pups delivered by cesarean section (Table 2; $p = 0.04$ and 0.05, respectively). The sample volumes in CDH pups were so small that the protein concentration could only be measured in eight samples (n=4 per group).

The ratio of 6-keto-$PGF_{1\alpha}$ and TxB_2 in BAL fluid was significantly higher in CDH pups than in controls who were delivered by cesarean section (7.93 ± 2.95 and 2.22 ± 0.5, respectively; $p = 0.02$). A similar tendency was observed for the spontaneously born rat pups (3.63 ± 1.13 for CDH, and 1.48 ± 0.32 for controls; $p = 0.06$).

5.5 Discussion

In the present study higher levels of 6-keto-$PGF_{1\alpha}$, the stable metabolite of the pulmonary vasodilator PGI_2, were found in the lungs of CDH pups than in those of controls, irrespective of the mode of delivery. Lungs of CDH pups had similar or lower levels of TxB_2, PGE_2, and LTB_4 than control pups. All eicosanoids studied were higher in the lungs of control pups delivered by cesarean section than in those born spontaneously; this was not the case in CDH pups.

The lower lung weights in spontaneously born controls compared to those delivered by cesarean section probably indicate that lung fluid was absorbed to a large extent during the first adequate breaths. The gasping, irregular breathing movements in the spontaneously born CDH pups have been insufficient to

***Table 2:** Eicosanoids in BAL fluid of controls and CDH pups after delivery by cesarean section or after spontaneous birth.*

		Cesarean section	Spontaneous birth
6-keto-$PGF_{1\alpha}$ (pg/ml)	Control	171 (64-303)	278 (102-743)[a]
	CDH	207 (110-521)	243 (172-617)
6-keto-$PGF_{1\alpha}$ (pg/µg protein)	Control	1.72 (0.58-14.8)	1.43 (1.06-4.04)
	CDH	5.53 (1.88-38.9)	1.81 (1.12-3.52)
TxB_2 (pg/ml)	Control	100 (23-372)	182 (78-496)
	CDH	65 (6-140)	96 (19-305)[a]
TxB_2 (pg/µg protein)	Control	1.19 (0.47-5.33)	1.18 (0.3-2.75)
	CDH	1.62 (0.16-6.72)	0.69 (0.43-2.5)
LTB_4 (pg/ml)	Control	15 (5-105)	162 (25-661)[a]
	CDH	42 (14-194)	100 (51-200)[a,b]
LTB_4 (pg/µg protein)	Control	0.24 (0.08-6.9)	0.92 (0.12-2.57)[a]
	CDH	0.23 (0.19-2)	0.87 (0.38-0.99)

All values are expressed as median (range). Data shown per ml BAL fluid are n=10 for each CDH group and n=13 per control group. Data shown per µg protein are n=4 for each CDH group, n=13 for controls delivered by cesarean section, and n=10 for spontaneously born controls. [a] *significantly different from cesarean section in the same group; $p < 0.05$;* [b] *significantly different from spontaneously born CDH pups; $p < 0.05$.*

overcome the pressure that is needed to initiate lung expansion and to provide adequate lung aeration and absorption of lung fluid[26,27], thus explaining the similar lung weights in both CDH groups.

We studied the eicosanoid concentration both in lung homogenates and in BAL fluid to determine whether the concentration in BAL fluid adequately reflects the situation in the lung tissue. We found widely varying eicosanoid concentrations in BAL fluid of the neonatal rat pups. After correction for protein, only the concentration of 6-keto-$PGF_{1\alpha}$ in control pups showed comparable results between BAL fluid and lung homogenates. The concentration of TxB_2 was generally 10 times higher in lung homogenates than in BAL fluid, which suggests that

thromboxane is mainly present in the pulmonary vasculature and not into the airspaces. The same may be true for the concentration of LTB_4 during intrauterine life. Our data support earlier observations that the eicosanoid content in the pulmonary vasculature is more adequately reflected in tissue homogenates than in BAL fluid[7]. However, the ratio of 6-keto-$PGF_{1\alpha}$ and TxB_2 was significantly higher in lung homogenates and in BAL fluid of CDH pups than in that of controls, suggesting that this parameter in BAL fluid reflects the values in lung tissue. A high ratio of 6-keto-$PGF_{1\alpha}$ and TxB_2 was also found in BAL fluid of two CDH patients with evidence of PPH (unpublished data).

During normal transition from intrauterine to extrauterine life, the pulmonary vascular resistance rapidly declines within the first 30 s, and declines more slowly over the next 10-20 minutes.[2] The first phase occurs irrespective of prostaglandin synthase blockade by indomethacin[2], but several studies in lambs and goats indicate that a transient prostacyclin production in the lungs, which is stimulated by tissue stress during establishment of gaseous ventilation and rhythmic ventilation[4], is important to sustain further pulmonary vasodilatation within the first hours after birth.[2-4,7]

The lower levels of all eicosanoids in lung tissue of normal controls compared to CDH pups following spontaneous birth in this study seem to contradict the earlier findings in newborn lambs and goats.[2-4,7] Perhaps the described loss of prostacyclin from the lungs shortly before birth[3] continued immediately after birth, and the rat pups died before the prostacyclin concentration began to increase. However, the CDH pups could not survive much longer without artificial ventilation and supplemental oxygen.

Persistent pulmonary hypertension is a serious problem in neonatology which largely contributes to the neonatal mortality and morbidity in isolated cases of PPH[28], and in children with CDH.[13] Improvement of oxygenation parameters in some children with PPH has been reported after intravenous or inhaled administration of prostacyclin[29,30], although other patients seem irresponsive to vasodilator therapy like prostacyclin[29] or inhaled nitric oxide.[28]

Increased levels of 6-keto-$PGF_{1\alpha}$, TxB_2, PGE_2, and leukotrienes have been reported in plasma and in bronchoalveolar lavage fluid of PPH patients[14,17,19,21] and in plasma of CDH patients with PPH.[15,16,21] A decrease in all eicosanoid levels was observed during clinical improvement, especially in patients who were being treated with extracorporeal membrane oxygenation.[16,21] It has been suggested that LTC_4, LTD_4, and TxB_2 have a pulmonary vasoconstricting activity, whereas 6-keto-$PGF_{1\alpha}$ opposes the hypoxic vasocontriction.[31]

In a fetal lamb model of chronic intrauterine pulmonary hypertension[32] increased pulmonary levels of 6-keto-$PGF_{1\alpha}$ and TxB_2 were detected shortly before, and 2 h after birth[7]. Our study did not reveal significant differences in eicosanoid content in the lungs of the CDH rats during transition from intrauterine to extrauterine life; this may be due to the short period of survival after birth and the lack of adequate respiratory movements.

We found increased levels of 6-keto-$PGF_{1\alpha}$ per μg protein, and decreased levels of PGE_2 and LTB_4 in lung tissue of CDH pups in the cesarean section group. Surprisingly, the concentration of TxB_2 per μg protein was similar in CDH pups and in controls. We assumed that a certain total amount of eicosanoids is important to exert a local effect, and we therefore determined the total eicosanoid content in both lungs of CDH and control pups. The results were similar to the data that were corrected for protein. The increased ratio of 6-keto-$PGF_{1\alpha}$ and TxB_2 in CDH pups compared to control pups confirms the presence of a dysbalance in vasoactive mediators, which is in favour of the pulmonary vasodilator. It has been suggested that prostaglandin generation in the pulmonary vasculature may reduce the pulmonary vascular pressure response to hypoxia.[31] Cott and coworkers[33] showed in adult rats that alveolar type II cells are capable of producing high levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 in vitro, whereas TxB_2 and LTB_4 are mainly produced by alveolar macrophages. Brandsma and coworkers[34] reported more type II cells that showed retarded differentiation in CDH lungs. The relatively higher number of type II cells may be responsible for the increased 6-keto-$PGF_{1\alpha}$ content in the CDH lungs.

In conclusion, the present study shows different eicosanoid profiles in lungs of perinatal rats with and without CDH. The most striking findings are the elevated concentration of 6-keto-$PGF_{1\alpha}$, and the increased ratio of 6-keto-$PGF_{1\alpha}$ and TxB_2 in CDH lungs. This is the first study of lung eicosanoids in perinatal animals with abnormal lung development. From the present data it is not clear whether the balance which is in favour of 6-keto-$PGF_{1\alpha}$, the stable metabolite of the pulmonary vasodilator PGI_2, compensates for an increased pulmonary vascular tone or that it reflects delayed cell differentiation in CDH. Our findings give reason to assume that lungs of CDH patients with PPH already contain increased levels of prostacyclin at birth and that the administration of exogenous vasodilators will not be helpful to decrease the pulmonary vascular resistance in those patients.

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Part III

Aspects of lung injury in CDH

Chapter 6

Prospective evaluation of prostanoid levels and inflammation markers in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age-matched controls*

6.1 Summary

Objective: To analyse whether vasoactive prostanoids which may be involved in the pathogenesis of persistent pulmonary hypertension (PPH) in children with congenital diaphragmatic hernia (CDH) have increased levels in bronchoalveolar lavage (BAL) fluid. Because inflammation induces the production of prostanoids, the correlations between the prostanoid levels and various inflammatory parameters were studied as well. *Design*: We measured the concentrations of two vasoactive prostanoids in BAL fluid of CDH patients treated with conventional ventilation or with ECMO and compared these with levels in BAL fluid of ventilated matched controls without PPH and pulmonary abnormalities. *Setting*: Surgical intensive care unit in a pediatric university hospital. *Methods*: BAL was performed in 18 CDH patients, five were treated with ECMO, and in 13 controls without CDH and concentrations of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), thromboxane B_2 (TxB_2), protein, albumin, total cell count, and elastase-α_1-proteinase-inhibitor complex were measured. Ventilatory parameters were recorded at the time of BAL. *Results*: We found different concentrations of prostanoids in BAL fluid of CDH patients with PPH: infants who died, or those who needed ventilatory support for a period of more than four weeks had either high levels of both 6-keto-$PGF_{1\alpha}$ and TxB_2 compared to controls, or high levels of 6-keto-$PGF_{1\alpha}$ only. The concentrations of TxB_2 correlated positively with both albumin and protein in BAL fluid of all CDH patients, and with the cell counts in CDH-ECMO patients. *Conclusion*: In some CDH patients with PPH high prostanoid concentrations in BAL fluid were associated with death either within a few hours after birth, or from

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recurrent episodes of therapy-resistent pulmonary hypertension several days after ECMO.

6.2 Introduction

The high mortality and morbidity in children with congenital diaphragmatic hernia (CDH) is largely determined by the severity of lung hypoplasia and persistent pulmonary hypertension (PPH).[1]

In the lung, arachidonic acid metabolites regulate the bronchial and vascular tone, and are involved in inflammatory processes.[2] Increased levels of eicosanoids have been reported in plasma and in bronchoalveolar lavage (BAL) fluid of children with PPH who were treated with conventional ventilation or with extracorporeal membrane oxygenation (ECMO).[3-6] Increased plasma levels of the stable metabolites of the pulmonary broncho-, and vasoconstrictor thromboxane A_2 (TxA_2) and the pulmonary vasodilator prostacyclin (PGI_2), TxB_2 and 6-keto-$PGF_{1\alpha}$ have been observed in CDH patients during episodes of hypoxemia[7], and in the immediate postoperative period.[8-10] BAL has been used to evaluate different aspects of inflammatory responses, such as the number of neutrophilic granulocytes, albumin, elastase, and α_1-proteinase inhibitor activity in ventilated preterm infants with respiratory distress syndrome who were likely to develop chronic lung disease.[11-13] We hypothesized that in CDH vasoactive prostanoids are involved in the pathogenesis of PPH, which might be reflected by increased levels in BAL fluid.

The aim of the present study was to establish the concentrations of 6-keto-$PGF_{1\alpha}$ and of TxB_2 in BAL fluid of CDH patients who were treated either with conventional ventilation or with ECMO and to compare these with levels in BAL fluid of controls without PPH who have similar gestational age and birth weight. Because inflammation induces the production of prostanoids[2], the correlations between the prostanoid levels and various inflammatory parameters were studied as well.

6.3 Patients and methods

6.3.1 Patients

The study was performed in our Pediatric Surgical Intensive Care Unit between December 1993 and January 1996. A group of 18 CDH patients was studied; 13 children underwent conventional ventilation (referred to as the CDH-CV group), and five children were treated with venoarterial ECMO (referred to as the CDH-ECMO group) using standardized treatment protocols.[14-16] Four CDH patients had been diagnosed prenatally. Ten patients in the CDH-CV group and all five ECMO patients suffered from respiratory insufficiency within six hours after birth; the other three patients were respiratory insufficient after 10 hours, 36 hours, and 28 days, respectively. Operative repair by an abdominal approach was performed in 11 conventionally ventilated children and in three ECMO patients after preoperative clinical stabilization;[14] four of the five patients who died had not been operated on. All 18 patients routinely received antimicrobial prophylaxis. They underwent echocardiography on admission: right-to-left shunting was diagnosed in six children of the CDH-CV group and in all CDH-ECMO patients.[10] Clinical evidence for right-to-left shunting was obtained by preductal and postductal transcutaneous O_2-saturation differences of > 10% in five CV-patients and in all ECMO patients.

For CDH patients in our institution the entry criteria for ECMO were: gestational age at least 34 weeks, birth weight at least 2000 grams, artificial ventilation for less than 7 days, alveolar-arterial oxygen difference (A-aDO_2) > 600 torr;[15,16] maximal PaO_2 at least 10 kPa. During ECMO ventilatory settings were usually reduced to PIP 12-16 cm H_2O, PEEP 5-6 cm H_2O, rate 10-15/min, and FIO_2 0.25-0.3.

Thirteen other children without pulmonary abnormalities, who were mainly ventilated perioperatively, served as age-matched controls. They were selected for the best possible match for gestational age and birth weight. They all received antimicrobial therapy. Echocardiography was performed in five controls to exclude structural cardiac anomalies; none of these had evidence of right-to-left shunting.

In all 31 patients sputum cultures were routinely performed every three days. The study was conducted according to the principles established in Helsinki and was approved by the Medical Ethical Committee of our hospital.

6.3.2 Study design

Of each patient the following data were recorded: gestational age, birth weight, diagnosis, survival, age at admission and at discharge or death, age at start of respiratory insufficiency, duration of ECMO and/or artificial ventilation, and duration of supplemental O_2 therapy.

Bronchoalveolar lavage was performed on day 1, day 3, and day 7 and once every week thereafter as long as endotracheal intubation was continued and the child remained in our Intensive Care Unit. However, an unstable clinical condition or the inability to obtain parental informed consent during the first days were reasons to modify this schedule.

The following data were recorded at each BAL procedure: medication, clinical events such as surgery, mean airway pressure (MAP), oxygenation index (OI), and $AaDO_2$.[14] Arterial blood gases and serum urea concentration, to correct BAL parameters for dilution, were obtained within six hours from the time of BAL.

6.3.3 BAL procedure

The procedure was performed directly after routine endotracheal suctioning by the nursing staff using a technique described by Grigg and coworkers.[17] The patient's head turned left, a 5 Fr open-ended catheter (outer diameter 1.7 mm; Sherwood Medical, Petit Rechain, Belgium) was passed down the endotracheal tube and placed in wedge position. Warmed NaCl 0.9% was instilled in 2 aliquots of 1 ml/kg each. Gentle manual suctioning with a 20 ml-syringe was directly performed after each aliquot. In most cases the ventilatory circuit was not broken. The whole procedure took always less than one minute. The recovered fluid was immediately processed; 20 µl was aspirated for cell counting, the remaining fluid was centrifuged at 900 g. The supernatant was frozen at -80°C, the cells were resuspended in NaCl 0.9% and processed for cytocentrifugation.

6.3.4 Measurements in BAL fluid

BAL fluid was diluted in Türk stain (1:10) for cell counting in a Bürker-hemocytometer. Differential cell counts were carried out on cytospin slide preparations with May-Gruenwald-Giemsa stain. In the supernatant 6-keto-$PGF_{1\alpha}$ and TxB_2 were measured by radioimmunoassay as described previously, using standard prostaglandins from Sigma Co. (St Louis, MO, USA) and antibodies from

Advanced Magnetics Inc. (Cambridge, MA, USA).[18] Elastase-α_1-proteinase inhibitor-complex (E-α_1-PI) was determined using a commercially available kit (PMN Elastase; Merck Immunoassay 11332; Merck, Germany). Albumin was measured by immunoprecipitation using N-antiserum against human albumin (Behring OSAL 14/15; Behring, France). Humane albumin 20% (CLB; Amsterdam, the Netherlands) was used to obtain standard curves. Total protein was measured as described by Lowry[19], standard curves were obtained using Preciset 6 g/100 ml (Boehringer 125610; Boehringer, Germany).

To correct for dilution, urea was determined in serum with a routinely used in-house urease-based assay. Urea in BAL fluid was measured using a commercially available urease-based kit (Merck 3334; Merck, Germany) that was more sensitive than the assay for measurement of plasma concentrations. The volume of epithelial lining fluid (ELF) was calculated from the formula: ELF = (BAL fluid urea/serum urea) x BAL fluid volume.[17]

6.3.5 Data analysis

Data were expressed as median (range) unless stated otherwise. Because a low yield of BAL fluid, not all measurements could be performed in all samples, which resulted in incomplete data. Ventilatory parameters were compared between groups using the non-parametric Mann-Whitney U-test, and statistical significance was assumed at 5% level. Two tailed Spearman's rank correlation coefficient was used to study the relationship between different parameters in BAL fluid and between ventilatory parameters and BAL parameters and statistical significance was assumed at at 1% level, because of the many possible comparisons.

6.4 Results

6.4.1 Patient characteristics

The characteristics of all patients are summarized in Table 1. Ventilated controls had the following diagnoses: meconium peritonitis (n=4), esophageal atresia (n=4), M. Hirschsprung, necrotizing enterocolitis, vesical exstrophy, hiatus hernia with Ehler-Danlos syndrome, wet lung (each n=1). None of the controls showed evidence of PPH. Ten CDH-CV patients had left-sided CDH and three had a right-sided defect. Six CDH-CV patients had PPH: two of those, who never met the

Table 1: *Patient characteristics of controls and CDH patients*

	Controls; n=13 (13)[a]	CDH-CV; n=13 (11)[a]	CDH-ECMO; n=5 (2)[a]
gestational age (weeks)	38 (36-41)	38.5 (34-41)	39 (34-42)
birth weight (grams)	2710 (2350-3910)	3140 (1550-4340)	3000 (2380-3630)
ventilation (days)[b]	3 (1-5)	10 (3-33)	42.5 (34-51)
supplemental O_2 (days)[b]	4 (1-12)	15 (3-47)	58 (35-81)[f]
age at surgery (days)[b]	----	4 (2-28[e])	9.5 (6-13)
age at start ECMO (hours)	----	----	16 (6-42)
duration of ECMO (days)	----	----	14 (6-25)
MAP[d] (day 1-2)	10 (7.7-25)	20 (4.6-62)[e]	----
(day 3-5)	8.9 (5.1-19)	17.7 (9.7-25)[e]	----
OI[d] (day 1-2)	3.3 (2.3-13)	5.5 (1-250)	----
(day 3-5)	1.9 (1.1-4.8)	3.9 (2.6-13)	----
A-aDO_2[d] (day 1-2)	51 (81-230)	110 (17-638)	----
(day 3-5)	26 (18-81)	64 (24-235)	----

Footnote Table 1: The median (range) values are shown for different patient characteristics. CDH-CV = conventionally ventilated CDH patients, CDH-ECMO = ECMO-treated CDH patients. [a] the total number of patients per group are shown, the number of survivors are shown in brackets; [b] only data from survivors are shown; [c] one child was diagnosed at the age of 27 days, surgery was performed one day after diagnosis, no documented PPH; [d] parameters calculated only at the time that BAL was performed, day 1-2: controls n=6, CDH-CV n=9; day 3-5: n=6 for both groups; [e] significantly different from controls, $p < 0.05$; [f] one child again received O_2-therapy one month later and is still O_2-dependent at 27 months of age.

ECMO entry criteria, died during the first day of life. Four CDH-CV patients were O_2-dependent at the age of 28 days (one patient without documented PPH). Of the CDH-ECMO patients, who all had documented PPH, one had a bilateral diaphragmatic defect, the other four had left-sided CDH. Two survivors were operated on while undergoing ECMO, and they were both O_2-dependent at 28 days. The other three ECMO patients died from recurrent episodes of therapy-resistent pulmonary hypertension 5 to 11 days after decanulation; one of these patients was operated on during ECMO on Day 20. In these three patients BAL was not performed after decanulation because their clinical condition was unstable and deteriorated rapidly.

6.4.2 Correction for dilution

To correct for dilution, the urea concentrations in BAL fluid and in serum were used to calculate the volume of epithelial lining fluid (ELF). In CDH-CV and control patients the concentrations of all parameters in BAL fluid and of those in ELF showed a strong positive correlation ($p < 0.001$ for all parameters). In CDH-ECMO patients this was true for the total cell number, protein, albumin, and E-α_1-PI ($p < 0.01$), but not for the prostanoids. We therefore decided to present the uncorrected data, i.e. per ml BAL fluid.

6.4.3 Prostanoid concentrations in BAL fluid of CDH patients

In all but one patients the prostanoid concentration in BAL fluid could at least be measured once; the sample volume of one CDH-CV patient who died during the first day of life was too small to measure prostanoid concentrations. The results are shown in Figures 1 and 2. Two CDH-CV patients with PPH had high levels of 6-keto-$PGF_{1\alpha}$, one of those had also high TxB_2 concentrations. One of these patients

died during the first day of life; the other patient remained O_2-dependent at the age of 28 days.

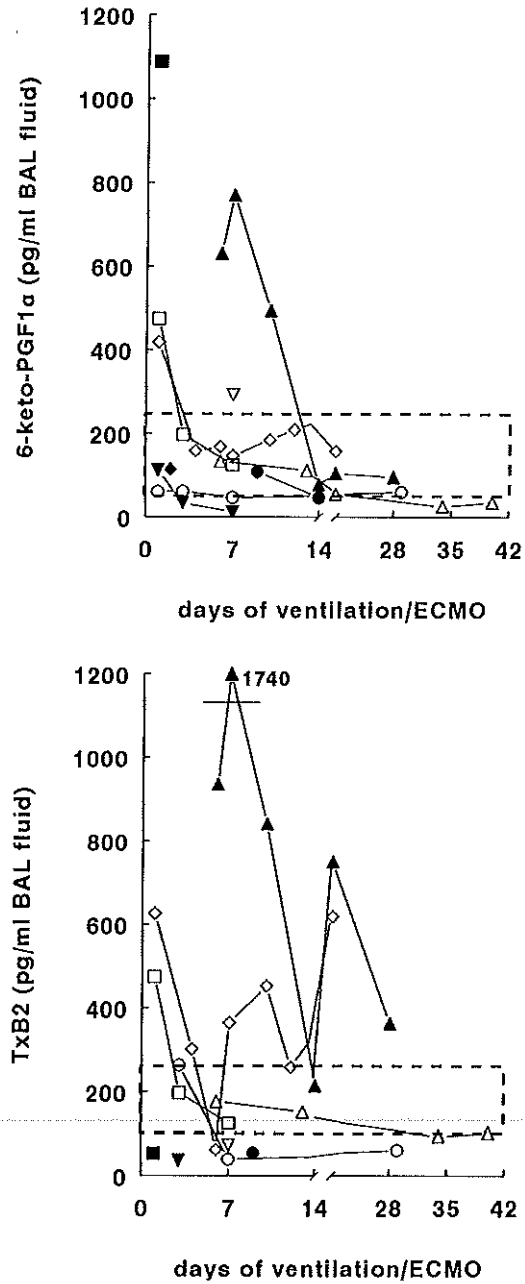
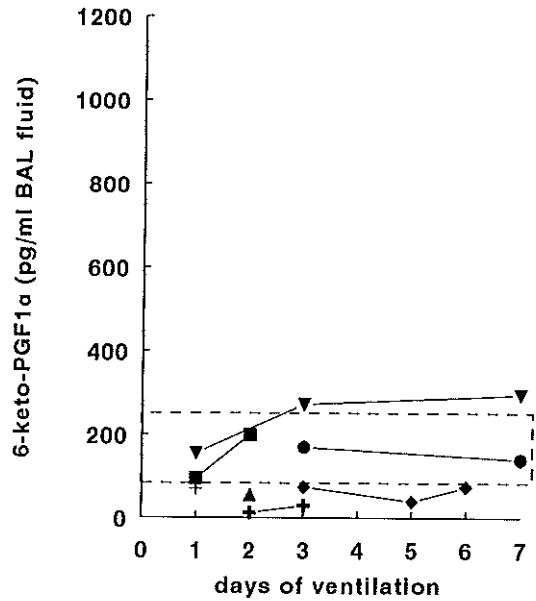


Figure 1: The concentrations of 6-keto-$PGF_{1\alpha}$ (Figure 1a) and TxB_2 (Figure 1b) in BAL fluid of CDH patients with documented PPH are shown. Closed symbols represent conventionally ventilated patients; open symbols with ECMO-treated patients. Each patient is indicated by an individual symbol, a line through the symbols indicates that more BAL procedures were performed in that patient. The box represents the interquartile range concentration of 6-keto-$PGF_{1\alpha}$ and TxB_2 during the first two days of ventilation in 10 control patients without PPH.



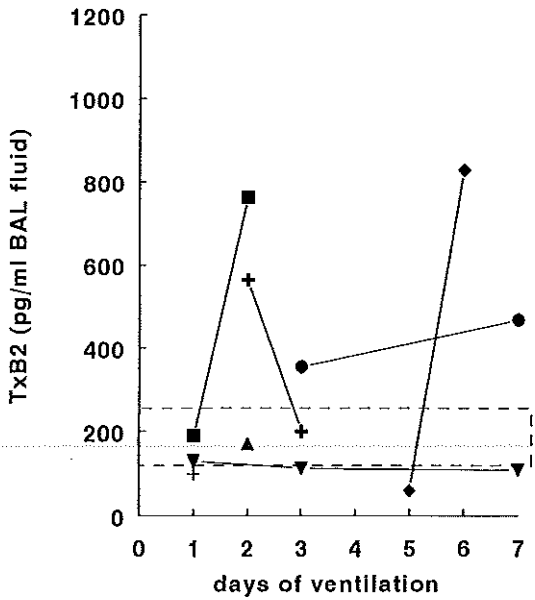


Figure 2: The concentrations of 6-keto-$PGF_{1\alpha}$ (Figure 2a) and TxB_2 (Figure 2b) in BAL fluid of conventionally ventilated CDH patients without documented PPH are shown. Each patient is indicated by an individual symbol, a line through the symbols indicates that more BAL procedures were performed in that patient. The box represents the interquartile range concentration of 6-keto-$PGF_{1\alpha}$ and TxB_2 during the first two days of ventilation in 10 control patients without PPH.

The median ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 of the CDH-CV patients with PPH did not differ from that of those without PPH: 0.52 (0.14-20) versus 0.51 (0.02-2.5), respectively; controls without PPH had a median ratio of 0.6 (0.14-1.6). The ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was high in two infants with CDH: in one conventionally ventilated patient who died during the first day of life it was 20, and in one CDH-ECMO patient who had a poor outcome it was 5.2. In the five CDH-ECMO patients, who all had PPH, the ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was 0.7 (0.23-5.2). Two infants in this group with high initial levels of 6-keto-$PGF_{1\alpha}$ had a poor outcome. They also had high levels of TxB_2 that remained high.

The concentrations of TxB_2 were variable (Figure 1b and 2b), whereas the 6-keto-$PGF_{1\alpha}$ levels remained more or less constant (Figure 1a and 2a). Deterioration of the clinical condition in one patient with PPH who developed sepsis on day 10, followed by thrombosis of the inferior vena cava, was not reflected by an increase in prostanoids initially, but by an increase in TxB_2 a few days later, whereas 6-keto-$PGF_{1\alpha}$ remained low.

6.4.4 Inflammatory markers in BAL fluid and correlation between different parameters

Cultures of sputum were negative in all patients at the time of BAL. The total cell count, the percentage of neutrophilic granulocytes and macrophages, and the concentrations of protein, albumin, and E-α_1-PI in BAL are shown in Table 2. During the first days the median cell count and the levels of protein, albumin, and E-α_1-PI were high in the CDH-ECMO group.

In the CDH-CV group a positive correlation was found between the % neutrophilic granulocytes and MAP, OI, and $AaDO_2$ ($r = 0.48$, 0.49, and 0.55, respectively; $p < 0.01$); these ventilatory parameters correlated negatively with % macrophages. Albumin correlated positively with A-aDO_2 in CDH patients ($r = 0.48$; $p = 0.008$), and with MAP and OI in control patients ($r = 0.58$ and 0.74; $p = 0.01$).

To determine the relation between the prostanoid levels in BAL fluid and inflammation, we evaluated the correlation between the concentrations of prostanoids and a number of markers of airway inflammation. In CDH-CV patients TxB_2 correlated positively with albumin ($r = 0.69$; $p < 0.001$), and with total protein ($r = 0.66$; $p < 0.001$); 6-keto-$PGF_{1\alpha}$ correlated positively with the % neutrophils ($r = 0.44$; $p < 0.01$). In CDH-ECMO patients TxB_2 correlated

Table 2: *Inflammatory parameters in BAL fluid of ventilated controls and CDH patients, and in CDH patients treated with ECMO*

		Controls-CV[a,b]	CDH-CV[a,c]	CDH-ECMO[d]
cells (x 10^4)[e]	Day 1-2	8.7 (1.7-90)	3.5 (1.7-63)	186 (28-227)
	Day 3-5	13 (1.7-38)	10 (1.7-38)	43 (23-69)
% neutrophils	Day 1-2	70 (2-90)	73 (10-85)	79 (36-93)
	Day 3-5	26 (11-86)	35 (3-72)	53 (33-86)
% macrophages	Day 1-2	22 (8-87)	12 (2-82)	14 (3-55)
	Day 3-5	61 (8-84)	60 (13-92)	46 (9-63)
albumin (mg)[e]	Day 1-2	0.1 (0.005-0.31)	0.1 (0.04-1.11)	0.29 (0.02-1.5)
	Day 3-5	0.09 (0.03-0.16)	0.05 (0.03-0.15)	0.11 (0.02-0.6)
protein (mg)[e]	Day 1-2	0.31 (0.02-1.67)	0.26 (0.12-2.03)	1.48 (0.09-2.9)
	Day 3-5	0.25 (0.1-0.42)	0.16 (0.08-0.34)	0.41 (0.26-4.14)
E-α_1-PI (µg)[e,f]	Day 1-2	0.04 (0-1.95)	0.03 (0-0.63)	0.24 (0.004-0.9)
	Day 3-5	0.04 (0-0.21)	0.03 (0-0.07)	0.07 (0-0.57)

[a] CV = conventional ventilation; [b] n = 9-12 on Day 1-2 and n = 6 on day 3 (no lavage data were obtained in controls after Day 3); [c] n = 7-9 on Day 1-2 and n = 5-7 on day 3-5; [d] n = 3 on Day 1-2 and n = 5 on Day 3-5; [e] concentrations are expressed per ml bronchoalveolar lavage fluid; [f] E-α_1-PI = elastase-α_1-proteinase inhibitor complex. Values are indicated as median (range). Statistical analysis between the groups was not performed because of the missing data and the different time points.

positively with albumin ($r = 0.86$; $p < 0.001$), with total protein ($r = 0.74$; $p < 0.001$), and with the total cell number ($r = 0.67$; $p < 0.01$). Prostanoid levels did not correlate with E-α_1-PI. In CDH patients E-α_1-PI correlated positively with the % neutrophils ($r = 0.56$ and 0.63 for CV and ECMO, respectively; $p < 0.01$), and negatively with the % macrophages in CDH-ECMO patients ($r = -0.64$; $p = 0.006$).

6.5 Discussion

In the present study we found different concentrations of prostanoids in BAL fluid of CDH patients with PPH: infants who died, or those who needed ventilatory support for a period of more than four weeks had either high levels of 6-keto-$PGF_{1\alpha}$ and TxB_2 compared to controls, or high levels of 6-keto-$PGF_{1\alpha}$ only. The ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was high in two CDH patients who died.

Increased levels of TxB_2 and 6-keto-$PGF_{1\alpha}$ have been reported in plasma of neonates with PPH who were treated with conventional ventilation or with ECMO.[4,5,7-10] Dobyns and coworkers described increased levels of TxB_2, 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, LTB_4, and LTE_4 in BAL fluid of neonates with PPH.[6] Prostanoid levels in plasma and in BAL fluid were shown to decrease during the course of treatment in these children[5,6] and in one CDH patient treated with ECMO.[9] Our findings are in accordance with the findings of Dobyns and coworkers[6], who found persisting high levels of 6-keto-$PGF_{1\alpha}$ and TxB_2 in BAL fluid of ECMO-treated children with PPH and a poor outcome, whereas the prostanoid levels decreased rapidly in PPH patients with a good outcome. We observed that conventionally ventilated CDH patients with documented PPH and a relatively mild course of disease, and those who survived following ECMO had similar or lower prostanoid concentrations than CDH patients without PPH.

To determine whether the high levels of prostanoids in BAL fluid of some CDH patients with PPH are a specific feature of the abnormal pulmonary vasculature in CDH, these levels should be compared with prostanoid levels in BAL fluid of children without CDH who need ventilatory support to the same extent as the most severely ill CDH patients. Our control population did not allow for such comparison.

The relatively high and variable TxB_2 levels in our ventilated CDH patients without evidence of PPH suggest that the clinical situation in this group of patients is not adequately reflected by the TxB_2 concentrations in BAL fluid. An earlier study from our group showed a correlation between plasma prostanoid levels and

ventilatory parameters in CDH patients.[10] We were not able to demonstrate such correlations in BAL in our study. This may indicate that prostanoid levels in the pulmonary vasculature are not adequately reflected in BAL fluid, as has been suggested by Abman and coworkers[20], and is supported by our finding that in neonatal rats with CDH the concentration of TxB_2 is tenfold higher in lung tissue than in BAL fluid. However, in these rat pups the ratio of 6-keto-$PGF_{1\alpha}$ to TxB_2 was consistently increased in lung tissue and in BAL fluid compared with controls directly after birth, suggesting that this parameter in BAL fluid may be representative for the values in lung tissue (unpublished observation, submitted).

Increased cell counts, neutrophil numbers, albumin, and elastase activity have been reported in tracheal aspirates or BAL fluid of prematurely born, ventilated patients who developed chronic lung disease.[11-13] In the present study concentrations of cells, protein, albumin, and E-α_1-PI complex in the BAL fluid of CDH-ECMO patients were high, compared with conventionally ventilated CDH patients and controls during the first days of treatment. However, statistical analysis of the data was not possible because of the incomplete data and the different time points of BAL. To our knowledge these parameters have not been reported before in lung lavages of ECMO-treated neonates. Increased vascular permeability with influx of cells may be responsible, and might be due to lung injury and activation of the inflammatory cascade before ECMO, or to neutrophil activation during ECMO.[21]

The positive correlation between TxB_2 and both albumin and protein in BAL fluid of all CDH patients, and between TxB_2 and the
total cell counts in CDH-ECMO patients indicate that increased vascular leakage may contribute to the high levels of TxB_2. New concepts in the management of CDH to avoid pulmonary overdistension might have a positive influence on these parameters and, thus, improve the survival of CDH patients and reduce the need for ECMO.[22]

In conclusion, we found that high prostanoid concentrations in BAL fluid were associated with a poor outcome in some CDH patients with PPH who died either within a few hours after birth, or from recurrent episodes of therapy-resistant pulmonary hypertension several days after ECMO. The high levels of 6-keto-$PGF_{1\alpha}$ might have been induced by the hypoxic vasoconstriction in these patients.[23] The variation in TxB_2 concentrations may reflect lung injury and increased vascular permeability. Our study does not allow for definite conclusions regarding the question whether the high cell counts, and the high levels of protein,

albumin, and elastase-α_1-PI-complex in ECMO-treated CDH patients result from the disease state or from the ECMO procedure.

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Chapter 7

Long-term pulmonary sequelae in children with congenital diaphragmatic hernia*

7.1 Summary

Neonates with congenital diaphragmatic hernia (CDH) often suffer from respiratory insufficiency due to lung hypoplasia and pulmonary hypertension. Artificial ventilation is frequently required, and this leads to a high incidence of bronchopulmonary dysplasia. Long-term follow-up studies have shown persisting airway obstruction. To evaluate the long-term pulmonary sequelae in CDH, we studied 40 CDH patients aged 7 to 18 years (median 11.7 years) and 65 age-matched controls without CDH and lung hypoplasia who underwent similar neonatal treatment. Mild airway obstruction was found in both groups with more peripheral airway obstruction in CDH patients than in controls. Both groups had normal TLC and single-breath carbon monoxide diffusion capacity (D_{LCO}). CDH patients had increased residual volume (RV) and RV/TLC compared with controls. Increased airway responsiveness to methacholine (MCH) was common but bronchoconstriction to inhaled metabisulfite (MBS) was rare both in CDH and control subjects. We conclude that this group of CDH patients has minor residual lung function impairment. Mild airway obstruction and increased airway responsiveness to inhaled MCH but not to MBS suggest that structural changes in distal airways are involved and not autonomic nerve dysfunction. Both artificial ventilation in the neonatal period and residual lung hypoplasia seem important determinants of persistent lung function abnormalities in CDH patients.

7.2 Introduction

The treatment of prematurely born neonates and children with congenital anomalies has improved during the past 20 years, owing to more sophisticated artificial

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ventilation techniques and neonatal intensive care. This has resulted in better chances to survive, at the cost of considerable secondary morbidity, e.g. due to bronchopulmonary dysplasia.[1] Bronchopulmonary dysplasia (BPD) is a chronic lung disease, which may occur following artificial ventilation in the first weeks after birth. The pathogenesis is multifactorial: barotrauma and high concentrations of inspired oxygen are important.[1,2] The incidence of bronchopulmonary dysplasia is highest in prematurely born infants with very low birth weight[2] (up to 85%). Long-term follow-up of prematurely born neonates has revealed chronic obstructive lung disease with increased airway responsiveness in adolescents and young adults who required artificial ventilation in the neonatal period.[3-9] Congenital diaphragmatic hernia (CDH) is associated with a decreased number of airway generations, and a decreased number of vascular generations.[10] Furthermore, pulmonary vascular abnormalities which lead to pulmonary hypertension have been described.[11] Artificial ventilation with high pressures and a high inspired oxygen fraction (FiO_2) is often required in the neonatal period.

Bronchopulmonary dysplasia has been described in 33% of CDH survivors, despite a mean birth weight of nearly 3000 grams.[12] Several follow-up studies found mild airflow obstruction in CDH patients.[13-15] Ventilation-perfusion lung scans show diminished perfusion on the ipsilateral side of the diaphragmatic defect, suggesting residual vascular abnormalities in the most hypoplastic lung.[14-17] Data on airway responsiveness in CDH are lacking. We hypothesized that children with CDH have an increased risk to develop chronic lung disease and increased airway responsiveness in later life, and that the prevalence and severity of lung function abnormalities might depend on initial lung hypoplasia. The aim of this study was to evaluate the long-term pulmonary sequelae in children with CDH and in age-matched controls without CDH and lung hypoplasia who underwent similar neonatal treatment.

7.3 Patients and methods

7.3.1 Patients

A group of 45 children survived neonatal operative repair of CDH in our department of Pediatric Surgery between 1975 and 1986. Forty could be traced and were willing to participate. Operative repair was performed immediately after diagnosis of CDH in all patients using an abdominal approach. Thirty-five children

with left-sided CDH and one with right-sided CDH had surgery within the first day of life. All patients routinely received antimicrobial prophylaxis perioperatively. Thirty-one children suffered from severe respiratory insufficiency within the first six hours after birth. We attempted to select two age-matched controls without CDH for each CDH patient. The controls were selected from files of the Neonatal and Pediatric Intensive Care Units of the Sophia Children's Hospital in Rotterdam, and the Neonatal Intensive Care Units of the Wilhelmina Children's Hospital in Utrecht and the Free University Hospital in Amsterdam. These controls were all matched for age at follow-up, and further selected to obtain the best possible match for gestational age, birth weight, duration of artificial ventilation, duration of supplemental oxygen, and sex. To be included in this study the following criteria had to be met by all patients: 1) ability to perform lung function tests reproducibly, that is, coefficient of variation in three consecutive measurements of FEV_1 < 5%; 2) clinically stable period of at least three weeks prior to the lung function tests. Exclusion criteria were: any previous thoracic surgery for other reasons than CDH; (congenital) heart disease; lung hypoplasia following prolonged rupture of membranes or renal anomalies with oligohydramnion, or other congenital or acquired disorders of the lungs or airways; inability to follow study instructions; inability to inhale medication adequately. The study was approved by the Medical Ethical Committees of all hospitals involved. Written informed consent was obtained from all parents.

7.3.2 Study design

During a prestudy visit a detailed medical history, including personal and family history of atopy and lung disease, was taken. Furthermore, the parents were asked to fill out a standardized questionnaire referring to respiratory symptoms, consultation of physicians and smoking habits. Physical examination was performed including pulse, blood pressure, respiratory rate, and auscultation of heart and lungs. Two weeks prior to the lung function tests daily peak flow measurements (Mini Wright Peakflowmeter, Airmed, Harlow, England) were performed at home in the morning and evening (before taking medication if required), and recorded on a daily record card. The highest value of three consecutive measurements was used for further analysis. The symptoms cough, wheezing, production of sputum, and dyspnea were recorded daily. For each symptom a score from 0 (absent) to 3 (severe) was given. In this way a maximum score of 96 could be obtained within an 8 day period. The mean diurnal variation of peak flow (which is the absolute

difference between morning and evening value divided by the mean value of morning and evening x 100%)[18] and the total cumulative symptom score from day 8 to day 15 were evaluated.

Lung function tests were performed on two separate days within a one week period. Any medication was discontinued 12 hours prior to the tests. To avoid diurnal variation in the measurements, all tests were done in the morning. Spirometry was performed on the first day between 9 and 11 AM, and followed by an inhalation provocation test with methacholine (MCH) when the baseline FEV_1/VC was at least 0.7. One hour later, when the baseline FEV_1 had returned to at least 90% of the initial baseline, an inhalation provocation test with metabisulfite (MBS) was performed. Methacholine is a bronchoconstrictor which acts directly at airway smooth muscle, whereas MBS acts indirectly, probably via neuronal pathways.[19] On the second day, spirometry and volume-flow curves were recorded before and after maximal bronchodilatation with terbutaline to study reversibility of airway obstruction. Helium dilution spirometry, bodyplethysmography, and single breath carbon monoxide diffusion capacity (D_{LCO}) were carried out after bronchodilatation only.

7.3.3 Lung function and bronchial provocation tests

Spirometry was performed using a water-sealed spirometer (Volutest model VLT, Mijnhardt, Zeist, The Netherlands). FEV_1 and VC were determined as the best of three reproducible measurements. Flow-volume curves were obtained with a heated Fleisch pneumotachograph (Number 3.1184; Godart Statham, Bilthoven, The Netherlands) connected to a computer. FEV_1, FVC, PEF and maximal expiratory flows at 25% of the FVC (MEF_{25}) were determined and the best of three consecutive measurements was recorded and expressed as % of predicted values.[20]

Spirometry and flow-volume curves were performed before and 15 minutes after 1 mg of terbutaline sulphate, delivered by turbuhaler (Astra Pharmaceuticals, Lund, Sweden) as two inhalations of 500 µg. After each inhalation the breath was held for 5 seconds. The change, expressed as % of predicted value, was calculated to evaluate reversibility of any airflow obstruction.

A water-sealed spirometer (Expirograph, Godart Statham, Bilthoven, The Netherlands) filled with a known concentration of helium was used to determine TLC_{He}, RV_{He}, VC_{He}, tidal volume (TV), inspiratory reserve volume (IRV), and expiratory reserve volume (ERV). Helium was allowed to wash in for at least 5 minutes during normal tidal breathing until a stable concentration was obtained.

Volumes were expressed as % of the actual TLC. Specific conductance of airways (sGaw), TLC_{pleth}, RV_{pleth}, and RV/TLC were determined by bodyplethysmography (Jaeger Masterlab, Würzburg, Germany), and the best of three consecutive measurements was recorded. All values, except RV/TLC, were expressed as a % of predicted values.[20] Carbon monoxide diffusion capacity (D_{LCO}) was measured using a single-breath method (Jaeger Masterlab, Würzburg, Germany). Reference values for D_{LCO} and D_{LCO} corrected for alveolar volume (D_{LCO}/V_A) were based on a study performed in our laboratory in 103 healthy Dutch children.[21]

Inhalation provocation was carried out with aerosolized methacholine bromide and sodium metabisulfite ($Na_2S_2O_5$, buffered to pH 7.4 by adding phosphate buffer). MCH was given in doubling concentrations of 0.15 to 39.2 mg/ml, MBS in doubling concentrations of 2 to 256 mg/ml as described previously.[22] The aerosols were generated by a calibrated De Villbiss 66 Nebulizer (De Vilbiss Co., Somerset, USA), with closed vent, attached to a French-Rosenthal dosimeter (Laboratory for Applied Immunology, Baltimore, Maryland, USA) driven by air at 138 kPa. The children were instructed to inspire slowly and as deeply as possible. During inspiration the dosimeter was triggered for 0.6 seconds. After full inspiration, breath was held for 5 seconds. A total of 20 microliters of aerosol was delivered to the mouth in 4 consecutive breaths. Mouth doses were 3 to 784 µg for MCH, and 40 to 5120 µg for MBS. Provocations with MCH and MBS were preceded by inhalation of normal saline. The interval between consecutive doses was three minutes. FEV_1 was measured in triplicate after each dose-step until the best value had fallen from baseline by at least 20%. The provocative dose that resulted in a 20% fall in FEV_1 (PD_{20}) was calculated by interpolation of the dose-response curve on a log-linear scale.

All lung function tests were performed with subjects in sitting position. All volumes were corrected to BTPS conditions. The equipment and procedures were in accordance with international recommendations.[23,24]

7.3.4 Data analysis

Where two controls were available for a CDH patient the mean value of two matched controls was used for paired analysis of the differences between CDH patients and controls. In the few cases where only one control patient was available, the data of this control patient were used. To exclude the influence of prematurity and atopy as confounding factors, separate analysis of data was performed after excluding all prematurely born infants, and after exclusion of all

atopic children. Data of CDH patients and controls were compared with paired t-tests, or Wilcoxon's signed-rank test if appropriate. Paired comparisons of percentages were done with the Mantel-Haenszel test. Logarithmic transformation was used in all analyses of $PD_{20}MCH$ to approximate a normal distribution. To evaluate the effect of age on $PD_{20}MCH$ logistic regression was performed. Since only few responded to MBS, these data were described without further statistical analysis. The relation between patient characteristics and lung function results was studied by least squares regression. Multiple regression analysis for continuous outcomes was used to study interaction between variables, i.e. whether the magnitude of the difference between both groups depended on certain other parameters. Correlation coefficients given are Spearman's. Data given are mean ± SEM unless stated otherwise. Statistical significance was accepted at 1% level for all tests.

7.4 Results

7.4.1 Patient characteristics, questionnaire, and physical examination

In the CDH group (n = 40) all patients except two had left-sided CDH. Two patients were small for gestational age; three children were born prematurely. Two CDH patients had only a prestudy visit in the hospital: One refused to perform the lung function tests, the second was not able to perform lung function tests due to neurodevelopmental impairment. The data on medical history, physical examination, and the standardised questionnaire of these two CDH patients were included in the analysis. Two matched controls could be recruited for 30 CDH patients, only one control patient could be found for 5 CDH patients, while for three CDH patients no suitable control patients could be selected. The 65 control patients had needed neonatal intensive care for: meconium aspiration (n = 29), pneumonia (n = 11), persistent fetal circulation (n = 8), asphyxia (n = 6), respiratory distress syndrome (n = 7), amniotic fluid aspiration (n = 3), and pneumothorax (n = 1). One control patient was small for gestational age at birth, and 24 were born prematurely. The characteristics of 38 CDH patients who performed lung function tests and 65 controls are shown in Table 1. The groups were similar with respect to sex, birth weight, duration of artificial ventilation and duration of oxygen supply. Older CDH patients had been ventilated shorter than younger CDH patients (r = -0.45; p = 0.005).

***Table 1**: Patient characteristics*

	CDH	Control without CDH
Number	38 (21 male)	65 (44 male)
Age at follow-up (years)	11.7 (7.4-17.6)	12.1 (7.7-18.2)
Gestational age (weeks)	40 (28-43)	38 (29-42)
Birth weight (grams)	3,275 (1,000-4,300)	2,950 (1,250-4,200)
Ventilation (days)	4.0 (0-49)	4.3 (0-25)
Supplemental oxygen (days)	11.0 (0-187)	10.0 (0-38)
Maximal FiO_2	0.5 (0.21-1)	0.95 (0.21-1)
Atopic history positive	3 (8 %)	15 (23 %)
Family history positive (atopy, lung disease)	20 (54 %)	33 (52 %)

Data given are numbers of patients or median (range). Duration of oxygen supply is expressed as the total number of days, including days of artificial ventilation.

Mean gestational age was 39.6 weeks in the CDH group which was slightly higher compared with 37.7 weeks in the control group. The median maximum FiO_2 was 0.5 in CDH and 0.95 in controls ($p < 0.001$). Ten CDH patients underwent pressure-controlled ventilation with median maximum inspiratory peak pressures of 37.5 cm H_2O (range 20-55 cm H_2O); all other CDH patients underwent volume-controlled ventilation, and peak pressure values could not be retraced. Median maximum inspiratory peak pressures available in 35 control patients was 35 cm H_2O (range 17 to 55 cm H_2O). Eight CDH patients (7 term) and six control patients (3 term) fulfilled the criteria of BPD according to Bancalari[2] (NS): They had been ventilated within the first week of life and were still oxygen dependent at the age of 28 days. Eight percent of the CDH children and 23% of control patients had a history of atopy (NS). The family history (first and second degree) for atopy or atopic lung disease was positive in 54% of CDH patients and in 52% of control patients (Table 1).

At the time of assessment one CDH patient and three controls were using inhaled corticosteroids. Two CDH patients and one control were using a $ß_2$-agonist

on demand. The medical history revealed lung symptoms within the last year in 11 of 40 CDH patients and 11 of 65 control patients (NS). Respiratory symptoms within the first three years of life were reported for 17 of 39 CDH patients and 24 of 64 controls (NS). Symptoms of wheezing and dyspnea during the past 12 months were reported by 23% of the CDH patients and 20% of controls (NS). Limitation of exercise endurance was mentioned by 18% of the CDH group and 6% of control patients (NS). Eighteen percent of CDH patients and 16% of controls had consulted a physician for respiratory symptoms during the past year (NS). Two children (one in each group) smoked themselves, whereas smoking occurred in 62% of the households in the CDH group and in 41% of the control group (NS). Separate analysis of all children who were born at term resulted in similar results for all items mentioned above. The median total respiratory symptom score was 0 in CDH (range 0-24) and 0.8 in control patients (range 0-32; NS). Physical examination showed mild funnel-shaped chest in 20% of CDH patients and in 12% of controls (NS). No wheezing was present in any of the children. Daily peak-flow registration showed a similar median variability of 4.4% in CDH (range 2 to 17.7 %) and 4.8% in control patients (range 1.9 to 12.9%; NS). The mean total respiratory symptom scores, the findings of physical examination, and daily peakflow registrations were not significantly different after exclusion of prematurely born children, or after exclusion of atopic subjects.

7.4.2 Lung function

Analysis was performed on data from 35 CDH patients and 65 matched controls. Spirometry showed a significantly lower FEV_1/VC in CDH compared with controls before bronchodilatation (Table 2; $p = 0.01$). Flow-volume curves showed normal values for FVC and PEF before and after bronchodilatation in both groups (data not shown). FEV_1 was significantly lower in CDH patients compared with controls before and after bronchodilatation (84 ± 3 versus 95 ± 2, and 92 ± 3 versus 101 ± 2 % predicted respectively). The percentage of patients with abnormally low FEV_1 and MEF_{25} values (< -1.96 SD from predicted) was high in both groups: FEV_1 was abnormal in 67% of CDH patients and 25% of controls before bronchodilatation, and in 47% of CDH patients and 22% of controls after bronchodilatation. MEF_{25} was abnormally low in CDH before and after bronchodilatation in 79% and 59% and in controls in 41% and 22% respectively.

***Table 2:** Lung function results in CDH and in matched controls*

			before broncho-dilatation	after broncho-dilatation	change % predicted
spirometry	CDH	FEV_1	89 ± 3	96 ± 3	7 ± 1
		FEV_1/VC	77 ± 2*	83 ± 1	
	Control	FEV_1	96 ± 2	103 ± 2	7 ± 1
		FEV_1/VC	82 ± 1	86 ± 1	
flow-volume curves	CDH	FEV_1	84 ± 3*	92 ± 3*	8 ± 1
		MEF_{25}	52 ± 4*	67 ± 5*	16 ± 2
	Control	FEV_1	95 ± 2	101 ± 2	6 ± 1
		MEF_{25}	70 ± 4	92 ± 3	21 ± 3
bodyplethysmography	CDH	TLC		104 ± 3	
		RV		125 ± 5*	
		RV/TLC		29 ± 1*	
	Control	TLC		99 ± 2	
		RV		105 ± 3	
		RV/TLC		23 ± 1	
diffusion capacity	CDH	D_{LCO}		100 ± 3	
		D_{LCO}/V_A		93 ± 3	
	Control	D_{LCO}		105 ± 2	
		D_{LCO}/V_A		101 ± 2	

Means ± SEM are shown. All data are expressed as % predicted, except FEV_1/VC, and RV/TLC. Spirometry and flow-volume curves are shown before and after inhalation of 1 mg of terbutaline, whereas bodyplethysmography was performed after bronchodilatation only.

** significant difference between CDH and controls in the same treatment group (with or without terbutaline), $p < 0.01$.*

After 1 mg of inhaled terbutaline flow-volume curves showed a significant increase in FEV_1 and MEF_{25} both in CDH and in controls (Table 2; $p < 0.001$). CDH patients and controls showed no significant differences in reversibility of airflow obstruction (Table 2).

Bodyplethysmography showed a significantly higher mean RV_{pleth} and RV/TLC_{pleth} in CDH than in control patients (Table 2; $p = 0.001$ and 0.006 respectively). SGaw was 181 ± 15% predicted in CDH and 156 ± 12% predicted in controls (NS). Air trapping was estimated from the difference between TLC_{pleth} and TLC_{He}: Mean trapped air was 5 ± 0.5% of TLC_{pleth} in CDH and 3 ± 0.6% in controls (NS). RV/TLC_{He} was 26 ± 1% in CDH and 23 ± 1% in controls ($p=0.006$). No differences in ERV, TV, and IRV were found between the groups.

D_{LCO} was measured in 17 matched couples and was 100 ± 3% predicted in CDH and 105 ± 2% predicted in controls (NS). D_{LCO}/V_A was 93 ± 3% predicted in CDH and 101 ± 2% predicted in controls (NS).

Separate analysis of term born patients showed similar % predicted values for all lung function tests. In controls spirometric FEV_1/VC before and after bronchodilatation were significantly lower (differences of means both 5) in the atopic children than in non-atopic children. The same was true for MEF_{25} (difference of means before and after bronchodilatation respectively 14% and 24%). Abnormal MEF_{25} was observed in 22% of term born controls without an atopic history, irrespective of bronchodilatation.

7.4.3 Airway responsiveness

In four CDH patients and in four controls FEV_1/VC was less than 0.7, and hence no challenge test was done. Inhalation provocation was performed in 32 CDH patients and in 56 controls. Inhalation of MCH resulted in a 20% or more decrease of FEV_1 in 56% of CDH patients and in 38% of controls (NS). The individual $PD_{20}MCH$ values are shown in Figure 1. $PD_{20}MCH < 150$ µg, which is more than 2 SD below the mean value in healthy children[25], was found in 38% of CDH patients and in 23% of controls (NS). Similar results were found after exclusion of prematures and atopic children. The prevalence of increased airway responsiveness to MBS was much lower than to MCH: Only two CDH patients and six controls showed a 20% or more decrease in FEV_1 after inhalation of MBS. $PD_{20}MBS$ was 1100 and 195 µg in the two CDH respondents; the median $PD_{20}MBS$ in six controls was 765 µg. After exclusion of prematurely born children two CDH and four control respondents were left; after exclusion of atopic children only one

respondent to inhalation of MBS was present in each group. No difference in the prevalence of increased responsiveness to MCH and MBS was found between CDH patients and controls with or without a history of atopy, and the same was true for PD_{20}MCH and PD_{20}MBS in respondents. No relation between positive challenge tests and a positive family history for lung disease or atopy was apparent in CDH and control patients.

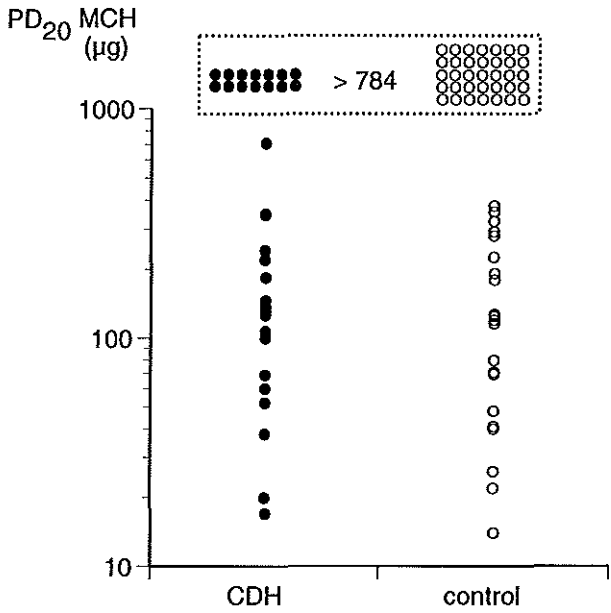

Figure 1: *Inhalation provocation with methacholine (MCH) in CDH and in controls. The provocative dose that resulted in 20% decrease of FEV_1 is indicated for MCH on a logarithmic scale. CDH patients are shown as closed circles and controls as open circles. The highest dose of MCH was 784 µg. Non-respondents are indicated in the box.*

7.4.4 Correlation between lung function results and other patient characteristics

This analysis was performed in 38 CDH patients and 65 controls. The results of the lung function tests and the prevalence of increased airway responsiveness on the one hand and gestational age, birth weight, maximum FiO_2 or parental smoking habits on the other hand did not correlate for either group. In CDH the duration of

artificial ventilation correlated negatively with spirometric FEV_1 before and after bronchodilatation (Figure 2), spirometric VC before and after bronchodilatation, FEV_1 and FVC in flow-volume curves after bronchodilatation, and VC_{pleth}. All

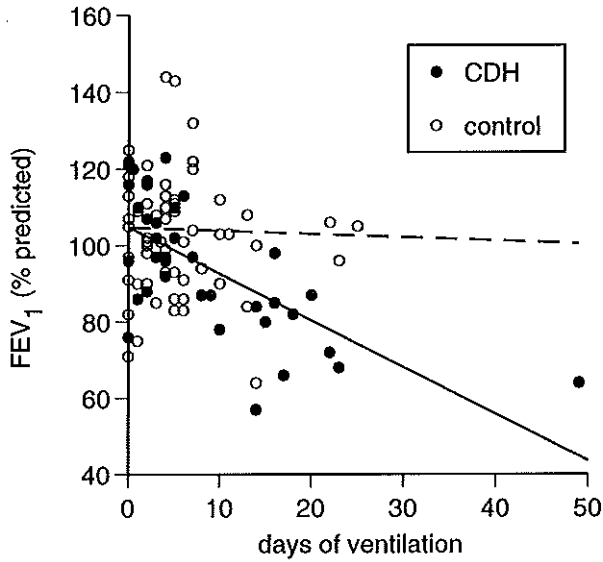

Figure 2: *Relation between duration of artificial ventilation (days) and FEV_1 (expressed as % predicted) after bronchodilatation with 1 mg of inhaled terbutaline measured by spirometry in CDH (closed circles) and controls (open circles). The regression lines are indicated for both groups: solid line for CDH (y = 103.03 - 1.12 * ventilation; p = 0.0001) and dashed line for controls (y = 103.14 - 0.02 * ventilation; p = 0.97).*

slopes resulting from linear regression analysis of lung function parameters against duration of ventilation in CDH patients were between -1.0 and -1.2% predicted per day of ventilation ($p < 0.01$ in all cases), whereas no significant slopes were found in controls (slopes varied from -0.3 to 0.3). In addition, CDH patients and controls were grouped according to whether or not they had been ventilated for at least 7 days. Controls who had been ventilated for less than 7 days had similar lung function results as those who had been ventilated for at least 7 days. However, CDH patients who had been ventilated for 7 days or more had significantly lower FEV_1 and VC (spirometry and flow-volume curves, before and after bronchodilatation), PEF before bronchodilatation, and MEF_{25} after

bronchodilatation than CDH patients who had been ventilated for up to 7 days. CDH patients ventilated for at least 7 days had significantly lower TLC_{He}, TLC_{pleth}, VC_{pleth}, and higher RV/TLC_{pleth} than those who had been ventilated shorter (Table 3). D_{LCO} was 93 ± 4% predicted in CDH patients ventilated for up

Table 3: Lung function in CDH patients ventilated for less than 7 days and 7 days or more

	parameter	< 7 days (n=23)	≥ 7 days (n=15)
duration of ventilation median (range)		2 (0-6) days	16 (7-49) days
spirometry	FEV_1 before BD	98 ± 3	74 ± 4
	FEV_1 after BD	105 ± 3	79 ± 3
	VC before BD	103 ± 3	83 ± 3
flow-volume curves	PEF before BD	100 ± 4	80 ± 5
	MEF_{25} after BD	76 ± 6	48 ± 7
bodyplethysmography	TLC	108 ± 3	96 ± 3
	RV/TLC	27 ± 1	33 ± 2

Means ± SEM are shown. All data are expressed as % predicted, except RV/TLC. Significant differences were found for all parameters ($p < 0.01$).

to 7 days and 98 ± 4% in those who had been ventilated for 7 days or more (NS). Both for CDH and control patients a negative correlation was found between the duration of oxygen supply and FEV_1 and VC before and after bronchodilatation in spirometry, FVC before and after bronchodilatation, FEV_1 before bronchodilatation in flow-volume curves, TLC_{pleth}, TLC_{He}, VC_{pleth} and RV/TLC_{pleth}. Similar correlations were found after exclusion of children who were born prematurely and/or had a positive atopic history. Age at follow-up in CDH and in controls correlated positively with PEF before and after bronchodilatation. In CDH, the probability to have a positive response to inhalation of MCH, i.e. $PD_{20}MCH < 784$ µg, correlated negatively with age ($p = 0.001$; Figure 3). The same was true for the probability to have a $PD_{20}MCH < 150$ µg ($p = 0.004$; Figure 3). No significant relation between the presence and the magnitude of $PD_{20}MCH$ and age could be shown for controls. The relations of these parameters with age, however, did not differ significantly between the two groups. Children who had respiratory

symptoms during the first three years of life or within the last year had significantly lower FEV_1/VC, FEV_1 (spirometry and flow-volume curves), PEF, and MEF_{25} before and after bronchodilatation compared with children without respiratory symptoms. Similar results were found after exclusion of prematures and atopic subjects.

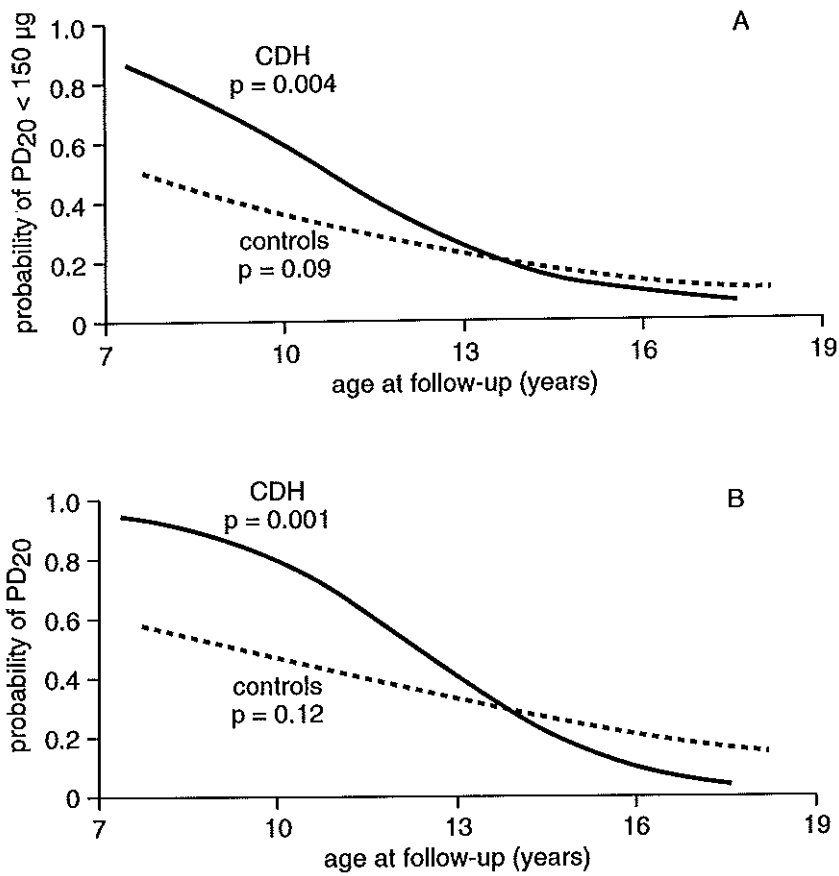

***Figure 3:** Relation between the probability to have $PD_{20}MCH$ < 150 µg (3A) or to reach $PD_{20}MCH$ (3B) and age at follow-up in CDH and in controls.*

7.5 Discussion

We found mild obstructive lung function abnormalities and a high prevalence of increased airway responsiveness to methacholine both in children with neonatal repair of CDH and, to a lesser extent, in age-matched controls. The controls underwent similar neonatal treatment but had not been operated on for CDH and did not have lung hypoplasia. Both groups showed normal total lung capacity and normal diffusion capacity under resting conditions, suggesting that no important lung function impairment had resulted from CDH.

Interpretation of long-term pulmonary sequelae after neonatal repair of CDH has been difficult because of the lack of comparative long-term data on lung function abnormalities in ventilated term neonates without CDH. The present study is the first in which lung function data of CDH patients are compared with those of age-matched controls who were selected for the best possible match for gestational age, birth weight, duration of artificial ventilation and oxygen supply, and sex. Gestational age was slightly, but significantly lower in the control group, and it could be argued that this may explain differences in lung function. However, mean gestational age was more than 37 weeks in both groups (37.7 versus 39.6 weeks), and similar results were found after separate analysis of term born children. Therefore differences in lung function between the two groups can not be attributed to differences in gestational age. The maximum FiO_2 was significantly lower in CDH than in controls, but did not correlate with the measured lung function parameters. The groups were similar with respect to atopic history, positive family history for atopy or lung diseases, and smoking habits. Therefore, these potential confounding factors cannot explain differences in lung function between CDH and controls either.

We found more peripheral airway obstruction in CDH patients than in controls, as was apparent from decreased FEV_1/VC and MEF_{25}, high percentages of abnormal FEV_1 and MEF_{25}, and increased RV/TLC. In controls mild airflow obstruction was suggested by decreased MEF_{25}, and abnormal FEV_1 and MEF_{25} in more than 20% of cases after exclusion of the prematurely born and atopic children. Separate analysis of term born children without a history of atopy indicated that in controls FEV_1/VC and MEF_{25} were higher in non-atopic children compared with atopic children. Such a difference could not be demonstrated for the CDH group (with only three children having a history of atopy). There was no difference in reversibility of airway obstruction between the groups. The mean reversibility of FEV_1 was within the normal range seen in healthy individuals.[24]

Mild airflow obstruction[13,14,26] or normal spirometry[17] were reported in school age children born before 1976. A number of these children were operated on after the perinatal period at ages up to two years. An unspecified number were ventilated post operatively. It is likely that the number was small. In patients born subsequently, Falconer[15] found evidence of reduced expiratory flows at 50% VC. Seven of his 19 children had been ventilated for 4 days or more.

Airflow obstruction has been described in several follow-up studies of premature children with and without BPD[3-5,9,27], but normal spirometric results have also been reported.[8] The airflow obstruction described in nonventilated CDH patients suggests residual hypoplasia of lungs or airways. Abnormal tracheal or bronchial cartilage has been described in CDH.[28] This may enhance airflow obstruction as a result of inadequate support, and, hence, dynamic compression of the intra-thoracic airway during forced expiration. In addition, functional impairment may have resulted from airway damage due to artificial ventilation and oxygen treatment in ventilated CDH patients.

TLC and VC were normal in our study, consistent with the observations of others[13,14], although restrictive defects have also been observed.[29] Residual volume was marginally elevated in our study and in that of Reid and Hutcherson.[29]

Normal TLC and VC in CDH suggest the absence of residual lung hypoplasia and it may well be that the compensatory lung growth takes place during the first year of life.[30] However, the number of alveoli may still be reduced. Therefore we also measured diffusion capacity, which is a measure of the diffusion surface and consequently of the alveolar surface. D_{LCO} and D_{LCO} corrected for alveolar volume were normal in CDH and in controls. This suggests the presence of a normal diffusion surface area under resting conditions at long-term follow-up in CDH, as described previously.[14] Exercise stress testing with measurement of D_{LCO} would be necessary to reveal the functional significance of a possible residual lung hypoplasia. In favor of residual lung hypoplasia are mild airflow obstruction, decreased lung perfusion on the ipsilateral side of the diaphragmatic defect[14,16,29], the observation that anatomic formation of further generations of airways and conducting vessels does not develop post 16 weeks gestation[31], and findings of long-term lung morphology after neonatal repair of CDH: Normal total lung volume, but an abnormal lung structure, has been described in 3 cases of CDH at autopsy after 2.5 to 64 months.[32,33] While the total number of alveoli was either decreased[32,33] or normal[33], the alveolar size was increased in all cases, especially on the ipsilateral side. The lower D_{LCO}/V_A in our CDH patients compared to controls may indicate persistence of such morphological abnormalities. The normal

D_{LCO} in CDH patients and in age-matched ventilated controls suggests that diffusion capacity is not severely affected by lung damage caused by artificial ventilation.

There are no data on airway responsiveness at long-term follow-up in children with CDH. We found a high prevalence of MCH responsiveness both in CDH (56%) and in controls (38%), irrespective of an atopic history or a positive family history for atopy or lung disease. Several authors have described increased airway responsiveness to inhalation of MCH or histamine in prematures following artificial ventilation.[6,5,27] A high prevalence of exercise-induced bronchospasm was found in a group of term born children with meconium aspiration syndrome following a short period of artificial ventilation.[34] The high prevalence of increased airway responsiveness to MCH, together with the small number of positive respondents after inhalation of MBS, suggests that the mechanism of airway narrowing in our patients is different from that in asthmatic subjects where both challenges correlate well.[35] Residual structural narrowing of distal airways or airway smooth muscle hypertrophy may explain our findings, since these abnormalities would lead to increased airway responsiveness only as a result of the altered airway geometry.[36] Since no differences were found between CDH patients and controls it can be assumed that artificial ventilation during the neonatal period is a more likely cause of these abnormalities than lung or airway hypoplasia in CDH. We found that the prevalence of airway responsiveness to MCH was significantly lower in older CDH patients. This may reflect the natural history of airway responsiveness following artificial ventilation in the neonatal period, although our cross-sectional data do not allow for this assumption. Another possible explanation is that older patients had been artificially ventilated for a shorter period of time because of the restricted intensive care treatment in those days. Therefore CDH patients born earlier, e.g. between 1975 and 1980, and who survived their neonatal period, may have had less severe lung hypoplasia with less structural small airway abnormalities than the children who were born later.

We found a negative correlation between the duration of ventilation and FEV_1 at follow-up in CDH. A negative correlation between FEV_1 and duration of ventilation has been described in follow-up studies of prematures as well.[4,9] Previous studies in ventilated prematures without BPD suggest that the effect of artificial ventilation is independent of gestational age.[8,9] However, most prematures with very low birth weight will require artificial ventilation for a longer period of time and are more likely to develop BPD.[2] A high incidence of BPD in CDH patients has been reported by Bos and coworkers[12] who studied a group of CDH

survivors born between 1980 and 1989 with respiratory insufficiency within the first six hours after birth. The children in our study group were born between 1975 and 1986 when extracorporeal membrane oxygenation and high frequency oscillatory ventilation were not available. It can be assumed that especially CDH patients with less severe lung hypoplasia survived at that time, whereas the children with severe lung hypoplasia and severe persistent pulmonary hypertension may have died even before reaching the Pediatric Surgical Intensive Care Unit. This assumption is supported by the fact that our group of CDH patients had a median duration of ventilation of only 4 days with a mean maximal FiO_2 of 0.5. That we found lung function abnormalities especially in CDH patients who were ventilated for at least 7 days suggests that respiratory morbidity may well increase in those children with more severe lung hypoplasia, who will require artificial ventilation for longer periods of time and who presently survive CDH.[3,8,9]

In conclusion, we found mild obstructive lung function abnormalities in CDH and also, but to a lesser extent, in carefully matched controls. The difference between CDH patients and controls could not be explained by differences in patient characteristics and could therefore be due to residual lung hypoplasia, and/or to anatomical and functional changes of the thoracic wall, the diaphragm, or the airway cartilage in the CDH patients. CDH patients showed no important reduction of lung volume and diffusion capacity under resting conditions. Airway responsiveness to MCH, but not to MBS, was increased in both groups, suggesting that the mechanism of airway narrowing was different from that in asthmatics. We speculate that structural abnormalities in distal airways are responsible for the high incidence of increased airway responsiveness. Our data suggest that not only residual lung hypoplasia, but also neonatal intensive care treatment contributes to the persisting airway obstruction and increased airway responsiveness in CDH patients.

7.6 References

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Chapter 8

Antioxidant enzyme profiles during artificial ventilation of neonatal rats with congenital diaphragmatic hernia and controls*

8.1 Summary

Infants with congenital diaphragmatic hernia (CDH) and lung hypoplasia often require artificial ventilation with high pressures and high oxygen fraction. Assumedly, a failing antioxidant enzyme (AOE) system may contribute to the high incidence of bronchopulmonary dysplasia in CDH patients. A previous study in neonatal rats with CDH showed normal antioxidant enzyme (AOE) activity at birth, and a decreased activity of glutathione peroxidase after 5 hours of ventilation with 100% O_2. We hypothesized that a failing AOE system would be reflected in different AOE profiles during artificial ventilation. Therefore, we measured the activities of catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase at birth, and after 2, 4, and 6 hours of ventilation with 100% O_2 in neonatal rats with CDH and in control pups. CDH was induced by oral administration of 60 mg of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) to pregnant dams on gestational day 12. All pups were born spontaneously at term. Ventilation was started directly after birth with PIP 25 cm H_2O (reduced to 17 cm H_2O after 30 minutes), PEEP 3 cm H_2O, 100% O_2, frequency 40/min. At birth CDH pups had a significantly higher catalase activity per mg DNA than controls. The responses of catalase and superoxide dismutase activities to ventilation with 100% O_2 differed between CDH and control pups. A transient increase in superoxide dismutase at t=2 hours was observed in CDH, whereas in controls the AOE activity increased slowly with maximal activities at t=6 hours. We conclude that in lungs of CDH rats the profiles of AOE activity during six hours of ventilation differ from those in controls. We speculate that an altered response of AOE activity in CDH contributes to the initiation of pulmonary O_2 toxicity.

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Submitted

8.2 Introduction

Congenital diaphragmatic hernia (CDH) is associated with a decreased number of airway generations and a decreased number of vascular generations.[1] Furthermore, pulmonary vascular abnormalities which lead to pulmonary hypertension have been described.[2] Artificial ventilation with high pressures and a high inspiratory oxygen fraction is often required in the neonatal period. A high incidence of bronchopulmonary dysplasia has been reported in CDH survivors despite a mean birth weight of nearly 3000 grams.[3,4]

Antioxidant enzymes (AOE) are regarded as the primary defense system of the cell against oxidative stress. The effects of hyperoxia on the AOE activity have been studied extensively in preterm and term born rats[5-8], rabbits[9], and guinea pigs.[10] In term born rats antioxidant enzyme activities increased significantly after hyperoxic exposure of 4 to 6 hours.[7] Deficiency of glutathione, a major antioxidant, has been reported in premature children with respiratory distress[11] and in those who developed chronic lung disease.[12] Furthermore, children who developed bronchopulmonary dysplasia had lower activity of superoxide dismutase than age-matched controls with and without respiratory distress syndrome.[13]

In a previous study normal developmental profiles of AOE have been reported in a rat model of CDH. After five hours of ventilation with 100% O_2 the activity of glutathione peroxidase was reduced in rat pups with CDH, whereas the activities of catalase and superoxide dismutase remained unchanged.[14] We hypothesized that a failing antioxidant enzyme system would be reflected in different AOE profiles during artificial ventilation, and that this might contribute to the high incidence of bronchopulmonary dysplasia in CDH patients. The aim of the present study was to examine the changes within time of glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase activity during a predetermined period of artificial ventilation in newborn rats with CDH and to compare them to those of control pups without CDH.

8.3 Materials and methods

8.3.1 Animal model

Female Sprague Dawley rats (Harlan Olac, England) weighing about 250 grams were mated during overnight (day 0 of gestation), and received 60 mg of 2,4-

dichloro-phenyl-p-nitrophenylether (Nitrofen: Rohm Haas Company, Philadelphia, U.S.A.) dissolved in olive oil by gastric tube on day 12 of gestation. This dose results in right-sided diaphragmatic defects with moderate lung hypoplasia.[15] Controls were obtained from litters not exposed to Nitrofen or olive oil. Previous experiments revealed that rat pups from control litters exposed to olive oil had similar birth weights compared to pups from litters not exposed to olive oil, and we were not able to show any evidence of other problems related to the procedure, such as fetal death (unpublished observations). Food and water were supplied ad libitum during the whole period of pregnancy. Spontaneous birth was awaited in all cases. The experiments were performed after approval of the Animal Care and Use Committee of the Erasmus University Rotterdam.

8.3.2 Artificial ventilation

Directly after birth the newborns were weighed and anesthetized with sodium pentobarbital (30 mg/kg) and paralyzed with pancuronium bromide (0.08 mg/kg) applied intraperitoneally. They were intubated with a 24G intravenous catheter (Neoflon, Viggo-Spectramed, Helsingborg, Sweden) with atraumatic stent, transferred to a multichambered bodyplethysmograph heated to 37°C, and connected to a modified Servo 900B ventilator (Siemens-Elema, Solna, Sweden).[16] Pressure-controlled ventilation was started using the following respirator settings: PIP 25 cm H_2O; PEEP 3 cm H_2O; frequency 40 cycles/min; fraction of inspired oxygen 1.0; inspiratory:expiratory ratio of 1:2. A previously performed pilot study revealed that opening pressures of 25 cm H_2O were needed to obtain a good lung aeration pattern, but that continuous ventilation with this peak pressure resulted in a high incidence of pneumothorax (unpublished data). Therefore, PIP was reduced to 17 cm H_2O after 30 minutes.

An attempt was made to ventilate newborn control pups for up to 24 hours. Only 3 of 27 rat pups from three different litters survived. Then we decided to ventilate the rat pups for the maximal duration of six hours.

8.3.3 Measurement of antioxidant enzyme activity

An overdose of pentobarbital (120 mg/kg intraperitoneally) was used to kill the rat pups. After removal of the heart-lung block, the lungs were stripped of nonpulmonary tissue, weighed, frozen with liquid nitrogen, and stored at -70 °C until further processed as described before.[14] All biochemical analyses were

performed on lungs from separate rat pups. After thawing, the lungs were diluted 1:15 (wt/vol) in ice-cold phosphate-buffered saline and homogenized with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) for 15 s at maximum speed. Next, the suspension was sonicated for 10 s on ice. In this crude suspension, the concentration of DNA was estimated by a sensitive fluorimetric assay.[17] To determine AOE, the crude suspensions were centrifuged at 20,000 x g for 30 min, and the pellets were discarded. Glutathione peroxidase activity was determined as previously described by Paglia and Valentine.[18] Glutathione reductase activity was determined as described by Goldberg and Spooner.[19] Catalase was measured according to Bergmeyer.[20] Superoxide dismutase activity was determined by using the SOD-525 method (R & D Systems, Abingdon, UK). Correction for blood contamination was performed as follows: From three different litters the blood of three to five rat pups was collected for measurement of hemoglobin[21], and antioxidant enzyme activity. The AOE activity per mg hemoglobin was thus determined. Lung suspensions from each group were pooled and the hemoglobin concentration was measured. The AOE activity resulting from blood contamination was calculated and subtracted from the total AOE activity in lung suspensions. Before correction, the mean percentages of total AOE activity represented by contaminating blood were 9.7 ± 0.2% for catalase, 3.7 ± 0.1% for superoxide dismutase, 34.9 ± 0.8% for glutathione peroxidase, and less than 1% for glutathione reductase activity, which was similar for CDH and control lungs (not shown).

8.3.4 Study design

We studied newborn rat pups for AOE measurement directly after birth (t = 0; CDH: n = 13; controls: n = 13), and after two (t = 2; CDH: n = 16; controls: n = 9), four (t = 4; CDH: n = 6; controls: n = 8) or six (t = 6; CDH: n = 10; controls: n = 11) hours of ventilation. Autopsy revealed the presence of a diaphragmatic defect in Nitrofen-exposed rats. To obtain a homogeneous study group Nitrofen-exposed rat pups without CDH (n = 2) were excluded. Pneumothorax, absent heart action, or other complications related to insufficient ventilation resulted also in exclusion.

8.3.5 Data analysis

All data were presented as means ± SEM, unless stated otherwise. As data were normally distributed, differences in AOE profiles were tested by two-way analysis of variance. If a significant F-value was found, Student's t-test with Bonferroni's correction method was used to identify the differences between the groups. Statistical significance was assumed at a 5% level.

8.4 Results

The Nitrofen-exposed litters were born after 22.6 ± 0.03 days of gestation, and control litters after 22.5 ± 0.13 days (NS). In the Nitrofen-exposed rat pups more than 95% had right-sided CDH. CDH rat pups had significantly lower birth weight, lung weight at birth, and lung-body weight ratio than controls (Table 1; $p < 0.001$).

***Table 1:** Growth parameters in newborn rat pups with CDH and controls*

	CDH	control
Birth weight (grams)	5.56 ± 0.04^{a}	6.12 ± 0.09
Lung weight (mg)	93.8 ± 4.4^{a}	138.9 ± 3.9
Lung-body weight ratio (mg/g)	16.7 ± 2.2^{a}	22.1 ± 1.8

All data are expressed as mean ± SEM. Birth weight is indicated for all rat ventilated rat pups (n=45 in CDH and n=41 in controls). Lung weight and lung-body weight ratios are indicated for rat pups studied at t=0 hours (n=13 for both groups) to eliminate possible effects of ventilation on the lung weight. [a]: significantly lower than in controls (p < 0.001).

During artificial ventilation a significant interaction between groups and time was observed for lung weights and DNA content per mg lung weight, indicating that the course of these parameters was different for CDH pups and controls (Table 2). The lung weights were significantly different between the groups ($p < 0.001$) and changed significantly within time ($p < 0.001$). The DNA content per mg lung changed significantly within time ($p < 0.001$) both for CDH pups and controls (Table 2).

At birth the activity of catalase expressed per mg DNA was significantly higher in CDH than in controls ($p = 0.02$; Figure 1a). The activities of superoxide

dismutase, glutathione reductase, and glutathione peroxidase expressed per mg DNA were similar at birth in CDH and in control pups (Figure 1b-1d).

Table 2: Changes in lung weight and DNA/mg lung weight during artificial ventilation in rat pups with CDH and controls

		CDH	control
lung weight (mg)	t=0	94 ± 4	139 ± 4
	t=2	74 ± 2	116 ± 5
	t=4	102 ± 3	107 ± 4
	t=6	74 ± 3	114 ± 2
mg DNA/mg lung	t=0	0.012 ± 0.0006	0.012 ± 0.0004
	t=2	0.016 ± 0.0005	0.014 ± 0.0006
	t=4	0.010 ± 0.0003	0.017 ± 0.0008
	t=6	0.017 ± 0.0007	0.014 ± 0.0003

Means ± SEM are shown. The numbers per group are mentioned in the materials and methods section.

Upon artificial ventilation a statistically significant interaction between groups and time for catalase and superoxide dismutase was observed (Figures 1a and 1b; $p < 0.01$), indicating that the course of these enzyme activities was different for CDH pups and control pups. For glutathione reductase and glutathione peroxidase no significant interaction between groups and time was observed, indicating that the courses of the activities of these enzymes during artificial ventilation were not significantly different between the groups (Figures 1c and 1d). The glutathione reductase activity showed a significant difference between the groups ($p = 0.005$) and a significant difference in time ($p = 0.008$). This indicates that the activity of glutathione reductase in CDH runs parallel with that of controls, but at a different level. For glutathione peroxidase only a significant difference in time was observed ($p = 0.02$), indicating that the course and magnitude of the activity of this enzyme were similar for both groups.

Further analysis showed that in CDH only the activity of superoxide dismutase increased transiently in response to artificial ventilation with a maximal activity at t=2 hours (Figure 1b), followed by a decrease which was significant at t=6 hours ($p = 0.02$). This tendency of an early, transient increase in AOE activity during

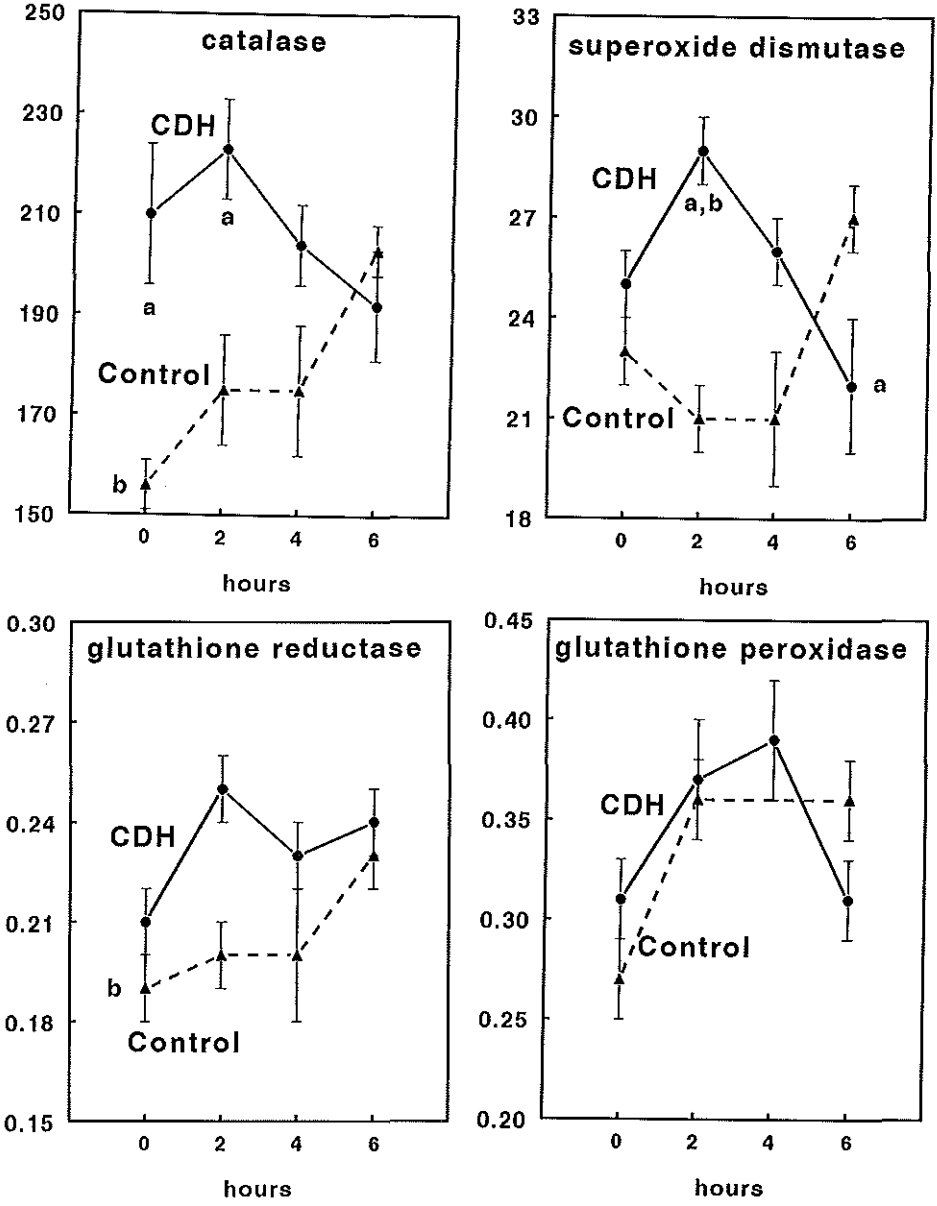

Figure 1: Activities of catalase (1A), superoxide dismutase (1B), glutathione reductase (1C), and glutathione peroxidase (1D) in units per mg DNA in CDH (circles with solid line) and controls (triangles with broken line) at birth and after 2, 4, and 6 hours of ventilation. 6-16 animals per group (see Materials and Methods for exact numbers per group). Means ± SEM are shown. a: significantly different from control, same time point ($p < 0.05$); b: significantly different from t=6 hours, same group ($p < 0.05$).

ventilation occurred also for the other enzymes in CDH, but these differences were not statistically significant (Figures 1a, 1c, and 1d). In controls, the activities of all enzymes increased slowly with maximal levels at t=6 hours, which was significant for the activities of catalase and glutathione reductase (Figure 1a and 1c; $p < 0.001$ and $p = 0.02$, respectively). The total AOE activities in the lungs of CDH pups and controls showed a significant interaction between groups and time for catalase and superoxide dismutase activities, but not for glutathione reductase and glutathione peroxidase activities. Both for glutathione reductase and glutathione peroxidase a significant difference between the groups ($p < 0.001$) and a significant difference in time ($p < 0.01$) was observed. At birth the total activities of catalase and glutathione peroxidase were similar in CDH lungs and control lungs, but after six hours of ventilation the total activities of all enzymes were significantly lower in lungs of CDH pups (Figure 2).

8.5 Discussion

In the present study we found decreased birth weight, lung weight and lung-body weight ratios in CDH rat pups. At birth lungs of CDH pups had higher catalase activity expressed per mg DNA, whereas no differences between CDH and controls were found for glutathione peroxidase, glutathione reductase, and superoxide dismutase. Expressed as total activity in both lungs, catalase and glutathione peroxidase activity reached control levels in CDH rats, whereas the activities of the other AOE were significantly lower in CDH. During artificial ventilation the profiles of catalase and superoxide dismutase activities in CDH rats were significantly different from those of control pups. An early, transient increase in superoxide dismutase activity occurred in CDH. In controls, the onset of the response to ventilation was slower, and maximal levels of AOE activity were reached after 6 hours of ventilation for catalase, glutathione reductase, and superoxide dismutase, whereas the activity of glutathione peroxidase showed an early increase to a constant level.

The finding that the activities per mg DNA of superoxide dismutase, glutathione reductase, and glutathione peroxidase were similar at birth, and that the catalase activity was even higher in CDH than in controls, suggests that the hypoplastic lungs in CDH are not immature with respect to the AOE activity, which is in accordance with our previous study.[14]

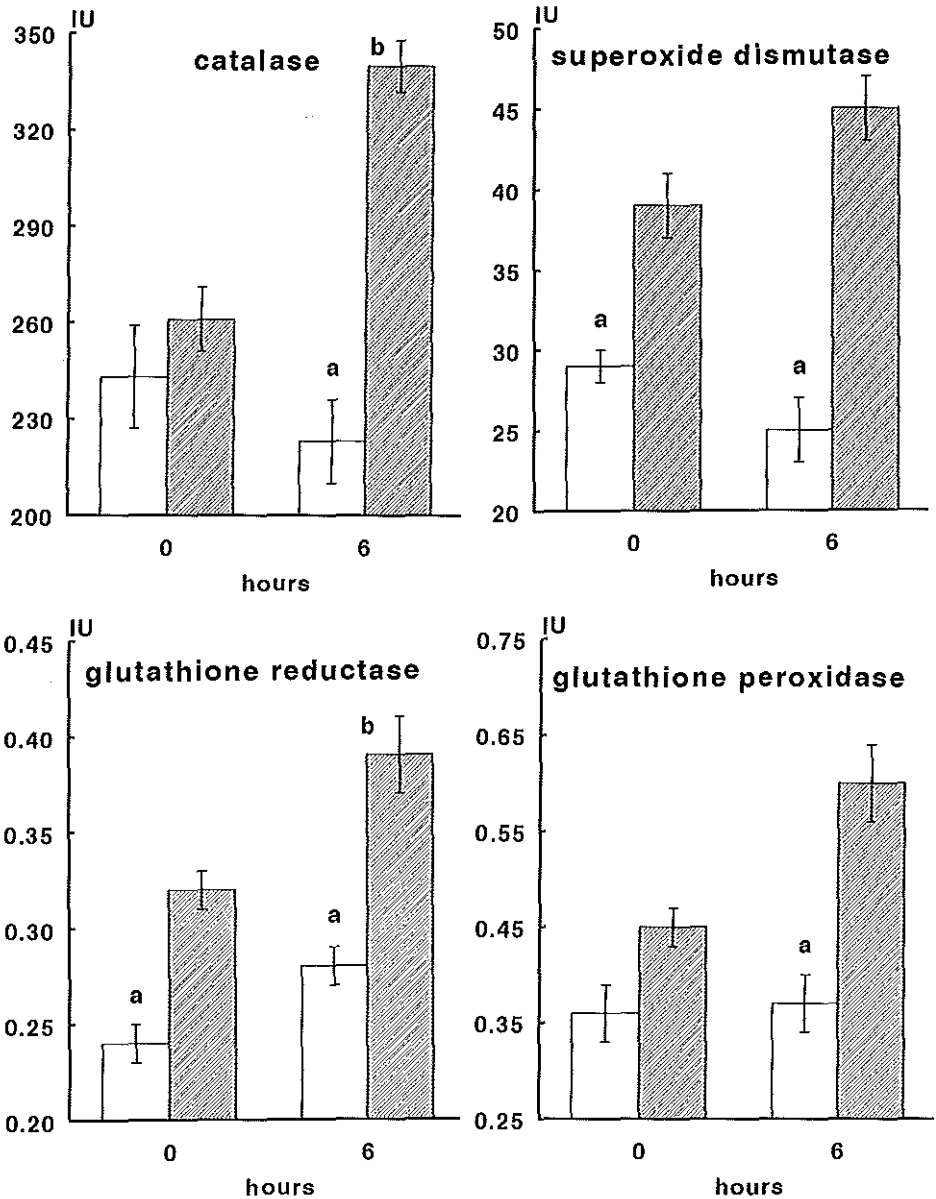

Figure 2: Total AOE activities of both lungs in CDH (open bars) and controls (striped bars). Mean ± SEM values are shown for catalase (2A), superoxide dismutase (2B), glutathione reductase (2C), and glutathione peroxidase (2D) at t=0 and t=6 hours. a: significantly different from control, same time point ($p < 0.05$); b: significantly different from t=0 hours, same group ($p < 0.05$).

The effects of hyperoxia on lung AOE activity of newborn animals have merely been studied after prolonged stay in exposure chambers.[5-10,22] Increased AOE activity has been described after at least 72 hours of exposure to 80% O_2 or more.[5-10,22] Hoffman and coworkers reported increased activity of superoxide dismutase after 4 hours exposure to 95% O_2, and increased glutathione peroxidase and catalase activities after 6 to 12 hours in 10-day old rat pups.[8] We were not able to study the effects of prolonged exposure to hyperoxia in our model of CDH and lung hypoplasia.

The effects of artificial ventilation on AOE activity have been studied previously in our rat model of CDH.[14] After 5 hours of ventilation with room air a slight but significant decrease of glutathione peroxidase activity was observed. This effect was more pronounced after 5 hours of ventilation with 100% O_2. Because the glutathione metabolism may be important to protect the lungs from hyperoxic injury, we decided to extend our study and measure the activities of glutathione reductase during artificial ventilation as well. This enzyme catalyzes the necessary reduction of oxidized glutathione, which is produced from reduced glutathione during the reduction of organic peroxides and hydrogen peroxide by glutathione peroxidase. A decreased activity of glutathione peroxidase may lower the production rate of oxidized glutathione, the substrate for glutathione reductase. Catalase can convert hydrogen peroxide to water, but is not able to catalyze the reduction of organic peroxides. A failing activity of glutathione peroxidase and/or glutathione reductase may thus result in accumulation of organic peroxides.[23]

The activity of glutathione reductase is increased in lung explants from fetal rat lungs during exposure to hyperoxia.[24] However, a recent paper reported no change in glutathione reductase activity in vivo in neonatal rats after 15 days of hyperoxic exposure.[6] In the present study we found that the response pattern of glutathione peroxidase and glutathione reductase to artificial ventilation and hyperoxia was similar in CDH rat pups and in controls, and that the activity of glutathione reductase per mg DNA was higher in CDH than in controls. This suggests that the glutathione system is frequently activated in rat pups with CDH. Whether the lower glutathione peroxidase activity in CDH after six hours, which is in accordance with a previous study after five hours[14], reflects a trend of decreasing activity of this enzyme, remains speculative.

The profiles of catalase and superoxide dismutase activity during six hours of ventilation in lungs of CDH pups differ from those in control lungs. However, from the present data it is difficult to reason whether the AOE response to hyperoxia and barotrauma fails. Two observations argue against an inadequate

AOE response in CDH. The AOE activity per mg DNA at birth is similar or even higher in CDH lungs than in controls. This may indicate that the lungs in CDH are well prepared for an initial attack by oxygen free radicals. Furthermore, the activities of all four enzymes rose transiently in CDH did not decrease below the initial levels of AOE at birth.

On the other hand, the altered response of catalase and superoxide dismutase in CDH lungs, which both decreased after two hours of ventilation, may be a reflection of failure indicating a trend of decreasing AOE activity. In normal neonatal rats the lungs may be adequately protected from lung injury, and resulting bronchopulmonary dysplasia, by the increase in AOE activity that is relatively slow in onset, as we have shown here, but that lasts for a long time, as described by others.[5,7,8,22] Adequate protection has been estimated by the relative AOE activity, that is on a cellular basis (i.e. corrected for DNA). However, to protect the intact lung it may well be that a minimal absolute activity is needed. We found that at birth the total activities of catalase and glutathione peroxidase were similar in CDH and controls, whereas the other two enzymes had a lower total activity in CDH. After six hours of ventilation with 100% O_2 the total AOE activity of both lungs for all enzymes was significantly higher in controls than in CDH. In CDH pups the total AOE activities after six hours of ventilation were similar as the initial levels at birth, whereas the activities of catalase and glutathione reductase had significantly increased in controls. If our hypothesis is correct that an absolute AOE activity is needed to protect the lungs adequately, these observations are indicative of a failing antioxidant enzyme system in CDH.

From the present data it is not clear why an early, transient increase in AOE activity occurs in CDH. It can be assumed that normally the initial protection of the lungs from oxygen free radicals during exposure to hyperoxia and barotrauma is being performed by non-enzymatic antioxidants.[25] A deficiency of non-enzymatic antioxidants may necessitate an early increase in antioxidant enzymes. The fact that prematures with a glutathione deficiency develop neonatal lung disease[11,12] supports this assumption. Moreover, in newborn infants with CDH at birth a lowered vitamin A status, which is another important antioxidant[25], has been reported recently.[26]

In conclusion, we showed that the response pattern of catalase and superoxide dismutase in CDH is different from controls during artificial ventilation with 100% O_2. In controls, but not in CDH, an increased activity of catalase and glutathione reductase was found after six hours of ventilation. We speculate that an altered response of AOE activity in CDH in combination with lower concentrations of

antioxidants, such as vitamin A[26], may contribute to the initiation of pulmonary oxygen toxicity[27], which in its turn may lead to the sequence of diffuse alveolar damage.[28] Supplemental therapy with antioxidants and antioxidant enzymes may be useful to prevent morphological changes following pulmonary oxygen toxicity, and this may contribute to lower the high incidence of bronchopulmonary dysplasia in CDH patients.

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Chapter 9

Intervention studies

9.1 Exogenous surfactant is distributed in both lungs of neonatal rats with congenital diaphragmatic hernia*

9.1.1 Summary

Exogenous surfactant therapy may improve survival in infants with congenital diaphragmatic hernia (CDH) and hypoplastic, immature lungs. We assumed that surfactant would be distributed unevenly, with less deposited in the ipsilateral, most hypoplastic lung. Therefore, we studied the distribution of exogenous surfactant in newborn rats with CDH, induced by oral administration of 100 mg 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen) on gestational day 10. Following a cesarean section at term newborns were ventilated for one hour in a standardized way with a pressure of 25 cm H_2O (reduced to 17 cm H_2O after 30 minutes), FiO_2 1.0 and PEEP 3 cm H_2O. Surfactant mixed with colored microspheres (diameter 15.5 μm) and administered endotracheally (dose 25 mg/ml; 50 μl). The number of microspheres in the lungs was measured by spectrophotometry. Exogenous surfactant was deposited in both lungs in CDH: The median number of microspheres per mg dry lung weight was 3710 (range 1080-5990) in the left, severely hypoplastic lungs versus 3510 (1520-4360) microspheres in the right lungs. This offers good prospects to apply surfactant in children with CDH in randomized trials not hampered by major differences in lung expansion and resulting pneumothorax.

9.1.2 Introduction

Children with congenital diaphragmatic hernia (CDH) have abnormal morphological development of lungs and intrapulmonary blood vessels.[1-3] Several

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publications suggest that in CDH the lungs are biochemically immature[1,4-6], but this is contradicted in other studies.[7,8] Exogenous surfactant therapy has been described to improve survival of children with CDH[9,10], although this has not yet been confirmed in prospective randomized trials. Lung compliance and oxygenation improved after exogenous surfactant in a lamb model of CDH.[11,12]

Positive effects of exogenous surfactant therapy in respiratory distress syndrome have been observed in preterm neonates[13-16] and, experimentally, in several models of premature animals.[15,17,18]

We previously studied the short-term effect of exogenous surfactant therapy following artificial ventilation in newborn rats with CDH and were not able to show benefit.[19] We suspected that the lack of a positive effect of exogenous surfactant therapy in our rat model of CDH might be due to the unequal distribution of surfactant in CDH, with less deposited in the ipsilateral, most hypoplastic lung. Therefore, we evaluated the distribution of exogenous surfactant during artificial ventilation in a rat model of CDH by means of colored microspheres.

9.1.3 Materials and methods

9.1.3.1 Animal model

Female Sprague Dawley rats (Harlan Olac, England) weighing about 250 grams were mated during one hour (day 0 of gestation), and received 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen: Rohm Haas Company, Philadelphia, U.S.A.) on day 10 of gestation, as described before.[20] Nitrofen results in a left-sided or sometimes a bilateral diaphragmatic defect with lung hypoplasia in 70-90% of the offspring.[19,20] Food and water were supplied ad libitum during the whole period of pregnancy. At gestational day 22 (term=22-23 days) the dams were anesthetized by inhalation of diethylether and a cesarean section was performed.

9.1.3.2 Artificial ventilation

After birth the newborns were weighed and anesthetized with sodium pentobarbital (30 mg/kg) and paralyzed with pancuronium bromide (0.08 mg/kg) applied intraperitoneally. They were intubated orally with a 24G intravenous catheter (Neoflon, Viggo-Spectramed, Helsingborg, Sweden) with atraumatic stent, transferred to a multichambered, pressure-constant bodyplethysmograph heated to 37°C, and in supine position connected to a modified Servo 900B ventilator

(Siemens-Elema, Solna, Sweden).[21] Pressure-controlled ventilation was started using the following respirator settings: PIP 25 cm H_2O; PEEP 3 cm H_2O; frequency 40 cycles/min; fraction of inspired oxygen (FiO_2) 1.0; inspiratory:expiratory ratio of 1:2. After 30 minutes, PIP was reduced to 17 cm H_2O in order to prevent pneumothorax. Tidal volume was not measured during these experiments, but in a previous pilot study with exogenous surfactant therapy tidal volume was measured in 15 CDH pups and 19 controls using a well established protocol.[19,21] This pilot study revealed that tidal volume after one hour of ventilation at 15 cm H_2O was 9.5 ml/kg in CDH and 11.3 ml/kg in controls using a similar ventilatory regimen (unpublished data).

9.1.3.3 Distribution of surfactant

Ten newborns from three litters exposed to Nitrofen and ten controls from two litters were randomly chosen to be studied according to the following protocol. Bovine surfactant and red microspheres with a mean diameter of 15.5 (SD 0.2) µm and a specific weight of 1.09 g/ml[23,24] (Dye-Trak Microspheres, Triton Technology, San Diego CA, U.S.A.) were mixed in NaCl 0.9% to obtain a suspension containing 25 mg/ml surfactant and 1.65 x 10^6 microspheres/ml. The surfactant consisted of approximately 90 to 95% phospholipids and 1% hydrophobic proteins (surfactant protein B and C), which had been prepared as described previously.[22]

After 15 minutes of ventilation a bolus of 50 µl of the freshly mixed suspension was instilled intratracheally. After one hour the rat pups were killed with 120 mg/kg pentobarbital intraperitoneally and the presence and size of a diaphragmatic defect in Nitrofen-exposed rat pups was revealed by autopsy. When more than half of the ipsilateral diaphragm was missing, the defect was considered to be large. After tracheotomy a cannula was fixed in the trachea. The heart-lung block was removed and dried for 4 hours using a continuous intratracheal inflation pressure of 20 cm H_2O. Lung weights obtained following this procedure were considered as dry lung weights. Tracheotomy or removal of the heart-lung block failed in five Nitrofen-exposed newborns and one control. Therefore, five Nitrofen-exposed rat pups (all with large left-sided CDH and severe lung hypoplasia) and nine control newborns remained. The dried lungs of control rat pups were divided into six pieces by two transversal cuts and one sagittal cut. This resulted in 12 pieces of lung from each control animal. The right lungs of CDH rat pups were cut likewise, but the severely hypoplastic left lungs could only be divided by one transversal cut in two halves, resulting in 8 pieces of lung in each CDH rat pup. All lung

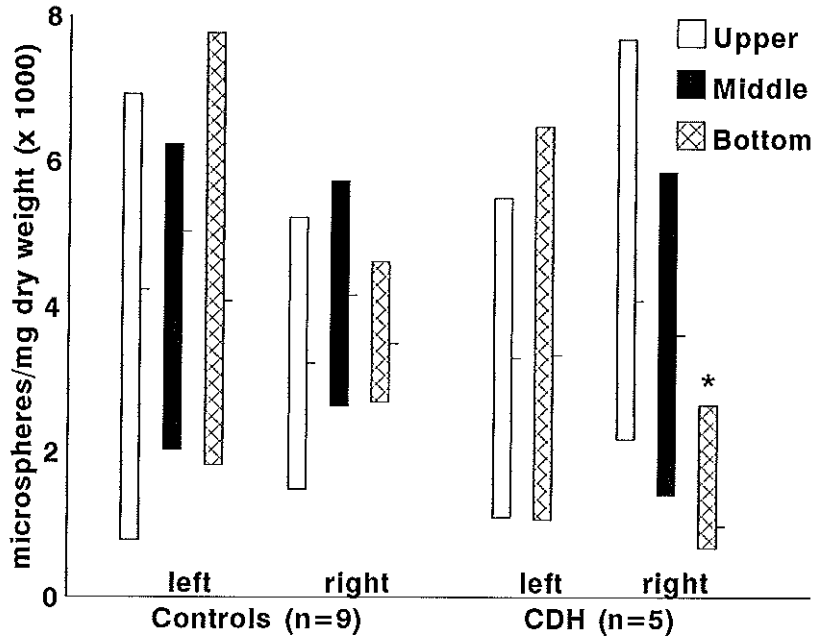

*Figure 1: Number of microspheres per mg lung weight in different lung sections. For controls (n = 9) and CDH rat pups (n = 5) median and range values for different lung parts are shown. The left lungs of CDH rats could only be divided into two pieces (upper and bottom) due to their small size. The data from frontal and posterior lung sections are pooled in the right lungs of CDH rat pups and the lungs of controls. *: significantly different from the upper and middle part of the right lung in CDH; p = 0.05.*

fragments were weighed and treated for tissue digestion with 4 M KOH as described before.[23] The microspheres were recovered by vacuum filtration and the red dye was extracted with dimethylformamide, according to the techniques described for quantification of colored microspheres.[24] After centrifuging, 100 µl of the colored fluid was transferred to a quartz microcuvette and the absorbance was measured by spectrophotometry at 530 nm (Spectrophotometer DU 7400, Beckman Instruments, Fullerton CA, U.S.A.). The microsphere number was calculated using the standard curve equation that was provided by the manufacturer and confirmed by measurement of serial dilutions.

9.1.3.4 Histology

To visualize the microspheres in the sacculi the lungs of 5 additional randomly chosen CDH rats and 3 additional controls were used for histological examination.

The ventilatory regimen and surfactant administration were performed as described above. The lungs were fixed without inflation in 3.7% formalin, embedded in paraffin, and cut into 10 μm sections.

9.1.3.5 Data analysis

All data are presented as median (range). Differences between the groups were tested by Kruskal-Wallis one-way-analysis of variance. Statistical significance was assumed at 5% level.

9.1.4 Results

The median birth weight in CDH rat pups was 4.72 (range 4.20-4.90 grams) and 5.72 (5.42-6.33) grams in controls ($p < 0.001$).

The median number of microspheres recovered from both lungs of CDH rat pups was 38,065 (16,790-45,503), which is significantly smaller than the number of 86,295 (50,645-104,605) in controls ($p = 0.003$). The dry lung weight was significantly lower in CDH pups than in controls: 11.4 (9.2-13.0) mg versus 21.8 (18.9-24.6) mg; $p = 0.003$). The mean number of microspheres corrected for dry tissue weight was 3580 (1400-4930) in CDH pups and 3830 (2060-5220) in controls (NS).

The median number of microspheres per mg dry lung weight was 3710 (1080-5990) in the left lungs in CDH, and 3510 (1520-4360) in the right lungs (NS). The lung weights were 3.32 (2.78-4.44) and 8.51 (5.99-8.66) mg respectively ($p = 0.009$). In CDH the bottom part of the right lungs contained significantly less microspheres per mg lung compared with both other parts: 980 (680-2650) versus 4080 (2180-7680) and 3610 (1420-1550) in the upper and middle parts respectively ($p = 0.05$; Figure 1). The hypoplastic left lungs in CDH, divided into two pieces, showed no difference between upper and bottom parts: 3290 (1100-5490) and 3330 (1070-6470) microspheres per mg lung respectively (Figure 1). The left and right lungs in controls showed no differences in microspheres per mg dry lung weight between the upper, middle and bottom parts (Figure 1), but significantly more microspheres were found in posterior than in frontal sections (Table 1).

Histological slides showed "alveolar" aeration in all control rat pups (Figure 2A). In all CDH cases widely expanded bronchioles with collapsed terminal airspaces were found (Figure 2B). Although the red colour of the microspheres was lost during the fixation procedure the microspheres could clearly be identified in the lung tissue (Figure 2C).

Table 1: *The number of microspheres per mg dry lung weight in different lung sections in CDH and controls*

	CDH (n=5)		Control (n=9)			
	right		right		left	
	frontal	dorsal	frontal	dorsal	frontal	dorsal
upper	2780 (1170-9330)	3700 (2760-6510)	2740 (570-4320)	4510 [a] (1570-6790)	3760 (3330-6540)	5280 (1320-8900)
middle	2860 (1370-6850)	3360 (1480-4820)	3060 (1900-5190)	4400 [a] (2650-7030)	3080 (635-6330)	6050 [a] (2720-7560)
lower	1250 [c] (380-2680)	1020 [b,c] (710-2620)	3180 (1590-4500)	4362 [a] (2370-6170)	3110 (4800-7280)	4910 [a] (2790-8000)

The median (range) number of microspheres per mg dry lung weight are shown in different lung sections in CDH and controls following administration of exogenous surfactant mixed with colored microspheres. The severely hypoplastic left lungs in CDH could only be divided in two pieces; see Figure 1 for the number of microspheres in these lungs. a: significantly different from frontal counterpart in the same group ($p < 0.05$)
b: significantly different from upper and middle dorsal sections in CDH ($p = 0.01$); c: significanly different from the same lung section in controls ($p < 0.01$).

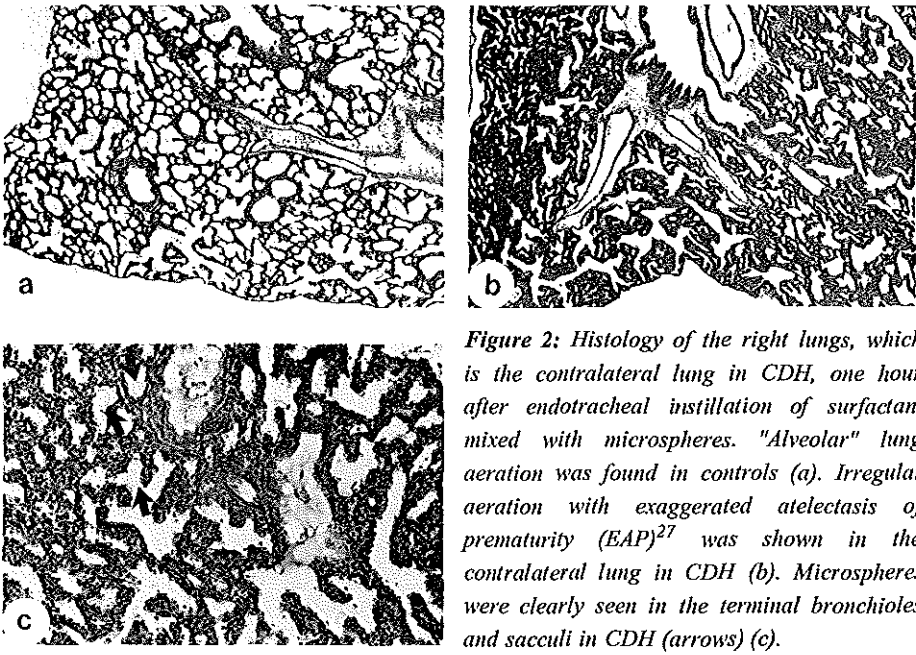

Figure 2: Histology of the right lungs, which is the contralateral lung in CDH, one hour after endotracheal instillation of surfactant mixed with microspheres. "Alveolar" lung aeration was found in controls (a). Irregular aeration with exaggerated atelectasis of prematurity (EAP)[27] was shown in the contralateral lung in CDH (b). Microspheres were clearly seen in the terminal bronchioles and sacculi in CDH (arrows) (c).

9.1.5 Discussion

We evaluated the distribution of exogenous surfactant in hypoplastic lungs in CDH rat pups following a standardized ventilatory regimen. Surfactant was deposited in both lungs indicating that exogenous surfactant reaches even the most hypoplastic, ipsilateral lung in CDH. The bottom parts of the right, contralateral lungs in CDH contained less microspheres than the upper and middle parts, and the anterior parts of the control lungs contained less microspheres than the posterior parts.

We performed this study since no positive effect of exogenous surfactant therapy on lung volume could previously be demonstrated in our CDH model.[19] We assumed that this could be due to different reasons: First, because surfactant was lost during the instillation, due to the relatively large fluid volume compared to the lung volume in these rat pups. Second, the timing of surfactant administration may not have been optimal, so that surfactant was inactivated through protein leakage.[25] Third, surfactant was spread unevenly over the hypoplastic lungs.

The first explanation may have contributed to our findings but it is unlikely that this is the only responsible factor. We found in this study that the number of

microspheres recovered from both lungs was lower in CDH than in controls, which confirms loss of surfactant. The relatively large fluid volume compared to the lung volume in CDH may be responsible for fluid leakage during the instillation procedure. However, 125-150 mg/kg surfactant would have reached the lungs in these rat pups even if half of the surfactant was lost. This dose is frequently used in human trials with exogenous surfactant therapy with positive effects on oxygenation and lung function.[13,15]

The second explanation is supported by Seidner et al[26], who in preterm rabbits observed a decreased dose-response, and a less uniform distribution of exogenous surfactant after delayed administration. They also found that lung lavages from fetal rabbits receiving surfactant after 15 or 30 minutes of ventilation contained more protein compared with the group that was treated directly after birth. Inactivation of surfactant due to early onset of protein leakage[25] may have contributed to the lack of benefit of exogenous surfactant therapy in our previous study.[19]

The third explanation seems unlikely, because we found that in CDH the number of microspheres corrected for lung weight was similar in the left and right lung. Besides the spread of surfactant over both lungs, we studied the distribution in the separate lungs. In CDH the left lungs were so small that these could not be cut into more than two parts. Therefore, the homogeneity of distribution in the left lung was not easy to estimate, but we found a similar number of microspheres per mg lung weight for both lung parts. The number of microspheres per mg lung weight in the bottom part of the right lungs was decreased in CDH. This may be explained by different reasons. First, the right, contralateral lung might have been compressed by abdominal viscera that migrated intrathoracally, or by the heart shifted to the right. Second, the aeration pattern which was demonstrated by histological examination in lungs of CDH rat pups might have been responsible. This pattern has been described as exaggerated atelectasis of prematurity (EAP) in premature children with respiratory distress syndrome.[27] However, it was not only found in the bottom parts of the right lungs, but in other parts of both lungs as well. That the posterior parts of the lungs contained more microspheres than the frontal parts may be explained by the supine position of the rat pups during the procedure of ventilation.

A remaining question is whether the distribution of the microspheres adequately reflects the distribution of surfactant. It could well be that in the lung the microspheres migrate separately from the surfactant suspension. However, in an earlier study exogenous surfactant mixed with colored microspheres with similar

properties showed the same distribution seen with radioactively labeled surfactant preparations without microspheres.[23]

Our study shows that the initial hypothesis that less surfactant is deposited in the ipsilateral, most hypoplastic lung in CDH should be rejected. That surfactant spreads evenly in the ipsilateral and contralateral lung in CDH is supported by the presence of microspheres in terminal bronchioles and sacculi in both lungs of ventilated rat pups, and therefore other factors such as a non-optimal ventilatory strategy or a non-optimal timing of surfactant administration may be responsible for the lack of a beneficial effect of exogenous surfactant on the lung volume in the rat pups with CDH. The question whether exogenous surfactant may be beneficial in the human cases of CDH has not been clarified yet. We were not able to estimate the distal delivery and alveolar recruitment by evaluation of gas exchange parameters or lung mechanics due to the small size of the animals in our model. However, improvement of arterial blood gases and lung compliance has been shown in a lamb model of CDH.[11] Our findings suggest that these positive effects of exogenous surfactant resulted not only from increased alveolar recruitment in the contralateral lung, but also in the ipsilateral lung. The possibilities to individualize the ventilatory treatment in children with CDH may be helpful to obtain optimal alveolar recruitment in both lungs following exogenous surfactant therapy. The uneven distribution of exogenous surfactant within the separate lungs of the rat pups may not be representative for the human situation. Differences in size and position, and hence in gravitational factors, between rat pups and children may result in an altered surfactant distribution within the lungs. However, an even spread of exogenous surfactant over both lungs is strongly supported by our findings. Another issue is that we administered exogenous surfactant in a large-volume fluid of about 10 ml/kg body weight. This is less than the optimal fluid volume of 16 ml/kg described by Van der Bleek et al. in three-month old rabbits in a study with radioactively labeled microspheres.[28] However, we agree with these authors that more studies are required before introducing such large fluid volumes clinically. Exogenous surfactant applied in a small-volume fluid may result in an altered surfactant distribution within the lungs of children with CDH.

In conclusion, we found that in CDH exogenous surfactant does spread in the ipsilateral and the contralateral lung. This offers good prospects to apply surfactant in children with CDH in randomized trials not hampered by major differences in lung expansion and resulting pneumothorax. Since the timing of surfactant administration as a rescue therapy in our experiments may not have been optimal,

we speculate that surfactant administration given as prophylaxis or in repeated doses using an optimal ventilatory strategy for each individual case might be useful to improve alveolar recruitment and to overcome a possible inactivation of surfactant by protein leakage.[25]

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9.2 Prenatal hormones alter antioxidant enzymes and lung histology in rats with congenital diaphragmatic hernia*

9.2.1 Summary

Prenatal administration of dexamethasone (DEX) and thyrotropin-releasing hormone (TRH) synergistically enhances lung maturity, but TRH suppresses the antioxidant enzyme activity. Prenatal hormonal therapy improves alveolar surfactant content and lung compliance in rats with congenital diaphragmatic hernia (CDH). In fullterm neonatal rats with CDH we studied the effects of prenatal DEX or DEX+TRH on antioxidant enzyme activity at birth, on survival, and on lung morphometry after 4 hours of ventilation with 100% oxygen. CDH was induced by administration of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) on gestational day 10. DEX+TRH-treated CDH rats had lower activity of glutathione reductase after birth than had sham-treated CDH pups. DEX-treated and sham-treated pups had similar antioxidant enzyme activity. Hormonal treatment did not change survival during ventilation. The average airspace volume increased in DEX-treated CDH pups after ventilation, with a small synergistic effect after addition of TRH. Based on our findings we speculate that prenatal administration of dexamethasone is the best choice to improve lung maturity and airspace volume in CDH patients.

9.2.2 Introduction

The effects of prenatal application of corticosteroids and thyroid hormones on lung maturity and on the antioxidant enzyme system have been studied experimentally in several animal models.[1-4] It appeared that dexamethasone and thyroid hormone have a synergistic effect on the development of the surfactant system, but a negative effect on antioxidant enzyme activity.[4] Clinically, the effects of prenatal hormonal therapy have been studied with respect to mortality and morbidity in preterm infants.[5,6] Administration of glucocorticoids to women at risk of preterm delivery was recommended[5], but the addition of thyrotropin-releasing hormone (TRH) to glucocorticoids is still debated because maternal and perinatal risks have been reported.[6]

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Infants with congenital diaphragmatic hernia (CDH) have abnormal morphological development of the lungs.[7] Several publications suggest that in CDH the lungs are biochemically immature[7,8], although normal lecithin/sphingomyelin ratios in amniotic fluid of CDH patients have been reported.[9] More recent data suggest, however, that infants with CDH do have surfactant deficiency.[10] Indications for lung immaturity in CDH have been found in several animal studies of CDH using sheep and rats.[11,12]

In our previously described rat model of CDH[13] and in lambs with CDH prenatal glucocorticoids improved the biochemical and morphological immaturity, and the compliance of the lungs.[14-17] Prenatal administration of dexamethasone in combination with TRH also improved lung compliance[14], and showed the best effects on disaturated phosphatidylcholine and glycogen content of the lungs in rat pups with CDH at birth.[17] In this CDH model the effects of prenatal application of glucocorticoids and TRH on the antioxidant enzyme system, and on the tolerance to hyperoxia and barotrauma have not been studied yet. We hypothesized that prenatal glucocorticoids would improve survival and lung morphology after artificial ventilation with 100% O_2, with synergistic effects on lung morphology after the combination of glucocorticoids and TRH. Moreover, we hypothesized that the combination of dexamethasone and TRH might have a negative effect on antioxidant enzyme activity. Therefore, in newborn rats with CDH we evaluated the effects of prenatal hormonal therapy with dexamethasone or the combination of dexamethasone and TRH on the antioxidant enzyme activity at birth, and on survival and lung morphometry following a short period of hyperoxic artificial ventilation.

9.2.3 Materials and methods

9.2.3.1 Animal model

Female Sprague Dawley rats (Harlan Olac, England) weighing about 250 grams were mated during one hour (day 0 of gestation). Ten of 17 pregnant rats received 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen: Rohm Haas Company, Philadelphia, NJ) on day 10 of gestation, as described before;[13] the remaining 7 rats provided control pups. Nitrofen results in a diaphragmatic defect with lung hypoplasia in up to 80% of the offspring.[13] Food and water were supplied ad libitum during the whole period of pregnancy. At gestational day 22 (term=22-23 days) the dam was anesthetized by inhalation of diethylether and a cesarean section was performed. The mean numbers of rat pups in the Nitrofen-exposed and control

litters were 13.1 ± 0.8 and 13.6 ± 0.8, respectively. All animal experiments were performed after approval of the Animal Care and Use Committee of the Erasmus University, Rotterdam.

9.2.3.2 Prenatal hormonal therapy

Six different study groups were created: sham-treated CDH rats (2 litters) and controls (2 litters), DEX-treated CDH rats (5 litters) and controls (2 litters), and DEX+TRH-treated CDH rats (3 litters) and controls (3 litters).

On day 19 and 20, i.e. 72 and 48 hours before delivery, pregnant dams received 0.25 mg/kg dexamethasone sodium-phosphate (DEX) in 0.2 ml saline intraperitoneally (i.p.), the lowest dose that is known to result in biochemical and morphometrical improvement with the least effects on somatic and pulmonary growth.[16] TRH (Calbiochem Corporation, La Jolla, CA) was administered i.p. to the pregnant dams as a loading dose (25 µg/kg in 0.5 ml of saline) and by an implanted osmotic mini-pump (Alzet pump; model 1003D: Alza Corporation, Palo Alto, CA) through which TRH was administered continuously i.p. in a dose of 100 µg/kg/day.[4] This pump was implanted i.p. on day 20 of gestation under short anesthesia with diethylether through a small midline incision using a sterile technique, and provided TRH continuously for 48 hours. Sham treatment was given using i.p. injections of saline and implantation of an osmotic pump filled with saline.

9.2.3.3 Measurement of antioxidant enzyme activity

Five to eight of the newborn rats per group were sacrificed for antioxidant enzyme measurement directly after birth. The presence and size of the diaphragmatic defects were assessed in Nitrofen-exposed rats. To obtain a homogeneous study group, Nitrofen-exposed rat pups without CDH (n=8) were excluded. The numbers of rat pups studied per group were: n=5 in sham-treated CDH rat pups and controls, n=6 and n=5 in DEX-treated CDH pups and controls respectively, and n=5 in DEX+TRH-treated CDH pups and controls.

After removal of the heart-lung block, the lungs were stripped of nonpulmonary tissue, weighed, frozen with liquid nitrogen, and stored at -70 °C until further processed as described before.[18] All biochemical analyses were performed on lungs from separate rat pups. After thawing, the lungs were diluted 1:15 (wt/vol) in ice-cold phosphate-buffered saline and homogenized with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) for 15 s at maximum speed. Next, the suspension was sonicated for 10 s on ice. In this crude suspension, concentrations

of protein and DNA were estimated as described previously.[18] To determine antioxidant enzyme, the crude suspensions were centrifuged at 20,000 x g for 30 min, and the pellets were discarded. The activities of glutathione peroxidase and catalase were measured as described before.[18] Glutathione reductase activity was determined as described by Goldberg and Spooner.[19] Superoxide dismutase activity was determined by using the SOD-525 method (R & D Systems, Abingdon, UK).

Correction for blood contamination was performed as follows: From three different control litters the red blood cells of three to five rat pups were collected for measurement of hemoglobin[20], and antioxidant enzyme activity. The antioxidant enzyme activity per mg hemoglobin was thus determined. The hemoglobin concentration was also measured in the lung suspensions. The antioxidant enzyme activity resulting from blood contamination was calculated and subtracted from the total antioxidant enzyme activity in the lung suspensions. To facilitate comparison and exclude differences merely based upon differences in lung weight the activities of glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase were all expressed as units per mg lung DNA.

9.2.3.4 Artificial ventilation

After birth the newborns were weighed, and anesthetized with sodium pentobarbital i.p. (30 mg/kg) and paralyzed with pancuronium bromide i.p. (0.08 mg/kg), intubated with a 24G intravenous catheter (Neoflon, Viggo-Spectramed, Helsingborg, Sweden) with an atraumatic stent, transferred to a multichambered bodyplethysmograph heated to 37°C, and connected in supine position to a modified Servo 900B ventilator (Siemens-Elema, Solna, Sweden). Pressure-controlled ventilation was started using the following respirator settings: PIP 25 cm H_2O; PEEP 3 cm H_2O; frequency 40 cycles/min; FiO_2 1.0; inspiratory:expiratory ratio of 1:2. A previously performed study revealed that opening pressures of 25 cm H_2O were needed to obtain a good lung aeration pattern, but that continuous ventilation with this peak pressure resulted in a high incidence of pneumothorax (unpublished data). Therefore, PIP was reduced to 17 cm H_2O after 30 minutes. The incidence of pneumothorax, absent heart action, or other complications related to insufficient ventilation were recorded in ventilated rat pups. In case of death the lungs were processed immediately for histological examination. After four hours the surviving pups were sacrificed. Autopsy revealed whether a diaphragmatic defect in Nitrofen-exposed pups was present.

9.2.3.5 Histological studies

The trachea was cannulated, the thorax opened, and the lungs inflated at a constant pressure of 20 cm H_2O with 10% formalin as described by Burri.[21] The distended lungs were removed from the thoracic cavity and the trachea was ligated to maintain lung inflation during fixation for 24 hours. After 24 hours the lung volume (V_L) was measured by the volume displacement method.[22] The fixed tissue was dehydrated in a graded alcohol series, embedded in paraffin, and cut in 1 cm slices in the parasagittal plane. Random 1 cm^2 specimens were taken from the lung for histological examination, 8 μm coronal sections were cut, and stained with hematoxylin and eosin.

Morphometry was performed using techniques adapted from Weibel[23], and Emery and Mithal.[24] Histological sections were examined with the aid of a Nikon microscope (Microphot-FXA, Nikon Inc., Melville, NY) at 100x magnification via a CCD color video camera (DXC-151, Sony Inc., Park Ridge, NJ) linked to a Sony color television monitor overlaid with a 42 point equidistant counting grid, calibrated with an individual probe length line, Z, where Z = 83 μm. Lungs from CDH rats (sham n=7, Dex-CDH n=9, Dex+TRH-CDH n=5) in each treatment group were subsequently evaluated. The complete coronal sectional area of each lung was analysed using three different histological sections (6-10 fields/lung/slide) for each animal. Each coronal sectional area encompassed 18-30 fields/lung; the right and left lungs were studied separately, and these data were later combined because there were no significant differences between both lungs.

Volume fractions were established by counting test points falling on airspaces (volume fraction of airspaces, V_{Valv}), alveolar septa (volume fraction of airspace walls), airways (volume fraction of airways), and non-gas-exchanging elements - all other structures (volume fraction of other elements). The average inter-airspace wall distance (L_M) was calculated from the formula L_M = 2 x length of grid line (Z) / number of transections of septa within the 21 grid lines (or intercept number). The number of airspaces per unit area (N_A) was determined by dividing the airspace number into the area of the counting grid. Airspace number per unit volume (N_V) was calculated from the formula $N_V = K\ (N_A)^{3/2} / ß\ (V_{Valv})^{1/2}$, where K is the coefficient size distribution contstant (taken to be 1) and ß = 1.55, from the Weibel and Gomez shape constant.[23] Total number of airspaces (N_{AT}), total internal surface area (SA), and average airspace volume (AAV) were estimated from the following formulae: $N_{AT} = (N_V)\ (V_L)$, $SA = 4\ (V_L)\ (V_{Valv}) / L_M$, and finally $AAV = (V_{Valv})\ (V_L) / N_{AT}$. Radial saccular counts, a measure of the complexity of the respiratory acinus, were determined by counting the number

of airspaces lying on a line drawn perpendicularly from the center of a terminal or respiratory brochiole to the closest edge of the acinus (pleural or lobular connective tissue septum).[24]

9.2.3.6 Data analysis

All data are presented as means ± SEM, unless stated otherwise. As data were normally distributed, differences between the groups were tested by two-way analysis of variance, and one-way analysis of variance with the Student-Newman-Keuls and Bonferroni/Dunn tests. Chi-square test was used for proportions. Statistical significance was assumed at 5% level.

Table 2: *Mean birth weights (in grams) in newborn rats with and without CDH following prenatal hormonal modulation*

	CDH	control
sham	4.55 ± 0.11 (16)*	5.19 ± 0.06 (24)*
DEX	3.80 ± 0.07 (40)†	4.43 ± 0.09 (16)
DEX+TRH	3.62 ± 0.03 (47)	4.63 ± 0.09 (25)

*Means ± SEM are shown. The number of animals per group is shown in brackets. DEX = dexamethasone i.p. 72 and 48 h before delivery; DEX+TRH = addition of TRH i.p. during 48 h before delivery; * significantly higher than all other prenatal treatments in the same group ($p < 0.001$); † significantly higher than DEX+TRH in the CDH group ($p < 0.05$).*

9.2.4 Results

Both in CDH and in controls prenatal hormonal modulation resulted in lower birth weights compared with sham-treated pups (Table 2; $p < 0.001$). In the newborn rats that were used for antioxidant enzyme analysis CDH rat pups had lower lung-body weight ratios than controls (Table 3; $p < 0.001$), but hormonal modulation did not alter these ratios. The protein-DNA ratios were not different between the study groups (Table 3).

In controls, prenatal hormonal treatment with the combination of dexamethasone and TRH led to a significantly lower activity of glutathione reductase ($p < 0.001$), and to a higher activity of glutathione peroxidase ($p = 0.002$); the activities of catalase and superoxide dismutase were not impaired (Figure 3).

Table 3: *Lung-body weight ratios and lung protein-DNA ratios in newborn rats with and without CDH used for measurements of antioxidant enzyme activities*

	Lung-body weight ratio (mg/g)		Protein-DNA ratio (μg/μg)	
	CDH	control	CDH	control
sham	15.9 ± 0.8	26.6 ± 1.3*	4.5 ± 0.2	4.5 ± 0.1
DEX	13.8 ± 0.7	28.5 ± 0.5*	4.6 ± 0.1	4.9 ± 0.2
DEX+TRH	16.7 ± 1.1	28.2 ± 0.6*	4.6 ± 0.3	4.8 ± 0.2

*Data shown as mean ± SEM; n = 6 in the DEX-CDH group, n = 5 in all other groups. DEX = dexamethasone i.p. 72 and 48 h before delivery; DEX+TRH = Addition of TRH i.p. during 48 h before delivery; * significantly different from control, same treatment; p < 0.001*

In CDH only the activity of glutathione reductase was significantly lower in the DEX+TRH group ($p = 0.01$). The activity of glutathione peroxidase did not increase as was the case in controls (Figure 3). Prenatal treatment with dexamethasone alone led to a higher activity of glutathione peroxidase and catalase in controls ($p = 0.002$ and $p = 0.03$ respectively). A similar trend to a higher activity of glutathione peroxidase and catalase was present in CDH, but these differences did not reach the level of significance.

Intubation was successful in 50-70% of control rats irrespective of prenatal hormonal treatment. In the CDH rat pups intubation was successful in 70% (7 of 10) of the sham group; this was 26% (9 of 34) and 28% (12 of 43) in the DEX and DEX+TRH groups ($p < 0.01$). Birth weights from rat pups that could not be intubated successfully did not differ from those that could be intubated (data not shown). The mortality rate or the incidence of pneumothorax were not significantly influenced by prenatal hormonal modulation (Figure 4).

Lung morphometry of CDH rat pups showed a significant increase of the average airspace volume with a decrease in the number of airspaces in the DEX-group compared with sham-CDH (Table 4). The total internal surface area was unchanged. These significant effects were stronger in the DEX+TRH-group (Table 4). Representative pictures showing the effects of prenatal hormonal modulation on lung histology in CDH are shown in Figure 5; there was no evidence of septal disruption or diffuse alveolar damage in any of the treatment groups.

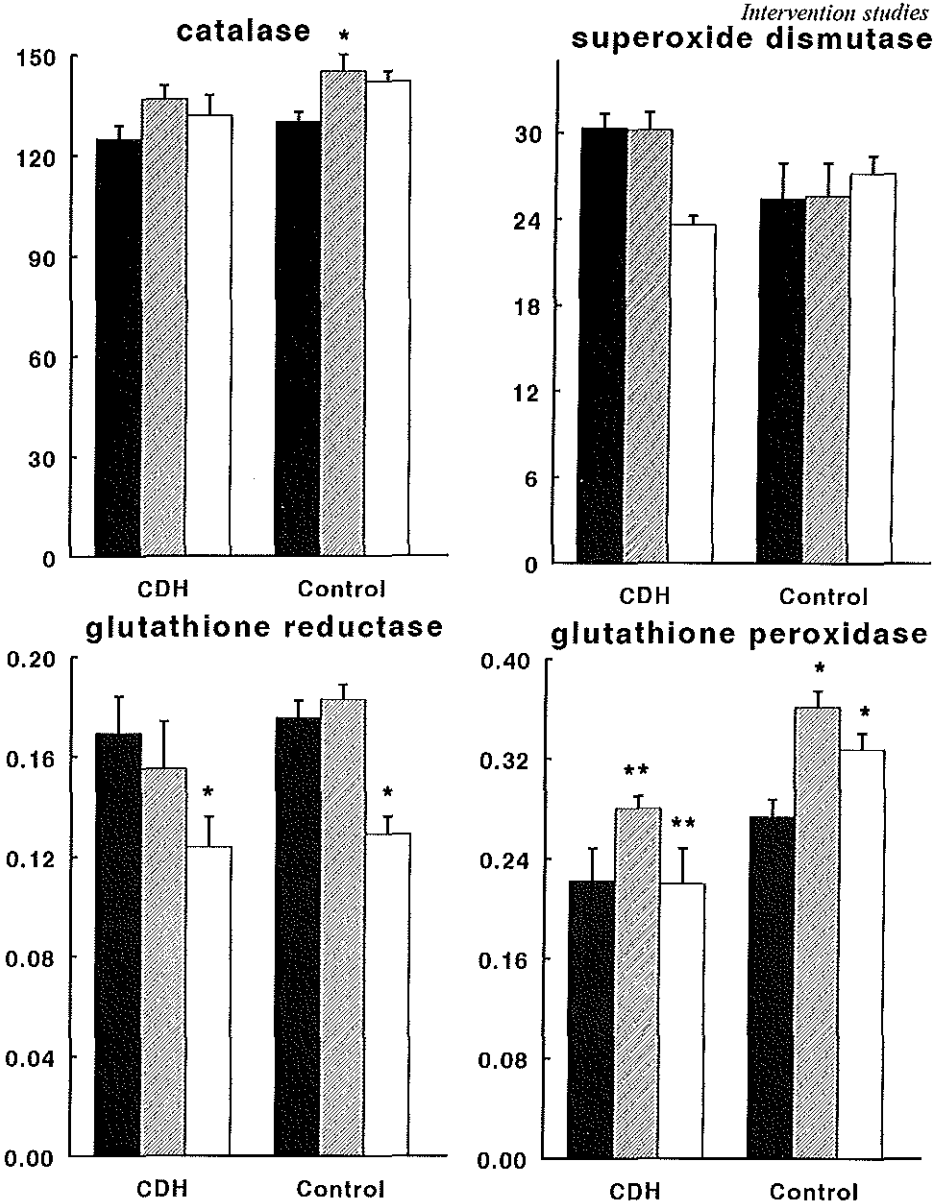

*Figure 3: Antioxidant enzyme activity after birth in lungs of newborn rats with CDH following prenatal hormonal modulation. All data expressed as IU per mg DNA (mean and SEM). Per group 5-6 animals were studied. Sham treatment: black bar; DEX-treatment: striped bar; DEX+TRH-treatment: white bar. * significant difference compared with all other treatments in the same group, $p < 0.05$; ** significant difference compared with the same treatment in controls, $p < 0.05$.*

9.2.5 Discussion

In this study we evaluated the effects of prenatal hormonal treatment on a number of parameters in a rat model of CDH. The major findings after artificial ventilation were that the survival rate was not influenced by hormonal therapy and that an increase in airspace volume without changes in the total internal surface area was observed after prenatal DEX, with stronger effects after the addition of TRH. In addition, it is noteworthy that prenatal DEX+TRH resulted in decreased glutathione reductase activity in CDH rat pups and controls at birth. Hormonal treatment led to a significantly higher glutathione peroxidase activity in controls, but not in CDH.

In the present study we confirmed the finding that prenatal hormonal modulation reduces birth weight, both in healthy, term born rats and in CDH rats[2,3,4,14,16], and found no effect on lung-body weight ratios and lung protein-DNA ratios. That prenatal hormonal therapy has no effect on lung-body weight ratios and protein-DNA ratios suggests that lung growth is reduced to the same extent as body weight, which is in agreement with earlier studies.[2,14]

The effects of prenatal hormonal therapy on antioxidant enzyme activity at birth have previously been studied in fullterm, spontaneously born, healthy rats.[2-4] Prenatal dexamethasone administered 48 and 24 hours before the expected time of delivery did not change the activities of catalase, glutathione peroxidase, and superoxide dismutase.[2,3] We found that in control pups the activities of catalase and glutathione peroxidase increased significantly after prenatal treatment with dexamethasone 72 and 48 hours before delivery by cesarean section. Our timing and dosage of dexamethasone administration, which was based on a previous experiment in rat pups with CDH[16] may probably explain the differences compared with other studies.

Rodriquez and coworkers reported in fullterm rats a negative effect of prenatal dexamethasone in combination with TRH on the activities of catalase, glutathione peroxidase, and superoxide dismutase compared with sham-treated rat pups.[4] Our observation that only the activity of glutathione reductase decreased after prenatal DEX plus TRH is not in accordance with their findings. The dosage and timing of TRH was similar in both studies, and it is not clear whether the difference in dexamethasone administration may explain the dissimilarity. However, both their study and ours showed negative effects of prenatal dexamethasone in combination with TRH on antioxidant enzyme activity.

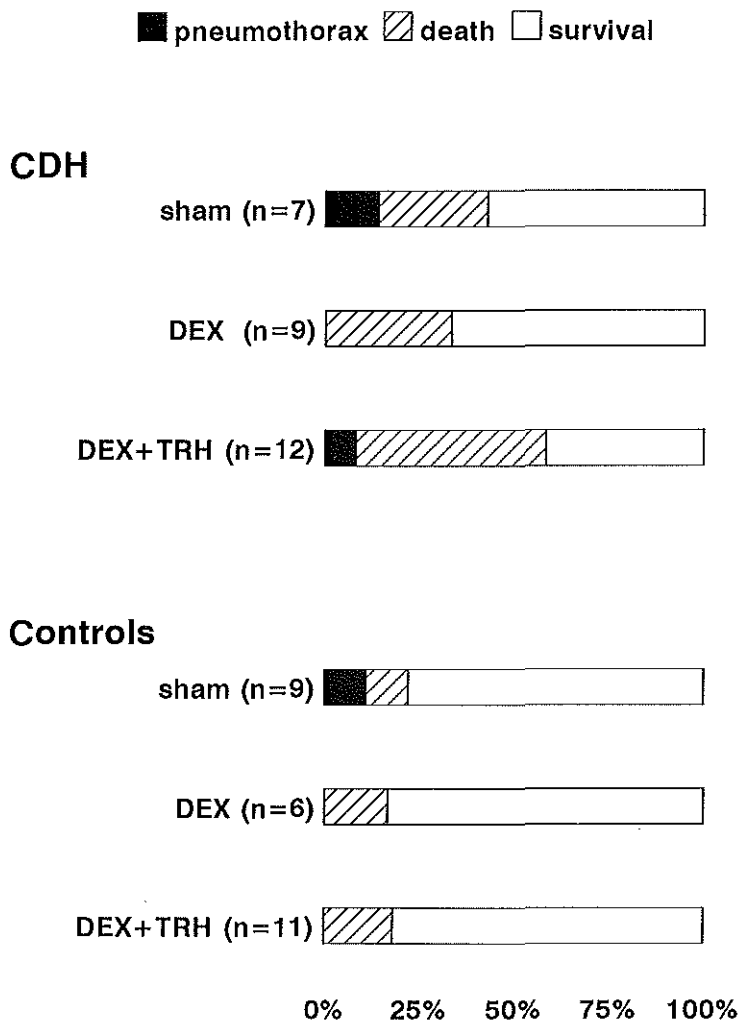

Figure 4: *The incidence of pneumothorax and mortality in ventilated rat pups with CDH and controls after prenatal hormonal modulation. The number of animals that died from pneumothorax or by other causes (indicated as 'death') and the survivors after 4 hours of ventilation are indicated for each group. No significant differences were found. DEX = dexamethasone i.p. 72 and 48 h before delivery; DEX+TRH = addition of TRH i.p. during 48 h before delivery.*

A lower activity of glutathione reductase may result in a lower production rate of reduced glutathione, which is the substrate for the reduction of organic peroxides and hydrogen peroxide, a process catalyzed by glutathione peroxidase. Catalase can convert hydrogen peroxide to water, but is not able to catalyze the reduction of organic peroxides. The decreased activity of glutathione reductase after prenatal treatment with dexamethasone and TRH, may thus result in accumulation of organic peroxides.[25]

In the present study up to 75% of CDH pups in the groups with prenatal hormonal therapy died during the procedure of intubation. This may be due to their low birth weights and not to structural changes induced by prenatal hormonal therapy, because controls, who all had a birth weight of more than 4 grams, could be intubated easily irrespective of the prenatal hormonal therapy. Although the number of ventilated newborns studied was small, the results of the present study indicate that prenatal glucocorticoids and TRH did not affect the mortality rate or incidence of pneumothorax. Previous studies in fullterm, healthy rat pups exposed to hyperoxia showed that dexamethasone improves survival[2], whereas TRH or the combination of dexamethasone and TRH have a negative effect on the survival rate.[26] However, these effects in non-ventilated rat pups were observed after at least one week.

Lung morphometry showed higher average airspace volume in DEX-treated and DEX+TRH-treated CDH pups than in the sham-CDH group, which is in accordance with previous findings in non-ventilated CDH rats.[16] Artificial ventilation may explain that the average airspace volume in the present study was three times higher than that in lungs of non-ventilated pups.[16] In neonatal rats and lambs with CDH prenatal treatment with glucocorticoids significantly improved lung compliance.[14,15] The addition of TRH to dexamethasone further improved alveolar stability and lung morphology in non-ventilated rat pups with CDH.[14] The increased lung compliance may result in an easier dilatation of airspaces and thus explain the observed higher average airspace volume. These beneficial effects on lung expansion may have implications for the treatment of CDH patients: ventilatory requirements may improve as soon as the lungs have been opened up well.

The volume fraction of airspaces and the total internal surface area did not change after prenatal hormonal therapy, as a result of a lower total number of airspaces in the DEX-treated and DEX+TRH-treated CDH rats.

Table 4: *Morphometric analysis of lungs after 4 hours of artificial ventilation in newborn rats with CDH; the effect of prenatal hormonal modulation*

	Sham (n=7)	DEX (n=9)	DEX+TRH (n=5)
Volume fraction of airspaces	0.63±0.01	0.61±0.02	0.62±0.01
Volume fraction of airspace walls	0.093±0.003	0.113±0.007*	0.125±0.007†
Volume fraction of airways	0.089±0.009	0.123±0.012*	0.142±0.011†
Volume fraction of other elements	0.187±0.009	0.156±0.008*	0.118±0.009‡,§
Average airspace volume ($\mu m^3 x10^5$)	3.06±0.12	4.53±0.30†	5.74±0.48‡,§
Total no. of airspaces ($x10^5$)	4.80±0.16	3.31±0.14‡	2.81±0.29‡
No. of airspaces/unit volume (μm^{-3})	2.11±0.07	1.45±0.06‡	1.23±0.12‡
Total internal surface area ($\mu m^2 x10^{10}$)	3.60±0.21	3.71±0.20	3.73±0.17
Inter-airspace distance (μm)	16.8±0.75	16.1±0.98	15.4±0.60
Radial saccular count	4.45±0.12	4.06±0.08†	4.45±0.08§

*Data shown as mean ± SEM; the number of animals per group is indicated. DEX = dexamethasone i.p. 72 and 48 h before delivery; DEX+TRH = addition of TRH i.p. during 48 h before delivery; * $p < 0.05$; † $p < 0.002$; ‡ $p = 0.0001$ compared with sham-treated CDH; § $p \leq 0.01$ compared with DEX-treated CDH, by Bonferroni/Dunn.*

The unchanged internal surface area together with the increase in volume fraction of airspace walls is consistent with the premise that prenatal hormonal therapy accelerates lung maturation, but not lung growth.[16] Larger airspaces with a decreased number of alveoli have been found in premature rhesus monkey fetuses following prenatal treatment with betamethasone.[27] In addition, a similar finding has been ascribed to inhibited septation in rats at the age of 28 days following exposure to hyperoxia in combination with postnatal dexamethasone administration.[28] Because septation in the rat starts around the third or fourth postnatal day[29] it seems unlikely that inhibited septation explains the present findings.

In conclusion, prenatal dexamethasone had a positive effect on the average airspace volume in ventilated newborn rats with CDH, and only a small synergistic effect was observed after the addition of TRH. However, the activity of glutathione reductase decreased significantly after prenatal treatment with DEX and TRH. We speculate that our findings of hormonal therapy in rat pups extend to CDH patients as well. If that hypothesis is correct, antenatal administration of dexamethasone as a monotherapy will offer better prospects for randomized trials in prenatally diagnosed children with CDH than the combination of dexamethasone and TRH.

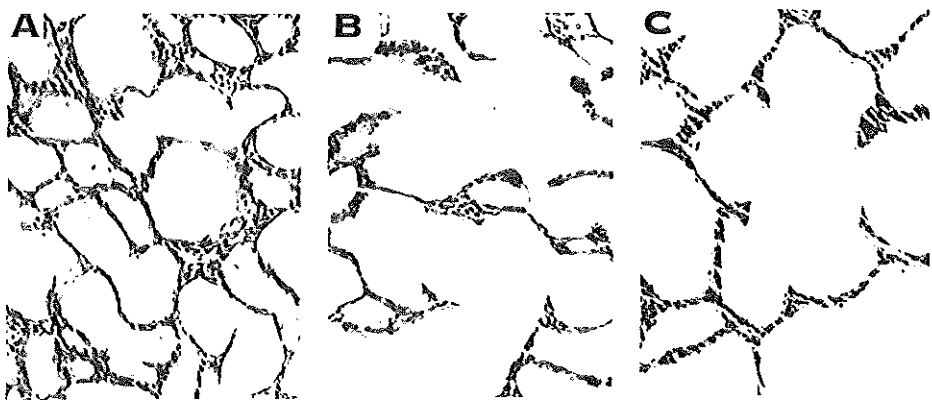

Figure 5: Representative pictures from lungs of CDH rats following 4 hours of artificial ventilation. The lungs were inflated at a constant pressure of 20 cm H_2O with 10% formalin. A: sham-treated CDH; B: DEX-treated CDH; C: DEX+TRH-treated CDH.

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Part IV

General discussion and summary

Chapter 10

General discussion and directions for future research

10.1 Introduction

The most important aims of the studies presented in this thesis were:

1. to study the surfactant status in the lungs of CDH patients;
2. to determine the expression of pulmonary neuroendocrine cells in CDH lungs;
3. to evaluate the role of eicosanoids in the pathogenesis of PPHN in CDH;
4. to study the long-term pulmonary sequelae of CDH patients and compare those with matched controls;
5. to measure antioxidant enzyme activities during artificial ventilation in CDH;
6. to study the distribution of exogenous surfactant in the hypoplastic lungs in CDH;
7. to evaluate the effects of prenatal hormonal therapy on antioxidant enzyme activity and lung histology after artificial ventilation.

This chapter discusses the way in which the results could be interpreted, as well as their implications and limitations. A section reviewing the management of CDH patients focuses on new developments. The chapter is concluded with directions for future research.

10.2 Interpretation and implications of the studies

We found no evidence of surfactant deficiency in CDH patients. However, increased vascular leakage during artificial ventilation may lead to higher concentrations of proteins in the alveoli, which in their turn may inactivate surfactant.[1]

Our data indicate that the hypoplastic lungs in rats with CDH show retarded differentiation. The expression of CGRP-positive pulmonary neuroendocrine cells is decreased in CDH during the late pseudoglandular, early canalicular stage of lung

development. This observation is in accordance with a previous study of Brandsma and coworkers.[2] However, some of our findings suggest that at birth developmental retardation is maximally compensated in CDH: 1. both in rat lungs and in lungs of infants with CDH the expression of pulmonary neuroendocrine cells is increased compared with lungs of controls; 2. no evidence of primary surfactant deficiency is found in BAL fluid of CDH patients; 3. the antioxidant enzyme (AOE) activity at birth is similar or even higher in lungs of CDH pups. Studies by others confirm these observations.[3-5]

Moreover, both in lungs of perinatal rats with CDH, and in BAL fluid of infants with CDH who died within a few hours after birth and in those who died from recurrent episodes of therapy-resistent pulmonary hypertension after decanulation from ECMO, we found high levels of 6-keto-$PGF_{1\alpha}$, the stable metabolite of the pulmonary vasodilator prostacyclin. Calcitonin gene-related peptide, a neuropeptide that shows increased expression in pulmonary neuroendocrine cells in lungs of fullterm rats with CDH, may not only act as a growth factor[6], but is also known as a vasodilator.[7] It can be speculated that the lungs try to compensate maximally for pulmonary vasoconstriction in CDH. This may also explain why some patients with CDH and PPHN fail to respond to therapy with intravenous prostacyclin.[8] The same explanation may be true for non-responders to inhaled nitric oxide (NO) shortly after birth.[9]

The long-term pulmonary sequelae in CDH patients and in matched controls without CDH revealed peripheral airway obstruction and increased airway responsiveness to inhalation of methacholine in both groups. These data show that artificial ventilation in the neonatal period contributes to the pulmonary sequelae in CDH patients.

The AOE activity in CDH lungs is not impaired at birth, but our observations suggest that it may fail during prolonged artificial ventilation with 100% O_2. Prenatal hormonal therapy with dexamethasone and TRH showed a lower activity of glutathione reductase at birth in CDH rats and in controls. We assume that a failing AOE system in CDH may contribute to the lungs' susceptibility to injury from artificial ventilation, especially after prenatal treatment with TRH. Prenatal hormonal therapy with dexamethasone did not impair the AOE activity, and the positive effects of this treatment on lung compliance and lung morphology may be helpful to prevent severe lung damage.

It can be assumed that exogenous surfactant especially reaches the contralateral, less hypoplastic lung in CDH. We demonstrated in rats with CDH that surfactant spreads evenly over both lungs, suggesting that in those cases where exogenous

surfactant is considered, it can be applied without inducing the risk of differences in expansion between both lungs and resulting pneumothorax.

10.3 Limitations of the studies

10.3.1 Limitations of the human studies

Three different kinds of studies were conducted in humans. First, ventilated CDH patients and age-matched controls underwent bronchoalveolar lavages during the course of treatment and different parameters in the recovered fluid were measured. Second, lung sections of autopsied CDH patients were studied immunohistochemically to evaluate the expression of bombesin-positive pulmonary neuroendocrine cells. Data were compared with those of control patients and those of infants who died from lung hypoplasia due to other causes. Third, children who had been operated on for CDH in the neonatal period and controls without CDH who suffered from neonatal respiratory insufficiency were recruited to perform lung function tests at ages ranging from 7 to 18 years.

The BAL procedure that was performed in ventilated patients was a blind procedure: the catheter was introduced via the endotracheal tube without the aid of a bronchoscope, and its position was not determined. However, this technique has been validated by Grigg and coworkers; they showed that the catheter entered the right main bronchus when the patient's head was turned left during the procedure. They also showed that the procedure itself is safe.[10] However, in our study some CDH patients were in a critical condition during the first days of treatment and it was not always possible to perform BAL at regular time points. Therefore, it was difficult to evaluate different parameters during the complete course of treatment. Also related to the procedure is the small amount of fluid that could be recovered. Not all parameters that we wanted to determine could be measured in each sample, and this resulted in missing data. Interpreting our results and comparing them between different study groups was, therefore, not easy.

Another limitation of this study was the validity of the control group. To determine whether the concentrations of the various parameters were abnormal in CDH, they had to be compared with data from control patients. We decided to study infants who were selected for the best possible match for gestational age and birth weight without CDH and without pulmonary abnormalities. Most controls were, therefore, infants with other congenital anomalies, such as esophageal atresia

and meconium peritonitis, who were ventilated perioperatively. However, it is debatable whether they could be considered true controls. On the one hand, infants who are not ventilated at all could be considered the best controls, but studying such a control group is obviously out of the question on ethical grounds. On the other hand, to study the specific features of CDH it may be useful to compare data of CDH patients with those of ventilated controls who have similar ventilatory requirements. Unfortunately, during a study period of several years, we were not able to recruit such a group of control patients. For CDH patients who were treated with ECMO it was even more difficult to find suitable control patients. We performed BAL in a group of infants without CDH who were treated with ECMO, but most of these infants suffered from diseases which may have influenced the surfactant status and the inflammatory parameters, apart from the effects of fluid extravasation caused by the ECMO procedure itself.

To study the expression of pulmonary neuroendocrine cells the airway epithelium needs to be well preserved. Several factors could adversely influence the epithelial preservation: artificial ventilation with high peak inspiratory pressures and high oxygen fraction leads to severe epithelial damage within a few hours; autolysis of lung tissue might occur when autopsy is performed a long time after the patient has died, and it is also frequently seen in tissue obtained from abortions. In addition, methodological factors, such a the fixative that has been used to preserve the lungs, are also important for the immunohistochemical procedure. Most infants with CDH died after a period of prolonged artificial ventilation and the lung tissue was severely damaged in most of these cases; the same held true for some patients without CDH who died from lung hypoplasia. We were able to select lung tissue samples from ten CDH patients and seven lung hypoplasia cases without CDH. Most of these infants died within two hours after birth, because they could not be ventilated adequately. Consequently, a kind of selection bias may inevitably have occurred. Furthermore, it would have been interesting to study the expression of bombesin-positive pulmonary neuroendocrine cells in the lungs of CDH patients at different stages of lung development. Unfortunately, it was not possible to find suitable autopsy material with well preserved airway epithelium for this experiment.

The immunohistochemical and morphometric techniques were performed in the Department of Pathology in the Hospital for Sick Children in Toronto, Canada, and have been well established by Cutz and coworkers.[11] The morphometric analysis was performed using a projection microscope and drawings. Because this work is very labor-intensive only a few slides per patient were studied. With the recently

developed advanced morphometric techniques it will be possible to study the expression of pulmonary neuroendocrine cells in more detail.

To evaluate the long-term pulmonary sequelae in CDH patients we performed lung function tests and bronchoprovocation tests in children born between 1975 and 1986 who had been operated on for CDH in the neonatal period. Most patients had been ventilated perioperatively. To determine the possible long-term effects of artificial ventilation on lung function, we studied a group of age-matched control patients who were selected for gestational age, birth weight, duration of artificial ventilation, duration of supplemental oxygen therapy, and sex. All children were studied once, and it is therefore not possible to draw any conclusions regarding the course of the lung function within time. One of the most characteristic long-term abnormalities that has been described in the literature is the decreased perfusion, especially in the ipsilateral lung, in CDH patients.[12,13] Our findings of a normal diffusion capacity suggest that the lung surface area available for diffusion is normal. However, we only tested our children under resting conditions and it may be that oxygen desaturation occurs only during exercise. Most ideally, diffusion capacity should be measured during exercise, but this might raise practical problems.

10.3.2 Limitations of the experimental studies

For all experimental studies we used the rat model of CDH. The characteristics of this model and the main advantages and disadvantages have been discussed in Chapter 1. We performed immunohistochemical studies to evaluate the expression of pulmonary neuroendocrine cells during lung development in CDH. The rat model is suitable to study different developmental stages. One of the disadvantages of this animal species is that only a few neuropeptides can be studied among which calcitonin gene-related peptide (CGRP). Other peptides such as serotonin and bombesin can not be studied immunohistochemically in rat lungs.[14] The limitations with respect to the morphometrical analysis, which have been discussed in the previous paragraph, are also applicable to the studies in the rat model.

The most important limitation of the CDH rat model is the small size of the neonatal rats. It is possible to intubate and ventilate them for a short period of time, but it is very difficult to obtain reliable data regarding their respiratory and metabolic status. This, and the fact that the experimental equipment was not suitable to ventilate the pups individually, may explain that we had to restrict the duration of our studies to six hours. Pilot experiments revealed that blood gas

analyses can be done only when the animal is sacrificed. Therefore, the methods to evaluate new treatments, such as exogenous surfactant therapy and prenatal hormonal modulation, are limited.

The severe lung hypoplasia in rat pups with CDH results in respiratory insufficiency directly after birth. We did not find any changes in the eicosanoid levels in lungs of rat pups during transition from intrauterine to extrauterine life, and this may be explained by their insufficient respiration at birth. The respiratory problems in rat pups with CDH is comparable to the situation in infants with CDH who need ventilatory support within six hours after birth, the so-called high-risk CDH patients.[15] Therefore, the rat model may be useful to study histological and biochemical aspects of CDH.

10.4 Management of CDH: new treatment modalities and future directions

The prenatal and initial postnatal management of patients with CDH has been extensively described by Weinstein and Stolar.[16] Other studies have brought to light that the following clinical parameters are associated with a poor prognosis: the presence of other anomalies — especially cardiac defects — , premature delivery, polyhydramnios, severe lung hypoplasia as assessed by preoperative measurements of the functional residual capacity, and preoperative arterial pCO_2 > 40 mm Hg in correlation with a high ventilation index.[17-22]

The mortality rate in CDH is still more than 50%[16-18,23], despite new concepts for managing CDH. These are discussed by Tibboel and coworkers and include: prenatal diagnosis followed by in utero repair of the diaphragmatic defect, delayed postnatal surgery, alternative ways of oxygenation such as extracorporeal membrane oxygenation (ECMO) and high frequency oscillatory ventilation, modulation of the pulmonary vascular tone, and prenatal modulation of lung growth.[24] This overview will highlight some recent developments.

10.4.1 Therapy focusing on the lung hypoplasia and immaturity in CDH

Fetal surgery consisting of the in-utero repair of the diaphragmatic hernia has been studied extensively in lambs: improvement of lung volume and pulmonary arteriolar muscle hyperplasia, and a normal pulmonary arterial tree were reported.[20] The first data on fetal surgery in CDH patients have been published in 1994: in a series

of 14 fetuses, five died intraoperatively, and four of the nine babies who underwent successful in-utero repair survived.[25] The results of a randomized trial will be published in the near future.

Tracheal ligation has been advocated as a new treatment modality to stimulate lung growth. In fetal lambs with surgically created CDH tracheal occlusion at the canalicular stage of lung development appeared to increase lung weight, DNA and protein content, to stimulate lung maturation, and to improve oxygenation.[26-28] Some studies describe the necessity to perform a total occlusion, e.g. using translaryngeal placement of water-impermeable polymeric foam, or a partial occlusion, which is called tracheal stenosis.[26-29] It has been suggested to use tracheal occlusion either as prenatal therapy, or as postnatal therapy in combination with ECMO. However, further studies need to be done to evaluate the effects and side-effects of postnatal tracheal occlusion on lung growth.[26]

Another therapy that can be applied prenatally is hormonal modulation with glucocorticoids and TRH. The positive effects of prenatal glucocorticoids on lung maturity in premature infants are no longer debated, but the effects of TRH still have to be elucidated.[30,31] Prenatal administration of glucocorticoids stimulates surfactant production and improves lung compliance and morphology in lambs and rats with CDH.[32,33,34] We found negative effects of combined glucocorticoids and TRH on AOE activity in neonatal rats with CDH. These observations suggest that prenatal therapy with glucocorticoids alone may be best.

Positive effects of exogenous surfactant therapy have been reported in studies with small numbers of CDH patients.[24] Recent studies in newborn lambs with CDH indicate that surfactant may improve oxygenation dramatically when administered prophylactically, but not when it is given as a rescue therapy. Therefore, exogenous surfactant should be administered as early as possible, preferably before the first breath.[35,36] However, the need to use exogenous surfactant therapy is still unclear, and our own findings of normal levels of surfactant phospholipids in BAL fluid of CDH patients contradict earlier reports of primary surfactant deficiency in CDH.

10.4.2 Therapy focusing on lowering the pulmonary vascular tone

Several drugs have been used in an attempt to decrease the pulmonary vascular tone in CDH patients with PPHN.[24] Bos and coworkers showed that intravenous administration of prostacyclin may be effective.[8] Positive effects of endotracheal

administration of prostacyclin have been reported in a few patients with PPHN, but it has never been tested in randomized controlled trials.[37]
Inhalation of NO has been tried in a few CDH patients. Henneberg and coworkers described three successfully treated CDH cases and propose that NO may be an alternative for ECMO in some cases.[38] On the other hand, Karamanoukian and coworkers reported that NO only had a positive effect in five CDH patients studied after decanulation from ECMO, but not in patients who had been treated with NO before ECMO.[9] The same group describes that in fetal lambs oxygenation could only be improved when inhaled NO was combined with exogenous surfactant.[39] North and coworkers observed in fetal rats with CDH that the gene expression of endothelial NO synthase was lower than in controls[40], and this could explain why in some patients with CDH inhalation of exogenous NO is successful. Nevertheless, as discussed in the previous paragraph, findings from studies in this thesis led to the hypothesis that the lungs in CDH compensate maximally for pulmonary vasoconstriction and that exogenous vasodilator therapy may, therefore, not be helpful.

Drugs that may be useful in the treatment of PPHN are almitrine bismesylate and bosentan.[41,42] Almitrine bismesylate is a peripheral chemoreceptor stimulant that improves oxygenation in ventilated patients with ARDS by enhancement of hypoxic pulmonary vasoconstriction. The combination of this drug together with inhalation of NO was shown to have additive effects on gas exchange[41]. Bosentan is an endothelin receptor antagonist which was shown to protect hypoxic rats from pulmonary hypertension.[42]

10.4.3 Therapies to reduce lung damage in CDH

The use of ECMO has been advocated both to prevent the lungs from being further damaged by artificial ventilation and to overcome episodes of PPHN.[24] The mean survival rate of CDH patients treated with ECMO between 1980 and 1992 is 62%, although some centers using ECMO report much higher survival rates.[16,43] Recently, a retrospective study showed that the survival rate from unstable neonates — who could not be stabilized by conventional therapy — was 0% in the pre-ECMO era and 61% in the ECMO era.[44] However, in ECMO survivors with CDH pulmonary, gastrointestinal, and neurological sequelae were frequently seen at the age of one year.[45] The UK Collaborative ECMO trial group has recently published their report on neonatal ECMO between 1993 and 1995: they showed that allocation to ECMO reduced the risk of death or severe disability in most patients, but that

the prognosis of infants with a primary diagnosis of CDH was poor, because 14 of the 18 ECMO-allocated CDH patients died.[46]

High-frequency oscillatory ventilation has been put forward as a therapy that may reduce the need for ECMO. A recent study showed that only 6 of 27 CDH patients responded to this ventilatory treatment. However, these CDH patients were infants transported from other level III neonatal intensive care units, in whom conventional ventilation had failed to produce adequate gas exchange.[47]

Partial liquid ventilation (PLV) with perfluorocarbon has only recently been tried in humans, and the first results in adults, pediatric patients, and prematures with respiratory distress syndrome are encouraging.[48,49] Lung compliance and gas exchange in lambs with CDH improved significantly during PLV.[50,51] The perivascular emphysema observed in control lambs and in those treated with PLV, is possibly caused by barotrauma developing during conventional ventilation in the first 30 minutes. It is, therefore, essential to conduct further studies in an attempt to define the ventilatory strategy that might avoid lung damage.[51] In addition, vasoactive drugs may be administered via partial liquid ventilation.[52]

A completely new concept with respect to avoidance of lung damage in CDH has been proposed by Wung and coworkers, who suggest that very long delayed surgery and a respiratory care strategy that avoids pulmonary overdistension will improve survival and reduce the necessity of ECMO.[53]

10.5 Directions for future research

We did not find evidence of a primary surfactant deficiency in CDH patients. However, our study does not allow for conclusions regarding the surfactant function. Further studies are needed to evaluate the endogenous surfactant function and the pool size, because pool sizes may be rapidly exhausted during artificial ventilation.

Altered expression of pulmonary neuroendocrine cells became apparent both in clinical and in experimental studies. To test the speculations that neuroendocrine cells have a role as oxygen sensors in CDH or that neuropeptides exert a role as pulmonary vasodilators, the rat model could be used to perform pharmacological studies on inhibition of oxygen sensors or calcitonin gene-related peptide.

Eicosanoids may be involved in persistent pulmonary hypertension in CDH, but also in inflammation caused by artificial ventilation. It would be interesting to study the effects of different ventilatory strategies and of therapeutic interventions

with pulmonary vasodilators on eicosanoid production in the future. However, it will be difficult to establish whether changes in eicosanoid concentrations are a reflection of pulmonary hypertension or of tissue damage. A prospective case-control study with control patients who are not only matched for gestational age and birth weight, but also for ventilatory requirements may be useful.

Evaluation of the long-term pulmonary sequelae is important. Most of the conducted studies so far concerned CDH patients who had not been ventilated at all, or only for a short period. To study the postnatal lung growth in CDH, follow-up should start early in infancy and continue until adulthood. Exercise testing is useful to study pulmonary abnormalities that are not apparent under resting conditions. Our data showed that children who underwent artificial ventilation in the neonatal period had mild peripheral airway obstruction and increased airway responsiveness, which was more pronounced in CDH survivors who had been ventilated for more than seven days. The effects of smoking and environmental factors, such as air pollution, have never been studied in these patients. It can be assumed that this group of patients is susceptible to develop chronic obstructive pulmonary diseases. With increasing possibilities of neonatal intensive care treatment more infants with CDH and severe lung hypoplasia will survive, and this may well lead to increased respiratory morbidity on the long term. Obviously, the same holds true for other neonatal respiratory disorders that require ventilatory support.

A preliminary report showed that infants with CDH are deficient of vitamin A.[54] It may be worthwhile to examine the status of other antioxidants, such as glutathione, in CDH patients. Further studies are needed to determine whether the AOE system fails during artificial ventilation in CDH, because several antioxidants with therapeutic potential are presently available.[55]

The controversial data regarding the occurrence of surfactant deficiency in CDH published so far suggest that a randomized trial with exogenous surfactant is not the first choice. However, this assumption may have to be changed when more data on the surfactant function or secondary surfactant deficiency become available.

Prenatal diagnosis of CDH usually occurs in the second or third trimester of gestation[18], and studies for prenatal therapy should be focused on improvement of lung maturity, morphology and compliance. From experimental studies it has become clear that prenatal glucocorticoids in CDH may improve lung maturity, compliance and postnatal gas exchange. As long as the benefit of TRH has not been clearly established in preterm deliveries, randomized trials with prenatal glucocorticoids as a monotherapy may be the most desirable in CDH patients.

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Chapter 11

Summaries

11.1 Summary

Congenital diaphragmatic hernia (CDH) is a congenital anomaly manifesting itself in about one in 3,000 births. Despite improved neonatal intensive care, the overall survival rate is still not higher than 58%, and is even less in patients with associated anomalies. Inherent to CDH are hypoplastic lungs with a lower number of airway and vascular generations and greater muscularity of the peripheral arteries. These abnormalities may lead to respiratory insufficiency shortly after birth. Artificial ventilation with high peak inspiratory pressures and high O_2 fraction is often needed. This is associated with a high incidence of bronchopulmonary dysplasia in surviving CDH patients.

This thesis consists of four parts: *Part I* (Chapter 1) is the introduction, *Part II* (Chapters 2 to 5) describes studies focusing on aspects of lung development in CDH, *Part III* (Chapters 6 to 9) consists of studies focusing on aspects of lung injury in CDH, and *Part IV* (Chapters 10 and 11) includes the general discussion and summary.

Chapter 1 introduces the subject of this thesis by reviewing the literature. First, the fundamentals of normal lung growth and development are described, followed by the characteristics of abnormal lung development in CDH. Two different types of animal studies have been developed to study the specific features of CDH: models in which CDH is surgically created and those in which CDH is induced by prenatal exposure of the animal to a teratogenic agent, called Nitrofen. Fetal lambs are mainly used for models of the surgical type, whereas fetal rats are used for the non-surgical model. The etiology and natural history of CDH are still unknown. From studies in animal models of CDH it has been suggested that the diaphragm fails to close at an early stage of gestation (5-10 weeks gestation in humans), and that migration of the abdominal viscera, especially the liver, results in competition for space with reduction of lung growth. The lungs in CDH are not only hypoplastic, but differentiation is retarded as well. This may lead to deficiency of surfactant, although this is still debatable. The normal transition from intrauterine to extrauterine life does not occur in CDH, and many CDH patients suffer from

persistent pulmonary hypertension of the newborn (PPHN). Both structural and functional abnormalities of the pulmonary vasculature may be involved. At the end of this chapter six different aspects are mentioned that still have to be elucidated in CDH. The chapter concludes with the aims of the studies that are presented in this thesis.

The study described in *Chapter 2* was performed in 18 CDH patients and in 20 infants without CDH. Four different groups were studied: conventionally ventilated CDH patients (CDH-CV) n=13, ECMO-treated CDH patients (CDH-ECMO) n=5, conventionally ventilated age-matched controls who were ventilated perioperatively (C-CV) n=14, and ECMO-treated infants without CDH (C-ECMO) n=6. During the course of treatment bronchoalveolar lavage (BAL) was performed using a blind, standardized technique. The concentrations of phosphatidylcholine (PC), the major component of surfactant, phosphatidylglycerol (PG), and sphingomyelin (S) were measured in BAL fluid using thin-layer chromatography and a phosphorus assay. The ratio of PC to S (the l/s ratio) was calculated. In addition, the fatty acid composition of PC was studied by gaschromatography. The median concentrations of PC and PG, and the l/s ratios did not differ significantly between of the groups. The median percentage of palmitic acid in PC was slightly, but significantly lower in conventionally ventilated CDH patients compared with controls, 68% versus 73%, respectively ($p < 0.001$). In the conventionally ventilated groups there were no significant correlations between ventilatory parameters and the above-mentioned surfactant components in BAL fluid. We concluded that primary surfactant deficiency is an unlikely phenomenon in infants with CDH and possibly does not determine the clinical course in these patients. However, further studies may be necessary to confirm our findings.

Chapter 3 describes the expression of bombesin-immunoreactive PNEC and NEB in lungs of CDH patients, newborns with lung hypoplasia due to other causes, and controls without lung abnormalities. Bombesin is a neuropeptide produced by PNEC which has growth factor-like properties involved in lung development. The lungs of controls and infants with lung hypoplasia due to other causes did not differ with respect to bombesin-immunoreactivity. The mean NEB size was significantly increased in infants with CDH compared to both other groups. Some CDH cases with large NEBs also showed a high percentage of immunostained epithelium. We concluded that in lungs of CDH patients bombesin-immunoreactivity in PNEC and NEB differs from that of infants with lung hypoplasia due to other causes and controls. The increased bombesin-immunoreactivity observed in some

cases with CDH may reflect a compensatory mechanism related to impaired lung development and/or failure of neuropeptide secretion during neonatal adaptation.

In *Chapter 4* two studies on the expression of pulmonary neuroendocrine cells (PNEC) in the developing lung of Nitrofen-induced CDH are described. Amine and peptide producing PNEC are thought to be involved in lung development and in regulation of the pulmonary vascular tone. One of the peptides produced by PNEC is calcitonin gene-related peptide (CGRP). In the first study we report data on morphometric analysis of CGRP-immunoreactive PNEC clusters (neuroepithelial bodies, NEB) in lungs of term rats with CDH. The number of CGRP-immunostained NEB per surface area of lung parenchyma in CDH was significantly increased compared to that of controls. We speculated that this phenomenon may result in altered NEB function including imbalance in vasoactive mediators. The second study was performed to investigate the developmental pattern of CGRP-positive cells in lungs of fetal rats with CDH during different stages of lung development. The lungs of rats with and without CDH were examined on gestational day 16, 18, 20, and 22 (term), and immunostained with CGRP. We found that the CGRP-expression was negative on Day 16 in CDH and control lungs, whereas on Day 18 CGRP-positive immunostaining was observed in all lungs of controls, but in lungs of some CDH pups only. On Day 20 CGRP-immunoreactivity was similar in CDH and control lungs, and on Day 22 a higher proportion of CGRP-positive cells was found in CDH lungs. Supraoptimal dilution immunocytochemistry, applied to quantify the intracellular CGRP level on Day 22, yielded similar results in CDH and controls. We concluded that CGRP-expression in PNEC and NEB is delayed in CDH during the early stages of lung development. Because CGRP also exhibits growth factor-like properties on endothelium and epithelial cells, the lack of this factor during the canalicular stage of lung development may be causally related to lung hypoplasia. Whether the higher proportion of CGRP-positive cells in CDH observed at term reflects abnormal functioning of these cells remains to be determined.

In *Chapter 5* we report levels of several eicosanoids in BAL fluid and lung homogenates of rats with CDH. We hypothesized that a dysbalance of vasoconstrictive and vasodilatory eicosanoids is involved in persistent pulmonary hypertension (PPH) in CDH patients. Lungs of CDH rats and controls were studied after cesarean section or spontaneous birth. In lungs of controls, but not in those of CDH pups, the concentrations of 6-keto-$PGF_{1\alpha}$ — the stable metabolite of the pulmonary vasodilator prostacyclin —, thromboxane B_2 (TxB_2) — the stable metabolite of the pulmonary vasoconstrictor TxA_2 —, prostaglandin E_2, and

leukotriene B_4 decreased after spontaneous birth. The lungs of CDH pups, who showed respiratory insufficiency directly after birth, had higher levels of 6-keto-$PGF_{1\alpha}$ than those of controls. These observations indicate that in CDH abnormal lung eicosanoid levels are present perinatally. We speculated that the elevated levels of 6-keto-$PGF_{1\alpha}$ may reflect a compensation mechanism for increased vascular resistance at birth.

The study described in *Chapter 6* was performed to analyze whether vasoactive prostanoids which may be involved in the pathogenesis of PPH in infants with CDH have increased levels in BAL fluid. The correlations between prostanoid levels and various inflammatory parameters were also studied, because inflammation induces production of prostanoids. The concentrations of 6-keto-$PGF_{1\alpha}$, TxB_2, protein, albumin, total cell count, and elastase-α_1-proteinase-inhibitor complex were measured in BAL fluid of the following three groups which were described in *Chapter 2*: CDH-CV, CDH-ECMO, and C-CV. All CDH-ECMO patients and six CDH-CV patients suffered from PPH, whereas none of the C-CV patients had PPH. In CDH patients with PPH different prostanoid concentrations were measured: infants who died, or those who needed ventilatory support for a period of more than four weeks had either high levels of both 6-keto-$PGF_{1\alpha}$ and TxB_2 compared to controls, or high levels of 6-keto-$PGF_{1\alpha}$ only. The concentrations of TxB_2 correlated positively with both albumin and protein in BAL fluid of all CDH patients, and with the cell counts in CDH-ECMO patients. We concluded that in some patients with PPH high prostanoid concentrations in BAL fluid were associated with death, either within a few hours after birth, or from recurrent episodes of therapy-resistant pulmonary hypertension several days after ECMO.

The long-term pulmonary sequelae of CDH are reported in *Chapter 7*. A group of 40 CDH patients aged 7 to 18 years (median 11.7 years) and 65 age-matched controls without CDH and lung hypoplasia who underwent similar neonatal treatment were studied. Mild airway obstruction was found in both groups with more peripheral airway obstruction in CDH patients than in controls. Both groups had normal total lung capacity and diffusion capacity. Increased airway responsiveness to methacholine was common but bronchoconstriction to inhaled metabisulfite was rare both in CDH and controls. We concluded that this group of CDH patients has minor lung function impairment. Mild airway obstruction and increased airway responsiveness to inhaled methacholine but not to metabisulfite suggest that structural changes in distal airways are involved and not autonomic nerve dysfunction. We speculated that both artificial ventilation in the neonatal period and

residual lung hypoplasia are important determinants of persisting lung function abnormalities in CDH patients.

Chapter 8 describes the antioxidant enzyme (AOE) activities in lungs of neonatal rats with CDH and controls during six hours of artificial ventilation. Spontaneously born, term rat pups were intubated directly after birth and ventilated with 100% O_2, PIP 25 cm H_2O (reduced to 17 cm H_2O after 30 minutes), PEEP 3 cm H_2O, and frequency 40/minute. The activities of catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase were measured at birth and after 2, 4, and 6 hours of ventilation. At birth CDH pups had a significantly higher catalase activity per mg DNA than controls. The responses of catalase and superoxide dismutase activities to ventilation with 100% O_2 differed between CDH and control pups. An early, transiently increase in AOE activity was observed in CDH, which was only significant for superoxide dismutase at t=2 hours, whereas the AOE activity increased more slowly in controls with maximal activities after six hours. We concluded that in lungs of CDH rats the profiles of AOE activity during six hours of ventilation differ from those of controls. We speculated that an altered response of AOE activity in CDH contributes to the initiation of pulmonary O_2 toxicity.

In *Chapter 9* two different intervention studies performed in the rat model of CDH are described. In the first study we examined the distribution of exogenous surfactant. We assumed that surfactant would be distributed unevenly, with less amounts deposited in the ipsilateral, most hypoplastic lung. Term rat pups with and without CDH were ventilated in a standardized way for one hour (the settings were similar to those described in *Chapter 8*). Surfactant mixed with colored microspheres was administered endotracheally (dose 25 mg/ml; 50 µl). The number of microspheres in the lungs was measured by spectrophotometry. Exogenous surfactant was deposited in both lungs in CDH. These observations indicate that exogenous surfactant can be applied to infants with CDH without the risk that major differences occur in the expansion of both lungs, resulting in pneumothorax.

For the second intervention study dexamethasone and thyroid-releasing hormone (TRH) were administered to pregnant Nitrofen-exposed and control rats. A cesarean section was performed at term and in the lungs of the rat pups we studied the AOE activity at birth and morphometry after four hours of ventilation with 100% oxygen. At birth CDH pups exposed to the combination of dexamethasone and TRH had lower activity of glutathione reductase than sham-treated pups. The survival during ventilation was not influenced by prenatal hormonal therapy. Lung morphometry showed an increase of the average airspace volume in

dexamethasone-treated CDH pups, with a small synergistic effect after addition of TRH. On the basis of our findings we speculated that prenatal administration of dexamethasone as a monotherapy is the best choice to improve lung maturity and airspace volume in CDH patients without the risk to impair the AOE activity.

In conclusion, some of our findings suggest that at birth developmental retardation is maximally compensated in CDH: 1. both in rat lungs and in lungs of infants with CDH an higher expression of pulmonary neuroendocrine cells is observed as compared to control lungs; 2. no evidence of primary surfactant deficiency is found in BAL fluid of CDH patients; 3. the antioxidant enzyme (AOE) activity at birth is similar or even higher in lungs of CDH pups. Moreover, high levels of 6-keto-$PGF_{1\alpha}$, the stable metabolite of the pulmonary vasodilator prostacyclin were observed in perinatal rat pups with CDH and in infants with CDH who died shortly after birth, or from recurrent episodes of therapy-resistant PPH several days after decanulation from ECMO. Calcitonin gene-related peptide, a neuropeptide that shows increased expression in pulmonary neuroendocrine cells in lungs of fullterm rats with CDH, may not only act as a growth factor, but is also known as a vasodilator. From these observations it can be speculated that the lungs try to compensate maximally for pulmonary vasoconstriction in CDH. The lungs in CDH may be prone to injury by O_2 free radicals due to a failing antioxidant enzyme system during artificial ventilation. Both residual lung hypoplasia and the effects of artificial ventilation in the neonatal period may be responsible for long-term pulmonary sequelae in children with CDH.

11.2 Samenvatting

Congenitale hernia diaphragmatica (CHD) is een aangeboren afwijking van het middenrif die voorkomt bij ongeveer 1 per 3000 pasgeborenen. Ondanks het feit dat de intensive care voor pasgeborenen de laatste jaren een enorme ontwikkeling heeft doorgemaakt en het aantal mogelijkheden voor behandeling sterk is toegenomen, overleeft nog steeds slechts gemiddeld 58% van de pasgeborenen met CHD. Dit percentage is nog lager, wanneer naast CHD andere aangeboren afwijkingen — zoals hartafwijkingen — aanwezig zijn. Bij kinderen met CHD is sprake van een onvolledig aangelegd middenrif en in aanleg te kleine longen (longhypoplasie): het aantal vertakkingen van de luchtwegen en van de bloedvaten in de longen is sterk verminderd, evenals het aantal longblaasjes die voor de gaswisseling zorgen. Daarnaast zijn de bloedvaten in de longen afwijkend van

structuur: de spierlaag rond de bloedvaten is verdikt en hele kleine bloedvaatjes, die normaal alleen een elastische buitenlaag hebben, zijn eveneens omgeven door spierweefsel. Deze vaatafwijkingen kunnen leiden tot een verhoogde bloeddruk in longvaten (pulmonale hypertensie). De longhypoplasie en de pulmonale hypertensie veroorzaken vaak kort na de geboorte ademhalingsmoeilijkheden. Om toch voldoende zuurstof in het bloed te krijgen is dan kunstmatige beademing met hoge drukken en met een hoge concentratie extra zuurstof nodig. Hierdoor kunnen de longen beschadigd raken, hetgeen weer nare gevolgen voor de lange termijn met zich meebrengt. De behandeling bestaat uit het sluiten van het gat in het middenrif tijdens een operatie, die tegenwoordig pas wordt uitgevoerd na een aantal dagen, wanneer de toestand van een kind met CHD enigzins gestabiliseerd is. Het moge echter duidelijk zijn uit het voorgaande, dat niet het defect in het middenrif, maar met name de ernst van de longhypoplasie en de pulmonale hypertensie van belang zijn voor de prognose.

Dit proefschrift bestaat uit vier delen. *Deel 1* (Hoofdstuk 1) is de inleiding. In *Deel 2* (Hoofdstuk 2 tot en met 5) worden een aantal studies beschreven die zijn uitgevoerd om meer inzicht te verkrijgen in de afwijkende longontwikkeling bij CHD. In *Deel 3* (Hoofdstuk 6 tot en met 9) worden de resultaten van een aantal studies beschreven, die meer gericht zijn op de longbeschadiging ten gevolge van kunstmatige beademing bij CHD. *Deel 4* (Hoofdstuk 10 en 11), ten slotte, bestaat uit een algemene discussie en de samenvatting van het proefschrift.

In *Hoofdstuk 1* wordt een overzicht van de literatuur gegeven. Allereerst worden de grondbeginselen van de normale longontwikkeling besproken, gevolgd door een overzicht van wat bekend is over de abnormale longontwikkeling bij CHD. Om de afwijkingen bij CHD goed te kunnen bestuderen zijn een aantal diermodellen ontwikkeld. Deze zijn grofweg te onderscheiden in twee groepen: een model met een chirurgisch gecreëerd defect — veelal toegepast bij lammeren — en een model waarbij gebruik wordt gemaakt van een teratogene stof, Nitrofen. Wanneer Nitrofen tijdens de zwangerschap aan een rat of muis wordt toegediend, ontstaan bij de foeten aangeboren afwijkingen waaronder CHD. Van dit laatste model bij ratten is voor een aantal studies beschreven in dit proefschrift gebruik gemaakt.

De oorzaak van CHD bij de mens is nog steeds onbekend. Uit dierexperimenteel onderzoek meent men te mogen concluderen dat vroeg in de zwangerschap (voor de tiende week bij de mens) de normale sluiting van het middenrif niet plaatsvindt. Organen uit de buikholte, zoals lever, milt en darmen, verplaatsen zich vervolgens door het gat in het middenrif naar de borstholte. Hierdoor is er dan te weinig ruimte voor de longen om uit te groeien. Er blijkt echter meer aan de hand te zijn:

ook de uitrijping van de longen is gestoord, waardoor de longen bij de geboorte onrijp zijn. Onrijpheid van de longen is een probleem dat we vooral kennen van te vroeg geboren kinderen: de longblaasjes zijn nog zeer primitief ontwikkeld en surfactant, een stof die de binnenwand van de longblaasjes bekleedt en de oppervlakte spanning verlaagt, kan in te geringe hoeveelheid aanwezig zijn. Bij de normale geboorte vindt een verandering van de bloeddruk in de longen plaats: in de baarmoeder is de weerstand in de longvaten hoog en gaat er weinig bloed doorheen; zuurstofrijk bloed wordt voor de geboorte immers uit de bloedsomloop van de moeder verkregen. Tijdens de geboorte vinden een aantal veranderingen plaats die ervoor zorgen dat de bloeddoorstroming in de longvaten kan toenemen, zodat na de geboorte zuurstof, opgenomen via de longen, naar de rest van het lichaam kan worden getransporteerd. Bij CHD verloopt deze overgang van de bloedsomloop tijdens de geboorte vaak abnormaal: zowel de afwijkingen aan de vaten zelf (de toegenomen spierlaag), als mogelijk ook de productie van een aantal stoffen die invloed kunnen uitoefenen op de weerstand van de vaten, zorgen ervoor dat de vaatweerstand onvoldoende daalt en de bloeddruk in de longvaten toeneemt. Kort samengevat zijn er dus een aantal problemen in de longen van CHD patiënten die de opname van voldoende zuurstof bemoeilijken: de longblaasjes zijn te gering in aantal en primitief ontwikkeld, en de bloeddruk in de longvaten is vaak te hoog. In de loop der jaren is veel onderzoek verricht naar deze afwijkingen, in de hoop nieuwe behandelingen te ontwikkelen, die het sterftecijfer doen dalen. Aan het einde van het hoofdstuk wordt een opsomming gegeven van een aantal deelgebieden die nog niet uitvoerig onderzocht zijn. Tenslotte worden de onderzoeksdoelen vermeld van de studies die in dit proefschrift zijn beschreven.

In *Hoofdstuk 2* wordt een beschrijving gegeven van onderzoek dat verricht werd bij kinderen met CHD en kinderen zonder CHD die allen kunstmatig beademd werden. Doel van dit onderzoek was om te kijken of de abnormale ontwikkeling en uitrijping van de longen tot gevolg heeft, dat er te weinig surfactant in de longblaasjes aanwezig is. Daartoe werd een aantal malen een kleine hoeveelheid zout water in de longen gespoten en direct weer opgezogen. In het laboratorium werden vervolgens in het spoelsel de concentraties van een aantal vetten en vetzuren, die in surfactant zitten, gemeten. Wij vonden geen evident verschil in het gehalte van deze componenten van surfactant tussen kinderen met CHD en voldragen kinderen die om een andere reden kunstmatige beademing nodig hadden. Dit wijst er op dat kinderen met CHD waarschijnlijk geen tekort aan surfactant hebben.

Hoofdstuk 3 beschrijft de resultaten van een onderzoek dat werd verricht in longweefsel van overleden kinderen met CHD. In de bekleding van de luchtwegen

komt een bijzondere soort cellen voor, neuroendocriene cellen, die verschillende soorten eiwitten en andere stoffen produceren. Deze cellen zijn mogelijk van belang bij de longontwikkeling en bij het aanpassen van de weerstand in de bloedvaten van de longen. Bij dit onderzoek werd gekeken naar de bombesine-producerende neuroendocriene cellen. Bombesine is een eiwit dat bij de mens mogelijk een belangrijke rol speelt bij de longontwikkeling. De expressie van cellen in longweefsel van kinderen met CHD werd vergeleken met die van kinderen die ook te kleine longen hadden — bijvoorbeeld door te weinig vruchtwater tijdens de zwangerschap — en met die van kinderen met normaal ontwikkelde longen die kort na de geboorte overleden waren. We vonden dat de neuroendocriene cellen in de longen van kinderen met CHD groter waren dan die in de longen van beide andere groepen kinderen. Dit zou kunnen betekenen dat de toegenomen expressie van neuroendocriene cellen in de long specifiek met CHD samenhangt. Omdat bombesine een groeifactor is voor de long zou dit er op kunnen wijzen dat neuroendocriene cellen trachten om de groei van de in aanleg te kleine longen maximaal te stimuleren. Het zou echter ook mogelijk kunnen zijn dat de functie van deze cellen rond de geboorte ernstig gestoord is en dat onvoldoende bombesine wordt afgescheiden.

In *Hoofdstuk 4* worden twee studies beschreven die zijn uitgevoerd bij foetale ratten met CHD. Wij hebben naar het voorkomen van een speciaal soort eiwit, CGRP, in neuroendocriene cellen van de long gekeken. Dit eiwit zou mogelijk als groeifactor in rattenlongen kunnen fungeren en invloed kunnen uitoefenen op de bloedvaten. Om dit eiwit zichtbaar te maken in de longen is een speciale techniek nodig (immunohistochemie) en microscopisch onderzoek van weefselstukjes. Bij het eerste onderzoek hebben we de expressie van CGRP in pasgeboren, voldragen ratten met CHD bekeken en deze vergeleken met longweefsel van controleratten zonder CHD. We vonden meer neuroendocriene cellen met CGRP in longen van ratten met CHD, maar we konden uit deze gegevens niet afleiden of dit door de longhypoplasie wordt veroorzaakt. Daarom werd een volgend onderzoek verricht waarbij we longen van ratten met CHD en controles onderzochten op verschillende tijden van de zwangerschap. We vonden dat vroeg in de zwangerschap minder CGRP-positieve cellen in de longen van ratten met CHD aanwezig waren, en dat dit tekort in de loop van de zwangerschap werd ingehaald. Deze bevindingen wijzen er op dat het wel degelijk mogelijk is dat neuroendocriene cellen in de long een rol spelen bij de longhypoplasie bij CHD. Op grond van onze onderzoeken kunnen we geen uitspraak doen over de functie van de neuroendocriene cellen,

maar het zou goed mogelijk kunnen zijn dat een abnormale functie van deze cellen een rol speelt bij de problemen van CHD rond de geboorte.

In *Hoofdstuk 5* worden de resultaten van een onderzoek bij pasgeboren ratten met CHD beschreven. Er werd onderzocht of bepaalde stoffen (eicosanoiden) die mogelijk bij de geboorte een bijdrage leveren aan de normale veranderingen van de weerstand in de longvaten, in afwijkende hoeveelheden voorkomen bij CHD. Hiertoe werden longen van ratten met en zonder CHD die tegen het einde van de zwangerschap met behulp van een keizersnede geboren waren, en longen van ratten die spontaan geboren waren onderzocht. In deze longen werden vier verschillende eicosanoiden gemeten. We vonden dat bij controleratten de concentraties van alle vier eicosanoiden daalden na spontane geboorte, maar dit was niet het geval bij CHD. Verder vonden we in de longen van ratten met CHD een hoge concentratie van een specifieke stof, waarvan bekend is dat deze de weerstand van de bloedvaten in de longen doet dalen. We veronderstelden dat de hoge concentratie van deze stof een uiting kan zijn van een compensatiemechanisme om de hoge weerstand in de bloedvaten van de longen bij CHD te verlagen.

Hoofdstuk 6 beschrijft de resultaten van een onderzoek bij beademde kinderen met CHD. In de vloeistof, die door middel van de spoeling beschreven in Hoofdstuk 2 verkregen werd, werden de concentraties gemeten van twee verschillende eicosanoiden. We onderzochten de concentratie van een stof die de weerstand van de vaten in de longen kan verlagen en van een stof die juist een verhoging van de weerstand kan veroorzaken. In spoelsels van kinderen die veel problemen hadden van een verhoogde bloeddruk in de longvaten en die mede hierdoor kort na de geboorte of na een aantal weken overleden, vonden we hoge concentraties van de weerstandverlagende stof. De stof die de vaatweerstand kan verhogen — en waarvan we dus eigenlijk verwacht hadden dat die in hoge concentraties aanwezig zou zijn — was slechts in enkele gevallen in grote hoeveelheden aanwezig. Dit onderzoek laat dus resultaten zien die mogelijk vergelijkbaar zijn met de bevindingen in ratten met CHD (beschreven in Hoofdstuk 5), en een uiting zijn van een poging van de longen om maximaal te compenseren voor de hoge bloeddruk in de longvaten.

Hoofdstuk 7 beschrijft de resultaten van een onderzoek naar de longfunctie op de lange termijn bij 40 kinderen die als pasgeborene voor CHD behandeld werden. Om te kunnen beoordelen in hoeverre eventuele afwijkingen van de longfunctie het gevolg zijn van de afwijking zelf of van de kunstmatige beademing als pasgeborene, hebben we als controles een groep van 65 kinderen onderzocht die voldragen waren bij de geboorte — net als de CHD patiënten — en die ademhalingsmoeilijk-

heden hadden bij de geboorte, waarvoor velen van hen ook kunstmatig beademd moesten worden. Geprobeerd werd om voor elke CHD patiënt twee controle kinderen te vinden met eenzelfde zwangerschapsduur, geboortegewicht, leeftijd, duur van kunstmatige beademing en extra zuurstoftoediening, en geslacht. Voor veel, maar niet alle kinderen konden één of twee geschikte controlepatiënten gevonden worden. Onderzoek van de longfunctie liet zien dat in beide groepen kinderen een vernauwing van de luchtwegen aanwezig was. Deze vernauwing was ernstiger bij de kinderen met CHD. De totale longinhoud van de kinderen met CHD was normaal, dus de longen leken niet meer te klein te zijn. De gevoeligheid van de luchtwegen was bij veel kinderen in beide groepen toegenomen. Dat we in beide groepen kinderen afwijkingen van de longfunctie vonden wijst er op, dat de kunstmatige beademing en extra zuurstoftoediening bij de geboorte op latere leeftijd kan leiden tot luchtwegvernauwing en een toegenomen gevoeligheid van de luchtwegen. Omdat in de groep kinderen met CHD de vernauwing van de luchtwegen ernstiger was dan in die van de controles, mogen we aannemen dat dit wijst op blijvende afwijkingen in de in aanleg gestoorde longen.

In *Hoofdstuk 8* worden resultaten van een beademingsstudie bij pasgeboren ratten beschreven. In de long komen bepaalde enzymen voor die de long kunnen beschermen tegen te hoge concentraties zuurstof. Uit eerder onderzoek bij ratten met CHD was gebleken, dat een verminderde werking van één van deze enzymen bij CHD kan bijdragen tot de beschadiging van de longen tijdens kunstmatige beademing. Pasgeboren ratten met en zonder CHD werden kunstmatig beademd met 100% zuurstof en de activiteit van vier verschillende enzymen werd gemeten in longweefsel bij de geboorte en na 2, 4 en 6 uur beademing. We zagen dat in de longen van ratten zonder CHD de activiteit langzaam maar zeker toenam met maximale waarden na 6 uur. In de longen van CHD ratten nam de activiteit in de eerste uren toe, maar daalde hierna weer. Na 6 uur was de activiteit vergelijkbaar met de activiteit bij de geboorte. Dit kan er op wijzen dat de activiteit van deze enzymen bij CHD te snel uitgeput is en dat dit mogelijk een rol kan spelen bij de longbeschadiging door kunstmatige beademing bij CHD.

In *Hoofdstuk 9* worden twee studies beschreven die meer gericht zijn op behandeling van problemen bij CHD. Zoals beschreven in Hoofdstuk 2 vonden wij geen aanwijzingen voor een tekort aan surfactant. Er zijn echter ook een aantal onderzoeken gepubliceerd die erop wijzen dat er wel een tekort aan surfactant is bij CHD. Verder kan het zo zijn dat bij kunstmatige beademing de productie en de werking van surfactant verstoord worden. Het zou derhalve in een aantal gevallen nuttig kunnen zijn om extra surfactant aan de longen toe te dienen; dit is een

behandeling die veelvuldig wordt toegepast bij te vroeg geboren kinderen met een tekort aan surfactant. Bij CHD is de long aan de kant waar het defect van het middenrif zit kleiner en onrijper dan de andere long. De minst onrijpe long heeft vaak meer en beter ontwikkelde longblaasjes. De lucht zal daarom het gemakkelijkst in de best ontwikkelde long komen, dus aan de kant waar het defect niet zit. Wanneer extra surfactant wordt toegediend via de luchtwegen, zou men zich kunnen voorstellen dat deze zich het gemakkelijkst verspreidt over de best ontwikkelde long, die zich dan nog gemakkelijker laat beademen. Er kan dan een groot verschil optreden tussen de ontplooiing van beide longen, hetgeen een aantal problemen met zich meebrengt. Het doel van dit onderzoek was om te kijken of toegediend surfactant zich vooral verspreidt in de best ontwikkelde long. Hiertoe werden voldragen ratten met CHD beademd en werd surfactant gemengd met kleine gekleurde bolletjes (doorsnede 15 μm) in de luchtwegen gespoten. Vervolgens werd met een speciale techniek het longweefsel onderzocht om te kijken hoeveel bolletjes — en dus hoeveel surfactant — de verschillende delen van de longen hadden bereikt. Uit dit onderzoek bleek dat in beide longen evenveel surfactant per mg longgewicht terecht was gekomen. Wanneer behandeling van CHD patiënten met surfactant wordt overwogen, is het aannemelijk dat dit in beide longen terecht komt en dus geen grote verschillen in longontplooiing zal veroorzaken.

De tweede studie in dit hoofdstuk is gedaan om te kijken of het mogelijk is om voor de geboorte de longrijping te bevorderen. Deze behandeling kan dus alleen worden toegepast wanneer voor de geboorte bekend is dat het een kind met CHD betreft. Verschillende hormonen, zoals bijnierschorshormoon en schildklierhormoon, kunnen tegen het einde van de zwangerschap worden toegediend aan de zwangere vrouw, waardoor de longrijping van het kind bevorderd wordt. De behandeling met bijnierschorshormoon wordt veelvuldig toegepast bij dreigende vroeggeboorte. Op dit moment zijn vele onderzoeken gaande of toevoeging van schildklierhormoon aan bijnierschorshormoon een extra stimulans voor de longrijping kan veroorzaken. In onze studie hebben we vlak voor de geboorte van ratten met CHD bijnierschorshormoon alléén, of in combinatie met schildklierhormoon toegediend. Daarna hebben we gekeken naar de activiteit van de enzymen die de long tegen zuurstofschade moeten beschermen (eveneens beschreven in Hoofdstuk 8) en naar het microscopisch beeld van de longen na kunstmatige beademing. Het bleek dat de activiteit van één van de enzymen die we onderzochten negatief beïnvloed werd door de combinatietherapie van beide soorten hormonen, maar dat bijnierschorshormoon alleen geen nadelige gevolgen

had. De ontplooiïng van de longen werd in gunstige zin beïnvloed door bijnierschorshormoon en nog enigzins beter door de combinatietherapie. Gezien de ongunstige werking van schildklierhormoon op de ontwikkeling van de enzymen die de long moeten beschermen tegen zuurstofschade, lijkt het echter verstandiger om alleen bijnierschorshormoon te gebruiken om voor de geboorte de longrijping te bevorderen.

Samenvattend kunnen we concluderen dat uit de gepresenteerde onderzoeken een aantal aspecten naar voren zijn gekomen: Het lijkt er op dat de aanvankelijke achterstand in longrijpheid tegen het einde van de zwangerschap grotendeels is ingelopen, en dat er mogelijk zelfs sprake is van een 'maximale compensatie'. Dit blijkt uit het feit dat in longen van kinderen en van voldragen ratten met CHD de grootte van c.q. het aantal neuroendocriene cellen was toegenomen. Daarnaast vonden wij in voldragen kinderen geen directe aanwijzingen voor een tekort aan surfactant. De enzymen die de long tegen zuurstofschade moeten beschermen waren bij de geboorte in voldoende mate aanwezig in pasgeboren ratten. We vonden zowel bij voldragen ratten als bij een aantal beademde kinderen met CHD dat een stof, waarvan bekend is dat deze de weerstand van de bloedvaten in de longen kan verlagen, in hoge concentraties aanwezig was in de longen. Dit zou er op kunnen wijzen dat de longen bij CHD trachten om maximaal te compenseren voor de toegenomen bloeddruk in de longvaten. Het feit dat de activiteit van de enzymen, die de long tegen zuurstofbeschadiging moeten beschermen, daalt tijdens kunstmatige beademing kan een aanwijzing zijn dat de longen bij CHD gevoeliger zijn voor die beschadiging dan gezonde longen. Op latere leeftijd vonden we een toegenomen gevoeligheid van de luchtwegen en vernauwing van de kleine luchtwegen. Dit kan er op wijzen dat er nog steeds sprake is van afwijkingen, die het gevolg zijn van de gestoorde aanleg van de longen, maar daarnaast kan beschadiging van de longen door kunstmatige beademing met hoge drukken en hoge concentraties zuurstof een bijdrage aan deze afwijkingen hebben geleverd.

Naast het doen van onderzoek naar de oorzaak van CHD, is het belangrijk om te onderzoeken op welke wijze de beschadiging van de longen door kunstmatige beademing zoveel mogelijk beperkt kan worden. Naast de prenatale behandeling met bijnierschorshormoon om de longontplooiïng zo goed mogelijk te stimuleren zal verder onderzoek moeten worden verricht naar verschillende manieren van kunstmatige beademing. Een aantal nieuwe beademingstechnieken zijn recent door middel van dierproeven onderzocht, en worden nu in speciaal opgezette studies bij pasgeboren kinderen toegepast.

Abbreviations

$AaDO_2$	alveolar-arterial oxygen difference
AOE	antioxidant enzymes
BAL	bronchoalveolar lavage
BPD	bronchopulmonary dysplasia
C-CV	conventionally ventilated control patients
CDH	congenital diaphragmatic hernia
CDH-CV	conventionally ventilated CDH patients
CDH-ECMO	ECMO-treated CDH patients
C-ECMO	ECMO-treated control patients
CGRP	calcitonin gene-related peptide
CT	phosphocholine cytidylyltransferase
DEX	dexamethasone
D_{LCO}	carbon monoxide diffusion capacity
DNA	desoxyribonucleic acid
DSPC	disaturated phosphatidylcholine
EAP	exaggerated atelectasis of prematurity
ECMO	extracorporeal membrane oxygenation
EGF	epidermal growth factor
ELF	epithelial lining fluid
ERV	expiratory reserve volume
ET	endothelin
E-α_1-PI	elastase-α_1-proteinase inhibitor
FEV_1	forced expiratory volume in one second
FGF	fibroblast growth factor
FiO_2	fraction of inspired oxygen
FPF	fibroblast pneumocyte factor
FVC	forced vital capacity
GRP	gastrin-releasing peptide
IGF	insulin-like growth factor
IRV	inspiratory reserve volume
ITP	intratracheal pressure
l/s ratio	lecithin/sphingomyelin ratio
LTB_4	leukotriene B_4
MAP	mean airway pressure
MBS	metabisulfite
MCH	methacholine

MEF_{25}	maximal expiratory flow at 25% of the FVC
mRNA	messenger ribonucleic acid
NEB	neuroepithelial body
NO	nitric oxide
NOS	nitric oxide synthase
OI	oxygenation index
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PD_{20}	provocative dose resulting in 20% fall of FEV_1
PEEP	peak end expiratory pressure
PEF	peak expiratory flow
PG	phosphatidylglycerol
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PIP	peak inspiratory pressure
PLUG	plug the lung until it grows
PNEC	pulmonary neuroendocrine cells
PPH	persistent pulmonary hypertension
PPHN	persistent pulmonary hypertension of the newborn
RAC	radial alveolar count
RARs	retinoid acid receptors
RDS	respiratory distress syndrome
RSC	radial saccular count
RV	residual volume
S	sphingomyelin
SMC	smooth muscle cell
SP	surfactant protein
TGFß	transforming growth factor ß
THR	thyroid hormone receptor
TLC	total lung capacity
TRH	thyroid-releasing hormone
TV	tidal volume
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
V_A	alveolar volume
VC	vital capacity
VEGF	vascular endothelial growth factor
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$

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Curriculum vitae

Hanneke IJsselstijn werd op 14 juli 1965 te Wijk bij Duurstede geboren. Zij behaalde het eindexamen V.W.O. aan de Werkplaats Kindergemeenschap te Bilthoven in 1983. Het doctoraal examen geneeskunde werd afgelegd aan de Rijks Universiteit Utrecht in 1987. In de wachttijd voor de aanvang van de co-assistentschappen werd een klinische stage gedaan bij de afdelingen Kindergeneeskunde van de Universitaire Instellingen Antwerpen (hoofd: Prof. K.J. Van Acker; september-oktober 1987) en van het Algemeen Kinderziekenhuis Antwerpen (toenmalig hoofd: Prof. Dr R. Clara; november-december 1987) te België. Het artsexamen werd behaald in mei 1990. Van mei tot december 1990 was zij werkzaam als arts-assistent Interne Geneeskunde in Ziekenhuis "De Lichtenberg" in Amersfoort (toenmalig hoofd: Dr H.Ch. Hart). De Nederlandse bodem werd verruild voor het Zwitserse berglandschap van december 1990 tot januari 1992; in deze periode was zij werkzaam als arts-assistent bij de Divisie Jeugd van het Nederlands Astmacentrum Davos te Zwitserland (toenmalig hoofd: W.T.J. van den Brink; later: R. Aalbers). Van januari 1992 tot april 1993 werkte zij als arts-assistent Kinderchirurgie in het Sophia Kinderziekenhuis te Rotterdam (hoofd: Prof. Dr J.C. Molenaar). Intussen werd onder leiding van Prof. Dr D. Tibboel en Prof. Dr J.C. de Jongste een aanvang gemaakt met wetenschappelijk onderzoek, hetgeen resulteerde in een aanstelling als Assistent In Opleiding bij de Erasmus Universiteit Rotterdam sedert april 1993. Een deel van het onderzoek werd gedaan bij Prof. Dr E. Cutz (Department of Pathology of The Hospital for Sick Children, Toronto, Canada) in de periode van februari tot mei 1996. Vanaf 1 april 1997 zal de opleiding Kindergeneeskunde in het Sophia Kinderziekenhuis (opleider: Prof. Dr H.A. Büller) worden gevolgd.
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