Molecular and Structural Aspects of the Pulmonary Vasculature in Human Congenital Diaphragmatic Hernia, and Therapeutic Implications

Moleculaire en Structurele Aspecten van de Longvaten bij Congenitale Hernia Diafragmatica in de Mens, en Implicaties voor de Behandeling

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Back page: Represents the three pyramids of Kufu, Kafra and Menkaura at Giza (2500 BC) and the Erasmus University.

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"Who wants the current life, he has to take the science's way; who wants after-life, he has to take the science's way and who wants both lives, he has to take the science's way also"

***Prophet Mohamed** [allihe alsalat wa alsalam]*

To all the Sick Infants and Children in the World
To my Parents
To my Family
To my son: Mohamed
To EGYPT

Whom I Owe Much More Than I Can Give.

S M K Shehata

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Part. I

Introduction and Review of Literature

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Chapter 1

Introduction and Literature Review

1.1 General Introduction and History of CDH

Congenital diaphragmatic hernia (CDH) consists of a defect in the diaphragm and a variable degree of pulmonary hypoplasia with vascular abnormalities. In the whole clinical spectrum, surgical repair of the anatomical defect represents the lesser part of the problem[1]. Despite recent treatments, such as, preoperative stabilization, high frequency oscillation, partial liquid ventilation, extracorporeal membrane oxygenation and nitric oxide inhalation therapy, the survival rate still remains around 50%, taking into consideration that high-risk groups of neonates were included in all recent series of studies[2].

One of the most important factors determining survival is probably the degree of pulmonary hypertension[3,4]. Furthermore, pulmonary hypoplasia and stunted pulmonary vascular development represent other major problems in patients with congenital diaphragmatic hernia.

Despite the development in new therapeutic modalities, a number of questions regarding the pathogenesis and the optimal clinical management of CDH remain unanswered.

In 1575[5] Ambroise Pare first described CDH. Until the advent of its surgical correction, it was believed to be a purely anatomical defect. In the 1940s, it became clear that the diaphragmatic defect is only a part of the more complex CDH syndrome[6-10], which includes the diaphragmatic defect, pulmonary hypoplasia, underdevelopment of the pulmonary vasculature with surfactant deficiency in some cases. Indeed, postoperative follow-up studies revealed normalization of the ventilation lung scans without improvement of the perfusion lung scan[4].

The continuing low survival rate has been attributed mainly to pulmonary hypoplasia and the associated pulmonary hypertension (PH)[3,4,8,9,11,12]. Major efforts now aim to improve oxygenation in the peri-operative period and to reverse the persistent fetal circulation in order to mature the structurally and functionally immature hypoplastic lung[2,3,8,12].

To further improve management and care of patient with CDH, we need to understand the development of the pulmonary vasculature in detail. Unfortunately, our knowledge of the development of the normal human pulmonary vasculature is far from complete. Further research in the field of vascular biology of normal newborns and those with CDH is therefore mandatory in order to base our management protocols on a scientifically-sound knowledge of the disease process. The various aspects that should be evaluated are: vascular morphology and remodeling, angiogenic markers, and functional aspects including the vascular reactivity to vasoactive molecules as well as pulmonary stress response.

Knowledge of the molecular basis of changes preceding the histological ones in the pulmonary vasculature is of importance for possible early intervention as a therapeutic

strategy. Whenever possible, the modes of action of different therapeutic modalities should also be investigated at the molecular level.
I hope that the work presented here will clarify some points concerning the pulmonary vasculature in normal and CDH newborns, and may contribute to improved therapeutic outcome.

1.2 Anatomy of the Diaphragm

The diaphragm is the dome-shaped septum separating the thoracic and abdominal cavities. It consists of two portions: a peripheral muscular part that converges to an insertion in the central tendenious part[5]. Between the vertebral and costal parts on each side, there is always a space known as the vertebro-costal trigone. When such space is present, it is occupied by loose connective tissue that separates the pleura above from the suprarenal gland and upper pole of the kidney below. This structure is known as the embryological pleuro-peritoneal membrane.
The diaphragm forms between the fourth and eighth weeks of embryonic life[5]. The parts formed from the central tendon remain as connective tissue, but the remaining part becomes invaded through the transverse septum by muscle cells derived from the third, fourth, and fifth cervical myotomes. In embryos of the 8th week of gestation, the dorsal parts of the diaphragm have moved caudally, resulting into the domed-shape of the diaphragm as a whole. The pleuro-peritoneal membrane on each side starts to grow medially from the body wall and encroaches on the pleuro-peritoneal canal until it finally fuses with the septum transversum anterior to the esophagus and the dorsal mesentery posterior to the esophagus. During the process of fusion, the mesoderm of the septum transversum extends into and pervades the other parts, thus forming the entire muscle of the diaphragm[5,6]. Failure of fusion of the various elements that form the diaphragm results in different types of congenital diaphragmatic defect.

1.3 Prevalence of CDH

Estimations of the prevalence of congenital diaphragmatic defects vary widely from 1:1000 to 1:12,000[13,14]. A population-based study of CDH conducted by Torfs et al showed a prevalence of 3.13 per 10,000 live births, and 3.3 per 10,000 total births including stillbirths[15]. In another large population-based study in the southwest of England, an identical prevalence of 3.3 per 10,000 births was found[16]. Torfs et al reported that 95.8% of CDH were posterolateral, of which 84% were left-sided, 13% right-sided and 3% bilateral[15,17].
The overall mortality in babies born with CDH is about 50%. In severely affected infants presenting with severe respiratory distress at birth, mortality rate may be as high as 70%. In CDH cases with no clinical symptoms in the first 24 hours after birth (delayed presentation), the survival rate is about 95%[18].

According to the clinical presentation, infants with CDH can be classified into three groups:
Group 1: Neonates who present in the first 6 hours of life, constituting the *high-risk*

group. They have a poor prognosis as they have bilateral pulmonary hypoplasia, persistent pulmonary hypertension (PH), (fixed) right-to-left shunting, and systemic hypoxaemia. More than 50% of them do not respond to vasodilator and ventilation treatment and are called "non-responders".
Group 2: Neonates with a milder form of hypoplasia augmented by stress-induced, pulmonary vascular constriction resulting in right-to-left shunt. They may improve and respond to vasodilator and ventilator therapy, and achieve a higher survival rate. Usually these patients present between 6 hours and 24 hours after birth[19], and they are considered as "responders".
Group 3: Neonates and infants who present after the first 24 hours. They have no or minimal lung hypoplasia and normal or minimally elevated pulmonary blood pressure. Usually they have the best prognosis, with a survival rate about 95%[20].

1.4 Normal Lung Parenchymal Development

In a classic paper[21], Lynn Reid formulated three laws of human lung development:
1. The bronchial tree is fully developed by 16 weeks of gestation.
2. Alveoli continue to develop after birth, increasing in number until the age of eight years. Alveolar size increases until growth of the chest wall is completed in early adulthood.
3. The preacinar vessels (arteries and veins) follow the development of airways; the intraacinar vessels follow that of the alveoli. Muscularization of the intraacinar arteries does not keep pace with the appearance of new arteries[21]. So, the fetal lung does not simulate the adult lung in miniature.

Traditionally, lung development has been divided into five consecutive stages: the embryonic, pseudoglandular, canalicular, saccular, and alveolar period[22,23]. The first two periods are histologically similar and are therefore be referred together as the pseudoglandular period, thus leaving only four developmental stages[24,25]. Merkus and coworkers have provided a detailed review of the histological characteristics of these four stages[24]. In humans during the *pseudoglandular period*, the lung primordial system representing the branching lung bud develops until week 10-12 of gestation, followed by the differentiation of airways from weeks 12 to 16. Vascularization and further development of acinar tissue occurs in the *canalicular period*, resulting in the formation of the respiratory acinus, which is defined as the alveoli derived from one terminal respiratory bronchiole supplied by one pulmonary artery and drained by one pulmonary vein, from gestational week 16 to 28. During the *saccular period*, from weeks 26 to 36, the major event is the subdivision of sacculi, which are defined as the branches of the respiratory bronchioles lined with cuboidal epithelium. The *alveolar period*, from week 36 till about 18 years of life, is the stage of the emergence and enlargement of alveoli and the lining epithelium becoming one layer of flattened epithelium[24,26].

1.5 Morphological Aspects of Normal Pulmonary Vascular Development

In humans, angiogenesis is first detected in the developing trachea, esophagus and lung buds at about 32 days of gestational age. A vascular plexus is formed, which receives its blood supply from branches of the aortic sac as well as from numerous branches of the dorsal aorta. Primitive pulmonary arteries become incorporated into the sixth aortic arch, while the intersegmental arteries involute by the end of the 5th week of gestation. Connections with systemic arteries may persist in abnormal situations.

According to Reid's third law, preacinar vessels (both arteries and veins) develop at the same time as airways, so that after the 16th week all preacinar artery branches are present[21]. The relationship of the blood vessels to the airways and air spaces permits useful landmarking or timing of critical events in the development and function of the pulmonary circulation[27]. The preacinar arteries that accompany the airways are all present by the end of 16 week's gestation[21]. The intraacinar arteries supply the capillary bed and multiply very rapidly after birth in order to follow alveolar multiplication. Muscularization of non-muscular arteries however is a slow process, in the fetus and newborn, as muscular arteries are only found along airways[21].
Two morphogenic processes contribute to the development of the lung vasculature: vasculogenesis and angiogenesis[21,27-29]. In vasculogenesis, blood vessels develop de novo. The pre-existing endothelial cell precursors or angioblasts form primitive vascular channels, which subsequently remodel,producing arteries, veins and lymphatics, depending on local stimuli from the surrounding mesoderm[21-23,27]. In contrast, in angiogenesis, blood vessels develop from pre-existing ones by a process of budding and sprouting[23,24,27]. Angiogenesis is thought to be responsible for the formation of axial arteries[24,27].

The structure of the pulmonary arteries varies with vessel size and developmental stage of the lung. The muscular coat of the artery first becomes apparent in the canalicular stage. Axial arteries from the hilum to the 7th generation are elastic; more peripheral arteries are muscular, partial muscular or, at the level of intraacinar artery, predominantly non-muscular. By definition, an elastic artery has more than two elastic laminae in its media, whereas a muscular artery has only two elastic laminae[28-31]. A partially muscular artery has smooth muscle cell tissue in only one part of its circumference; at this level the continuous muscular coat has been replaced by a spiral of smooth muscle cells (SMC). A non-muscular artery (arteriole) is similar in structure to an alveolar capillary, exepting (larger) diameter[28,29]. Small muscular, and probably partially muscular, arteries, represent the -so called- *resistance arteries*. Muscularization decreases towards the lung periphery in the normal fetus. A newborn has one artery for every 20 alveoli. In humans, due to formation of new alveoli postnatally, this ratio is reduced to 8:1[28-31].
Two types of pulmonary arteries can be distinguished: axial arteries, which accompany airways and additional or supernumerary arteries. The latter are small lateral branches that arise from axial arteries and run a short course to supply the capillary bed of alveoli immediately adjacent to the pulmonary artery at the peribronchial parenchyma[27].

The latter are considerably more numerous and contribute considerably to the cross-section of the total recruited vascular bed. Supernumerary arteries constitute about 25% of the cross-sectional area at preacinar level, whereas at the intraacinar level they make up about 33%. According to Hislop and Reid the normal lung, counts 23 generations of conventional arteries along the posterior basal axial pulmonary artery branch, with 64 supernumerary branches, giving a ratio of 1:2.8 between conventional and supernumerary arteries for one axial branch[21,31-34]. Supernumerary arteries facilitate blood oxygenation by allowing passage of venous blood to the more remote alveoli adjacent to large arteries, veins and airways[27, 31,32]. The intraacinar arteries represent an important part of the resistance arteries in the pulmonary vascular bed. The external diameter (ED) of preacinar arteries usually exceeds 200 μm; the arteries running with the respiratory bronchioli represent the intraacinar arteries with ED 50-200 μm[33,34]. These intraacinar arteries together with the supernumerary arteries increase rapidly in number and dilate near term to accommodate the postnatal demands of the pulmonary circulation[35-37].

Following the description of morphological changes in the developing pulmonary vasculature, the role of growth factors has been investigated more recently, especially the family of fibroblast growth factor (FGF), transforming growth factor β (TGF- β) and isoforms of platelet derived growth factor (PDGF). Many animal models have been studied; differences between these models as well as between the techniques and culture systems used in the experiments make it difficult to define the role of different growth factors during pulmonary vascular development[38-42]. See sections 1.9, 1.10 and 1.13 of this chapter for a detailed description.

1.6 Developmental Anomalies of the Lung

Developmental defects of the lung include 1) agenesis or hypoplasia of one or both lungs, or of single lung lobes; 2) tracheal and bronchial anomalies; 3) vascular anomalies; and 4) hamartomatous malformations.

Pulmonary hypoplasia in newborns is known to occur in a number of malformation syndromes. It has been diagnosed in 7.8 to 10.9% of neonatal necropsies as, and in about 50% of necropsies of neonates with congenital anomalies[43-45].

This hypoplasia could be due to many factors unfavorably influencing the amount of intrathoracic space, as in the CDH *syndrome* and cystic malformations of the lung[46,47]. Also, diminished total amniotic fluid amount (oligohydramnios) due to rupture of the membranes or Potter syndrome[48-50] and decreased pulmonary arterial flow in cardiovascular malformations like tetralogy of Fallot or hypoplastic right heart result in lung hypoplasia[51,52]. Fetal airway obstruction is not accompanied by lung hypoplasia, indicating that tracheal fluid may play a role in the stimulation of lung growth[11].

Pulmonary weight expressed as a percentage of total body weight has been widely used as a parameter to define pulmonary hypoplasia[45]. In addition, so-called radial alveolar counts are useful to identify and quantitate the severity of lung hypoplasia[53,54]. In this method, a perpendicular line is drawn from a terminal bronchiole to the nearest septal division or pleural surface, and the alveolar septae intersected by the line are counted[55].

Normal lung weight or LW/BW ratios in different series ranged to be between 0.18 and 0.22. Emery and Mithal, after excluding lungs with edema and exudate, found that the mean LW/BW ratio of hypoplastic lungs was 0.13[55].

Wigglesworth and coworkers found that total lung DNA near term in many cases of pulmonary hypoplasia equalled to that in normal fetuses at about 20 weeks gestation[56,57]. They concluded that lung growth must have been impaired before 20 weeks and accordingly defined 2 groups of patients with pulmonary hypoplasia. The first group consisted of fetuses with oligohydramnios due to renal agenesis, urethral obstruction or amniotic fluid leakage in early pregnancy, without other malformations. The lungs show a characteristic histological pattern with narrow airways and impaired maturation of respiratory epithelium, associated with lack of interstitial tissue and failure of normal elastic tissue development around the airways and terminal sacs[26].
The second group (including CDH) had a normal or increased volume of amniotic fluid. In this group, although the lungs were small, they were usually of appropriate maturity for gestational age, with normal epithelial maturation, normal phospholipid content, and normal elastin development. So lung growth and maturation during the early period may be critically dependent on influences outside the lung, such as fetal breathing movements, or inside the lung, such as lung liquid secretion[58,59].

1.7 Abnormal Pulmonary Vascular Development

To study abnormal pulmonary vascular development, it is important to compare possible differences in the pulmonary artery structure in a standardized way. Four main features need to be assessed to determine vascular development: (i) branching pattern, (ii) number or density of arteries, (iii) wall structure, and (iv) arterial size (usually assessed as ED between the two external elastic laminae)[27]. Hislop and Davies described a way to process lung tissue into histological slides, resulting in barium-gelatin filled arteries, in which the dark stained elastic layers are easy to distinguish from the surroundings[31,60]. In these slides, external diameter, wall thickness, wall structure (muscular, partially muscular, or non-muscular) is registered for each artery, as well as the type of the accompanying airway. The percentage of wall thickness is measured as follows: 2 x wall thickness x 100 /external diameter. In addition, the use of a radio-opaque injection medium resulting in arteriograms on x-ray, allows rapid general assessment of the pulmonary vascular bed[9,28]. The use of this technique has been criticised because the relatively high pressures used to instill the barium-gelatin fluid result in marked distension of the arteries[35-37].
Geggel and Reid distinguished between maladaptation, maldevelopment, and underdevelopment in abnormal pulmonary vasculature (see table I)[34]. Maladaptation is represented by a structurally normal lung at birth, in which the normal increase in compliance of small resistance arteries fails to occur.

Table I: *Arterial and bronchial morphometrics in different cases of perinatal pulmonary hypertension.*
Adapted from Geggel and Reid, Clin Perinatol, 1984 [34]

	Airway		Intraacinar artery			
Cause	Number of bronchial generations	Number of alveoli per acinus	Muscle extension by position	External diameter	Medial wall thickness	Number
Excessive muscularization						
PPHN – idiopathic	N	N	↑	N	↑	N
Meconium aspiration	N	N	↑	N	↑	N
TAPVC-SD	N	N	↑	↑	↑	N
TAPVC-ID	N	N	↑	N	↑	N
Coarctation, VSD, PDA	N	N	↑	↑	↑1, ↓2	N
Underdevelopment						
CDH	↓	N	N,↑	↓3	↑	↓
Renal agenesis/ dysplasia	↓	↓	↑,N,↓	↓4	↑,N,↓	↓
Rhesus isoimmunization	↓	N,↓	N	↓3	N5	↓
Idiopathic (primary)	↓	↓	NA	NA	NA	NA
Maladaptation						
VSD	N	N	↑	↓	↑	↑,N

CDH = congenital diaphragmatic hernia, ID = infradiaphragmatic, PDA = patent ductus arteriosus, PPHN = persistent pulmonary hypertension of the newborn, SD = supradiaphragmatic, TAPVC = total anomalous pulmonary venous connection, VSD = ventricular septal defect, N = normal, NA = not available, ↑ = increase, ↓ = decrease.
Notes: 1, dependent on the severity of coarctation; 2, preacinar arteries; 3, small for age but appropriate for lung volume; 4, small for age but large for lung volume; 5, preacinar medial hypertrophy.

The pulmonary vascular bed therefor is highly reactive and a vicious circle of acidosis, hypoxia, hypercapnia may develop giving rise to vasoconstriction-induced pulmonary hypertension.

Maldevelopment indicates new and precocious muscularization as seen in idiopathic persistent pulmonary hypertension. Excessive muscularization is seen in hypoplastic left heart syndrome, chronic intra-uterine hypoxia, and in some cases of meconium aspiration syndrome. Underdevelopment represents the pathologic conditions with reduced size of arteries as seen in congenital anomalies associated with pulmonary hypoplasia, such as CDH, renal agenesis or dysplasia, and oligohydramnios.

1.8 Etiology of CDH

No definite cause is known for CDH. In all likelihood, it is a multifactorial process. Many theories have been postulated to explain the pathogenesis; these include the following:

***The mechanical theory*:**

This remains the most popular one. Normally, the diaphragm is complete at 10-12 weeks of gestation, by which time the gut returns from the umbilical cavity to the abdominal coelum. CDH develops either from early return of the gut to the abdominal cavity passing through the still opened pleural canals, or from failure of closure of the pleuro-peritoneal canals[61,62]. The herniated bowel compresses the developing lung, resulting in pulmonary hypoplasia in many cases[17,63]. In the postnatal period, two phenomena contribute to the herniation of the abdominal contents into the chest of CDH newborns. First, negative intrathoracic pressure, produced by the neonate' s first breathing, promotes the herniation of the bowel into the thorax. Secondly, the increased intraabdominal volume resulting from air entering the stomach and bowel on account of swallowing, leads to expansion of these organs[64].

***Other theories*:**

These are largely derived from different animal models of CDH. They include:

"Hereditary Theory": In humans, familial occurance of CDH is estimated to account for less than 2% of all cases. Although familial CDH is not frequent, the possibility of genetic factors in these familial CDH cases should be considered[11,13-17]. The mode of inheritance remains uncertain, but Passarge et al concluded that autosomal recessive inheritance was the most likely one, especially in familiar unilateral agenesis of the diaphragm[65]. The possibility of multifactorial inheritance has been stressed by other investigators[17].

"Chemical theory": Many drugs and environmental chemicals have been claimed to induce CDH. Maternal ingestion of drugs such as, Thalidomide, Phenmetrazine and Quinine during early gestation has been reported to be associated with CDH[17,66]. Vitamin A or Retinol deficient diets result in CDH in different strains of rats[17]. During the last decade, special attention has been paid to the herbicide 2-dichlorophenyl-p-nitrophenyl ether (Nitrofen). It is a teratogenic agent, which induces CDH and lung hypoplasia in rats. This results in a situation very similar to the human case[11,17,67,68] Iritani described the development of the so-called post-hepatic mesenchymal plate (PHMP) in association with growth of the lung bud into the pleuro-peritoneal cavity in a nitrofen-induced rat model of CDH and pulmonary hypoplasia[69]. The association between defective development of the left lung bud and underdevelopment of the left PHMP is well established in the rat model[69]. Closure of

the pleuro-peritoneal cavity results from development of the lateral and caudal parts of the PHMP. Although the nitrofen-induced CDH model is a toxicological one, studies using this model have brought forward the concept that CDH can be regarded a developmental disorder of the diaphragm, rather than a defect secondary to the persistence of the pleuro-peritoneal canal[69]. These data suggest a primary disturbance of mesenchymal growth in CDH[17].

1.9 Lung and Vascular Development in CDH

The lungs of CDH infants have reduced numbers of airway and vascular generations[46,53]. Since the bronchial tree is fully developed at 16 weeks gestation, it is likely that lung growth in CDH is affected before that period. It is believed that the pleuro-peritoneal canals fail to close at 8-10 weeks gestation and that the abdominal viscera herniate into the thoracic cavity, reducing lung growth through competition for space[70]. Competition persists during later gestational stages, so the development of the pulmonary acinus is impaired as well, resulting in decreased radial alveolar counts in CDH lungs[46,53].

As an integral part of the anomaly in CDH, lungs of CDH patients show a number of arterial abnormalities in the pulmonary vasculature consisting of a) reduced total pulmonary vascular bed and decreased number of vessels per volume unit lung; b) medial hyperplasia of pulmonary arteries together with peripheral extension of the muscle layer into small arterioles; and c) arterial adventitial thickening[53,71-73].

Newborns with CDH may show immediate severe respiratory distress on account of pulmonary hypoplasia with cardiac and lung compression by the herniated viscera[74]. Intraacinar arteries in the healthy newborn are virtually all non-muscular. In contrast, in persistent fetal circulation syndrome most of these arteries are completely muscularized.

Geggel et al gave a detailed morphometric analysis of the lungs in a series of 7 infants with CDH[33]. The authors distinguished two groups; 4 infants who could never be ventilated adequately (the so called no-honeymoon group), and 3 who did well initially following emergency repair of their diaphragmatic defect, but subsequently developed increased pulmonary vascular resistance leading to death (honeymoon group). The no-honeymoon patients had smaller lungs, increased muscularization of intraacinar arteries, and decreased luminal area of preacinar and intraacinar arteries[33]. Kitagawa and coworkers were the first to demonstrate abnormalities in both number and muscularisation of arterial branches in the pulmonary tree of CDH cases[46]. They reported that the number of conventional branches was reduced to 14 in the right lung and to 12 in the left lung. Furthermore a reduction of supernumerary branches to 17 in the right lung, but only to 36 in the left lung was noted by counting the branches of the posterior basal axial pulmonary artery. They also described thicker muscular walls in smaller diameter arteries (less than 300 μ). Other investigators reported reduction in the cross-sectional area of the pulmonary vascular bed and uniform thickening of the pulmonary artery muscle "mass" in CDH lungs[71,74-79].

Beside the morphological changes, functional abnormalities in the pulmonary arteries were reported also in the form of abnormal reactivity to changes in alveolar oxygen concentration, blood gases, and hormones. These factors may contribute to pulmonary

arteriolar vasoconstriction[11,74]. In CDH neonates, increased levels of the vasoconstrictor thromboxane A_2 have been reported[75]. In a recent study, a state of imbalance was reported in the rat CDH model with enhanced gene expresssion of endothelin 1(ET-1), a potent vasoconstrictor in CDH lungs[76]. At the same time, artificial ventilation or barotrauma may injure the fragile CDH lungs. Sometimes artificial ventilation leads to air dissection through the weak lung tissue and/or accompanied pneumothorax, which augments the compression of the pulmonary vessels, resulting in further increase in associated pulmonary hypertension[18,77]. These effects will particularly be more severe in preterm newborns[77].

1.10 Pulmonary Vascular Smooth Muscle

Previous studies focused on changes in the pulmonary vasculature during normal transition to extrauterine life. These include thinning of the arterial wall and luminal widening. Immediately after birth, pulmonary vascular resistance falls abruptly, and pulmonary blood flow rapidly increases approximately tenfold[22,27,35]. Adaptation of the pulmonary circulation to postnatal life requires growth and differentiation of blood vessels and a transition in smooth muscle cells (SMC), from a fetal to an adult phenotype[80-82]. Several studies have demonstrated interruption of the normal transition to postnatal life by hypoxia or increased pulmonary blood flow to result in marked proliferative changes in pulmonary artery SMC.

SMC derived from neonatal pulmonary arteries are less differentiated and exhibit enhanced growth responses to mitogenic stimuli when compared to the mature SMC derived from the adult pulmonary artery[83,84]. Thus, increased growth capacity of neonatal pulmonary artery SMC probably contributes to the marked pulmonary vascular remodeling which may occur as a reaction to injury in the neonatal period. Those changes are observed in idiopathic persistent pulmonary hypertension of the newborn, congenital heart disease, and lung hypoplasia with or without congenital diaphragmatic hernia[83, 85,86].

Among others, endothelin-1 (ET-1) and angiotensin-II (ANG II) are potent vasoconstrictors, which are generally believed to stimulate growth of adult SMC, derived from the systemic circulation[87,88]. Indirect evidence for the involvement of ET-1 and ANG II in medial thickening of pulmonary arteries has been shown in adult rats, but direct effects on pulmonary artery SMC proliferation are less clear[89]. Indeed, it remains unclear whether the pulmonary vasculature possesses a developmentally regulated response to ANG II and ET-1, or not. Intracellular signaling mechanisms involved in ET-1 and ANG II induced proliferation of neonatal pulmonary artery SMC are poorly understood. Previous studies have demonstrated that basal protein kinase (PKC) activity is higher in neonatal than in adult pulmonary artery SMC. In addition, reports documented that ET-1 and ANG II can activate PKC[87,89,90].

Several observations suggested that distinct mechanisms control vascular growth during development. Many factors have been postulated to modulate or alter vascular smooth muscle cell growth. Those with a documented function in this respect are listed in Table II.

Platelet-derived growth factor (PDGF) is a well-known inducer of DNA synthesis and mitogenesis. Previous work has demonstrated that PDGF activates mitogen activated protein (MAP) kinase, which in turn plays a crucial role in regulating the entry of cells into the growth cycle[41,42,84]. For a number of cells it was proposed that the kinetics of MAP kinase activation may dictate the relative efficacies of both growth factor and G-protein-coupled receptor agonists as mitogens.
Several reports indicated that differences between CDH and controls at the protein level and tissue localization of PDGF-AA, extracellular matrix protein, and myosine heavy chain isoforms are not significant[91,92].

Table II. *Factors modulating the growth of vascular SMC*
Adapted from Gibbons and Deau, N Eng J Med, 1994.

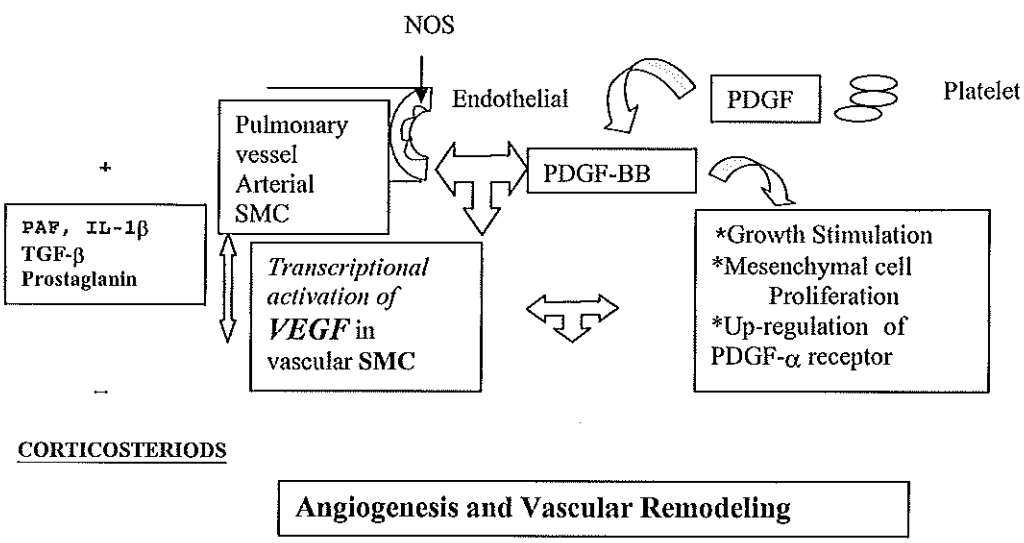
GROWTH PROMOTERS	GROWTH INHIBITORS
FGF	Heparine Sulfate
PDGF	Fibronectin
	TGF--β1 and -β2
TGF-β1 and -β2	Interferone-gamma
Heparine binding EGF	
EGF	
IGF-I	
Interleukin-1	
Interleukin-6	
Thrombin	
Serotonin	
Angiotensin II	Nitric oxide
Endothelin	Prostaglandin
Norepinephrine	Atrial Natriuretic Peptide
Vasopressin	Type C. NP
Substances P and K	
Leukotrienes	
Thromboxane	Shear Stress
Stretch or wall tension	

FGF = fibroblast growth factor, PDGF = platelet derived growth factor, TGF = transforming growth factor, EGF = epidermal growth factor, IGF = insulin growth factor.

Prostaglandins have been implicated in vascular SMC growth for many years; stimulation of pulmonary artery SMC growth by PGI_2 and PGE_2 has been demonstrated[93].

A growth factor of considerable interest, which may be regulated by prostaglandins, is the vascular endothelial growth factor (VEGF). VEGF is a potent angiogenic growth factor, which has been reported to have a narrow target cell specificity to endothelial cells. Moreover, VEGF regulates vasculogenesis and postnatal vascular remodeling[94,95]. Its effects are mitogenic and appear to be regulated at the receptor level[94,95]. Part of the interactions between the various growth factors and the arterial wall is shown in Figure 1. Fms-like tyrosine kinase (Flt-1) and Flk-1 are receptors for VEGF and are known to be expressed during early vascular development in mouse embryos.

Figure. 1: *The interaction of VEGF and PDGF in the vascular smooth muscle cells.*



PDGF = platelet derived growth factor, VEGF = vascular endothelial growth factor, SMC = smooth muscle cell, PAF = platelet activating factor, IL = interleukin, TGF = transforming growth factor, NOS = nitric oxide synthase, (+) = enhance, (-) = inhibit.

The expression of Flt-1 is restricted to vascular endothelial cells[95]. ET-1 has been demonstrated to stimulate the synthesis of VEGF-protein in human adult vascular SMC, which could regulate vasculogenesis and vascular remodeling in a paracrine manner. However, little is known about the molecular regulation of this endothelial-specific gene expression during development.
VEGF regulates vasculogenesis and plays a role in postnatal vascular remodeling[96-98].

It is expressed in a variety of cells, and a paracrine mechanism of action has been suggested whereby non-endothelial cells secrete VEGF, which modulates the vasculogenesis and angiogenesis in the adjacent vascular endothelium[99]. This important angiogenic role of VEGF is illustrated by the lethal abnormal vessel development occurring in embryos lacking a single VEGF allele[100]. The same has been reported following the experimental inactivation of the VEGF gene by replacing the coding sequence of exon-3 of the VEGF gene in embryonic stem cells[101]. An interaction between VEGF and PDGF-BB has been demonstrated, although further studies are necessary to elucidate their respective roles in more details[102,103].
Conclusions drawn from demonstration of signal transduction pathways in cultured cells must be re-evaluated in vivo, since inter-species variations, differences in experimental protocols, and other factors will influence growth responses.
The earliest and most striking proliferative changes in the neonatal pulmonary arterial wall occur in the adventitia, where the fibroblast resides[104,105]. Thus, the specific signaling mechanisms in the fibroblast need to be elucidated also, since they are evidently of importance in the (ab)normal development of the pulmonary vasculature. Even more, the differences in the physical properties between arterial medial muscle tissue and adventitial fibrous tissue must be relevant with respect to their respective roles in vascular reactivity in various hemodynamic situations[106].

1.11 CDH Studies in the Rat Model

Many studies were performed in the rat model of CDH in order to investigate the pulmonary arterial bed at histological and molecular levels. Administration of the herbicide 2,4 dichlorophenyl-p-nitrophenyl ether (Nitrofen) to pregnant littermates at day 9.5-10.5 in a dose of 100 mg/kg dissolved in 0.5 ml olive oil results in a rat model of CDH[67,68,107]. This protocol produces CDH in 60-70% of the offsprings with lung hypoplasia. The pathogenetic mechanism of the diaphragmatic defect and lung hypoplasia is probably the interruption of the post-hepatic mesenchymal plate and alteration of thyroid function prenatally, respectively, which are proven to be essential for lung maturation[68,69]. This model has been used in studying lung hypoplasia associated with CDH, morphologically[107], structurally[108] and biochemically[91,108,109]. The morphologic features in rats simulate those reported in human CDH cases

Functional studies

Histopathologically, abnormalities in the pulmonary vasculature in CDH have been demonstrated in detail. Experimental studies showed that reactions to various vasoactive agents including inhaled nitric oxide (NO) are highly unpredictable in CDH rats[109,110], as well as in human CDH newborns[111,112].
The exact mechanisms that control vascular tone in the neonatal pulmonary circulation are largely unknown. The medial SMC, and perhaps also the adventitial connective tissue in the vessel wall, play a key role. Various mediators (e.g. bradykinin, Ang II, ET-1, epinephrine, thromboxane B2, and metabolites of arachidonic acid) influence vascular tone in a complex and incompletely understood manner[103,105,107,113].

We hypothesized that in CDH altered pulmonary vascular reactivity might be related to differential expression of ET-1 and its receptors. A report by our group showed significantly enhanced levels of ET-1 mRNA in CDH rat lungs[76]. A 3.0± 0.9 fold increase in ET-A receptor mRNA was found in CDH as compared to controls, whereas ET-B receptor mRNA levels remained equal in CDH and control rats[76]. Interestingly, no significant difference in expression of ET-1 mRNA between the right and left lung (the most hypoplastic) was observed in CDH rats. These data suggest that the structural alterations of the pulmonary vasculature observed in infants with CDH may not necessarily cause exaggerated vasoconstrictor responses to normal stimuli.
Using the same rat model, Karamanoukian and coworkers detected both decreased NOS expression and NOS activity in CDH rat lungs using a 14 C-L-arginine to 14C-L-citrulline conversion assay and Western blots[109]. In another study, 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[113,114]. Elevated levels of 6-keto-PGF1α in CDH may reflect a compensatory mechanism for increased vascular resistance[115].

1.12 The Fetal Lamb Model of CDH

This is the most commonly used surgically induced model, developed by
Dr M. Harrison (San Francisco) in a series of experiments designed in the late sixties[116]. It has many advantages since it allows to measure the hemodynamic changes, pressure differences, and many of the previously mentioned structural, morphological and molecular studies[117]. Above all, the possibility for trials of fetal surgical[116,117] or non-surgical interventions remains its great advantage[118]. The hernia is produced at day 90 of gestation via hystrotomy and with surgical production of a diaphragmatic defect[117]. Now, this procedure can be conducted with fetoscopic assistance. In different studies, by near-term fetuses were delivered via a second hystrotomy[118]. Some of the disadvantages of this model are the differences in developmental lung stages as compared to humans and the degree of associated pulmonary hypoplasia and hypertension[11]. Trials of fetal surgical corrections were done around 110-120 days, as term in lambs is 135-140 days[116-118]. Moreover, special and expensive laboratory equipment is needed for this experimental technique.
The lamb model has been used mainly to study the effects of prenatal repair of the diaphragmatic defect and consequent catch-up of lung growth[118]. Glick and coworkers performed a series of experiments focusing on pulmonary vascular reactivity in newborn lambs with CDH and postulated that an abnormality and/or deficiency of the nitric oxide regulatory system may be present in CDH. These workers investigated the influence of nitric oxide on pulmonary vasodilatation in CDH lambs in utero. In their study, both the endothelium-dependent and endothelium-independent pathways appeared to be intact in this model, no difference could be demonstrated between control and CDH lungs[119], possibly since they investigated sections from the main pulmonary trunks only.

Wilson et al evaluated the pulmonary vasculature in newborn lambs with CDH by combining surgical induction of CDH with tracheal ligation (TL)[11]. Larger vessels in the pulmonary vasculature were analysed using a computerized digital system for the evaluation of angiograms of lung slices. This study showed that the total area of large vessels in the DH/TL group was increased, compared with normal control animals, or the DH group. The ratio of large vessel to lung area was similar in all groups. While the numbers of capillaries per alveolus were similar in all groups, microscopic morphometric analysis revealed that the total number of capillaries was increased in DH/TL lungs compared with both DH and control. In addition, the percentages of muscularized vessels less than 100 μm in diameter of the capillary wall and of the capillary-alveolar interface appeared to be normal in the DH/TL group as compared to the DH group.

1.13 Studies in Humans

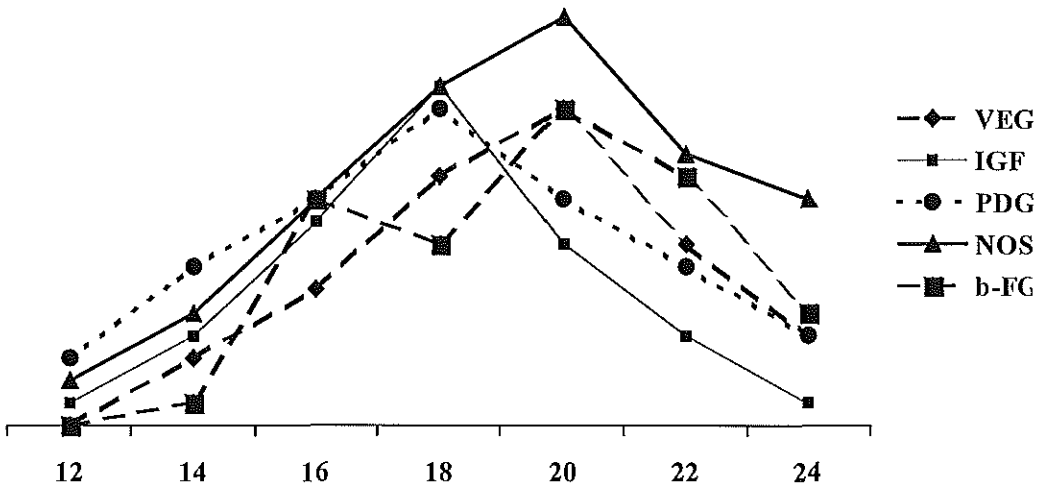
Despite the availability of a number of experimental CDH models, the human CDH situation is unique[111,114,120-122], as differences exist between two patients with the same disease. Our knowledge of human pulmonary vascular development is still incomplete, and the phenomenon of vascular remodeling occurring under different pathological and/or physiological situation remains incompletely understood.

Morphometric assessment of the pulmonary vasculature in human CDH began with observations of Kitagawa and coworkers[46]. Several later studies have documented the reduced number of arterial branches per unit lung volume with excessive muscularization of the normally non-muscular arteries leading to associated pulmonary hypertension and diminished lung perfusion in those infants[3,4,33,53,71-73]. Adventitial thickening has recently been reported in addition to the medial one constituting the main arterial morphometric changes in CDH[71-73]. It was documented that the intraacinar arteries together with the supernumerary arteries have a major role in regulating the pulmonary resistance and pressure[35-37].

Functional studies in humans

Recent studies of human cases of CDH suggest that the body attempts to overcome the vasoconstrictive state of the pulmonary vasculature[115,121]. Indeed, there may be an imbalance of vasoconstrictive and vasodilatory responses in CDH-associated PPH patients[121]. So far, few "vascular" molecules have been studied in detail in the human normal fetus (figure 2), and fewer in human CDH cases. High levels of circulating immunoreactive ET-1 have been reported in human neonates with persistent pulmonary hypertension (PPHN) and CDH[111].

Figure 2: *The" vascular" molecules studied in the human fetus.*



VEGF = vascular endothelial growth factor, IGF = insulin growth factor, PDGF = platelet derived growth factor, NOS = nitric oxide synthase, b-FGF = basic fibroblast growth factor, on the x-axis indicate gestational age in weeks. The Y-axis represents semiquantitave grading based on immunohistochemical stainig of tissue slides.

A variety of molecules of importance in vasculogenesis and vascular remodeling have been studied in CDH animal models as well as in human cases, these are shown in Table III. Since the human studies of CDH are few, the human situation is still incompletely understood.

Yamataka recently reported that newly synthesized type-I procollagen was detected in media and adventitia of pulmonary arteries of 21 newborns with CDH using M-57 antibody and also in the neointima of the included 2 patients with PPHN[72]. M-57 staining was not observed in the media of pulmonary arteries of the lungs of 8 SIDS patients. Angiogenic remodeling could be triggered through TGF- β and/or VEGF pathways in the fetal pulmonary vasculature[122].

Table III: *Different molecules in CDH vasculature as studied in human and lamb and rat models.*

Tissue	Human	Lamb	Rat
Muscle mass	↑ *46,71,72*	↑ 70	↑ 107
VEGF	↑ *123*	?	↓ *118,126*
α-actin	↑ 72	?	n 91,*126*
MHC-isoforms	?	?	n 91,*126*
Endothelin	↑ *111*	?	↑ *76*
NOS	?	n *119*	↓ 109,*110*
6-keto-$PGF_{1\alpha}$	↑ 75,*114,115,121*	?	↑ *113*
PDGF	?	?	n 91
FGF	?	?	?
TGF-α	?	?	?
TGF-β_1	n *140*	?	?
TGF-β_2	n *140*	?	?
TGF-β_3	↑ *140*	?	?
Procollagen type-I	↑ *140*	↑ *117*	↑ *141*
Tropoelastin	?	?	↑ *141*
IGF-I	?	?	↑ *142*
IGF-II	?	?	↑ *142*

VEGF = vascular endothelial growth factor, MHC = myosin heavy-chain, NOS = nitric oxide synthase, 6-keto-$PGF_{1\alpha}$ = stable metabolite of prostacyclin, PG = prostaglandin, PDGF = platelet derived growth factor, FGF = fibroblast growth factor, TGF = transforming growth factor, IGF = insulin like growth factor, ↑ = increased compared with normal controls, ↓ = decreased compared with normal controls, n = normal, not different from normal controls, ? = no data, and numbers in italic indicate the corresponding reference.

These observations suggest a potential role of TGF- ß3 but not of TGF- ß1 or TGF- ß2 in pulmonary vascular remodeling. Furthermore, SMC in muscular pulmonary arteries may actively synthesize collagen in patients with CDH and PPHN. Enhanced expression of VEGF in the pulmonary arteries in human CDH associated with PH has been reported[123]. A previous study reported that VEGF is not expressed in the normal endothelium of the developing fetus[124]. However, it was demonstrated that in

endothelial cells derived from microvessels, hypoxia and adenosine upregulate VEGF expression[124-126]. We and other investigators have reported that in the normal fetal rat lung, VEGF can be detected both at mRNA and protein levels, and that the expression is localized in bronchial epithelium and vascular SMC[126]. Paradoxically, immunohistochemical localization of VEGF was decreased in a rat CDH model[126].
The reported structural and molecular changes in the pulmonary vasculature of CDH hypoplastic lungs resemble in part that described in lungs of other forms of pulmonary hypertension such as PPHN[29,83,127,128].

1.14 Extracorporeal Membrane Oxygenation (ECMO)

Extracorporeal membrane oxygenation (ECMO) is a type of prolonged extracorporeal cardiopulmonary bypass using a membrane oxygenator. As one of the extracorporeal life support (ECLS) methods, ECMO is incresingly used to support infants and children with reversible pulmonary and cardiac failure[7-9]. The aim of ECMO institution is to maintain oxygen delivery and lung rest with low pulmonary arterial blood flow until the pathologic process of acute illness is resolved[8,9]. Since the introduction of ECMO as a therapeutic modality for CDH, this technique has been used as a last resort in many institutions. Many reports of its beneficial effect have appeared in the literature, although some controversy continues to exist[129,130]. The mechanisms underlying the observed benefits of ECMO are unknown. DeMello and Reid remarked that " an intriguing question is how ECMO in some cases allows resolution of pulmonary hypertension of the newborn. Whether this occurs by allowing growth or by (resting) the microcirculation and the small resistance arteries to avoid exposure to potential damage caused by increased blood pressure are not clear"[27]. Rather loosely, the term 'lung rest' has been put forward[7-9,27,129-133].
Recently, ECMO has been shown to decrease the stress to which the lung is exposed[9,134]. The structural and molecular changes influenced by ECMO largely remain to be identified. A better understanding of these underlying mechanisms may improve this therapeutic modality. Such improvement will be reflected directly in the management of newborns with CDH. Knowledge might be gained from models of lung injury in neonatal rats exposed to NO_2[135,136] or from human studies[9,134].

1.15 Objectives of the Studies

In spite of the detailed documentation of the morphological changes in CDH, no concepts are available concerning the pathogenesis of the vascular abnormalities. The question remains whether the pulmonary vascular changes described in CDH are the result of a maturational arrest or should be regarded an integral part of the abnormal parenchymal development of the lung[137]. Does the normal remodeling of the pulmonary vasculature occur near term? Or alternatively, have the genes responsible for this natural process not "switched off"?[138] Few comparative studies of human fetuses with different forms of pulmonary hypoplasia have been reported. The same holds true for cases of persistent pulmonary hypertension without underlying pulmonary hypoplasia[84,127,128,139].

The role of angiogenic growth factors in CDH has not yet been completely established. Based on several previous studies showing the unpredictable pulmonary reactivity to be as already was suggested by the variability in the clinical response to NO inhalation and ECMO institution, vascular reactivity in the CDH patients could be different. For these reasons, human CDH needs to be studied further, with especial attention to the pulmonary vasculature in the hypoplastic lungs, which leads to PPH in many patients.

The basic mechanisms of different therapeutic modalities to alleviate PPH are still unexplained. The stress conditions in CDH lungs reported in different studies, might be a result of ventilatory therapy. It is uncertain, whether anti-stress lung mechanisms might be of benefit to some patients. These unanswered questions, clearly show that our knowledge is still inadequate to understand the CDH problem. Clinical experience in many centers worldwide has revealed well-documented cases of responders and non-responders for the above-mentioned therapies. Whether this phenomenon can be correlated to distinct morphological changes or differences in expression of a variety of growth factors, known to be present in the different layers of pulmonary vascular wall, is one of the major challenges for the future[129-133].

In this thesis we investigated some of the deficiencies of the pulmonary vasculature in human newborns with CDH. The studies were conducted to investigate structural and molecular aspects of the pulmonary vasculature in CDH, correlating it to the functional response of the pulmonary vessels. In addition, studies were undertaken to assess the underlying mechanisms of ECMO as a therapeutic modality. Therefore, the scope of studies in this thesis is as follows:

1. Do the changes described in the pulmonary vasculature in CDH, result from developmental arrest or from lack of normal perinatal remodeling? [*Chapter 2*]
2. What is the role of various angiogenic growth factors, such as VEGF, PDGF-BB, in the pulmonary vasculature in CDH? [*Chapter 3*]
3. What is the expression pattern of nitric oxide synthase enzymes (NOS), which generate the vasodilator NO, in CDH lungs? [*Chapters 4,5*]
4. What is the state of molecular lung stress in CDH, as evidenced by expression of the relevant stress markers? [*Chapter 6*]
5. Does institution of ECMO therapy result in structural and molecular changes which may account for the improvement of the associated PPH? [*Chapters 7 totally and 2,4,5,6 partly*]

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Part II

Molecular and Structural Aspects of the Pulmonary Vasculature in Normal and CDH Newborns

Chapter 2

Developmental Structural Changes of the Pulmonary Vessels in Normal and CDH Human Neonates

Based on Article:

Impaired Structural Remodeling of Pulmonary Arteries in Newborns with Congenital Diaphragmatic Hernia: A Histological Study of 29 Cases

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2.1 Summary

Congenital diaphragmatic hernia (CDH) is associated with lung hypoplasia and pulmonary hypertension (PH) in many cases. The pathogenetic mechanisms underlying the pulmonary hypertension in CDH are not completely understood. In order to alleviate the pulmonary hypertension, new therapeutic modalities have been introduced including extracorporeal membrane oxygenation (ECMO). We studied the histology of the lungs of twenty-nine CDH autopsy cases, with special attention to the pulmonary arteries, and related our findings to gestational age and ECMO treatment. Formalin-fixed and paraffin-embedded specimens were stained with hematoxylin & eosin (HE) and elastic van Gieson (EvG) stains followed by morphometric measurements of the arterial media and adventitia. As expected, there was a significant decrease in adventitial percentage and total wall thicknesses of small pulmonary arteries with an external diameter $\leq$ 150 μm in term control newborns as compared to pre-term controls (*P*: 0.0004 and 0.05). In CDH newborns, all the measured values of the arterial wall remained significantly higher. The increase of adventitial thickness also affected the supernumerary arteries in CDH neonates. CDH newborns subjected to ECMO treatment showed a significantly thinner arterial adventitia than CDH cases who did not receive ECMO (*P*: 0.0001), the latter approaching normal values. These results indicate that in CDH, there is failure of the normal arterial remodeling processes occurring in the perinatal period. The adventitial thickening, which has been reported previously in term CDH patients only, the present study related it to difference in gestational ages. This appears to be partially reversed by ECMO treatment, thus constituting one of the mechanisms by which ECMO treatment aids in alleviating the associated PH in CDH newborns.

2.2 Introduction

Normal pulmonary arterial growth and development in the human fetus and neonate is not yet completely understood. Under normal conditions when birth is about to occur, the pulmonary arteries show rapid reduction in the medial thickness which continues in the first two weeks after birth followed by a gradual decrease till the age of 18 months[1-5]. In the fetus, pulmonary vascular resistance is high, pulmonary blood flow is low (about 35ml/min/kg fetal body weight at near-term), and the right ventricular outflow is thereby directed through the ductus arteriosus towards the placenta for gas exchange[3]. At the time of birth with the onset of extrauterine respiration, pulmonary vascular resistance falls abruptly, and pulmonary blood flow rapidly increases approximately tenfold[3]. A recent developmental study in rats revealed that in early gestation, the pulmonary vascular system develops by a combination of central angiogenic sprouting and the formation of peripheral vasculogenic lakes, which progressively communicate with each other as gestation advances[6]. A complete description of the early development of the human pulmonary vasculature is not yet available.

Congenital diaphragmatic hernia (CDH) is one of the major challenges in

perinatology with a high mortality rate approaching 50-60% in high-risk cases[7]. This high mortality is attributed to the abnormal vascular changes accompanied by lung hypoplasia in CDH, with resultant therapy-resistant pulmonary hypertension (PH) in many cases[8-10]. In CDH, pulmonary arterial abnormalities consist of: a) reduced total pulmonary vascular bed and decreased number of vessels per unit lung, b) medial hyperplasia of the pulmonary arteries together with peripheral extension of the muscle layer into the small arterioles[8,9], and c) arterial adventitial thickening, which has been reported recently[11,12]. The smaller arteries, with an external diameter (ED) less than 200 μm, are predominantly responsible for pulmonary vascular resistance[13,14]. These small arteries rapidly increase in number during the last trimester of gestation and dilate at term to accommodate the postnatal demands[15-17].

In order to decrease the PH associated with CDH and the resulting right-to-left shunt, extracorporeal membrane oxygenation (ECMO) resulting in diminution of the increased pulmonary flow for a period of time up to 3 weeks, with delayed surgery after patients' stabilization, has been advocated to "rest" the lung[18,19]. The exact effects of ECMO in CDH infants are not yet completely understood and the possible effects of ECMO on the pulmonary vascular architecture remain unknown[20].
The present study was carried out in order to investigate the structural changes of pulmonary arteries during pre-and post-natal development in CDH, and to study the possible arterial structural changes following ECMO treatment of CDH cases.

2.3 Materials and Methods

Tissue Specimens

We studied twenty-nine consecutive neonatal lung autopsy specimens of neonates with CDH and associated lung hypoplasia, confirmed by a lung / body weight index ≤ 0.012[21]. Materials were retrieved from the archives of the Department of Pathology, Erasmus University Medical Center, Rotterdam. The university ethical committee approved the experimental design and protocols. Tissue specimens were grouped into three groups: CDH-1 group: 5 CDH neonates with a gestational age below 34 weeks [mean 31.6±0.87 weeks]; CDH-2 group: 20 CDH neonates with a gestational age above 34 weeks [mean 38.6±0.41 weeks]. The later group included 5 newborns who died $\leq$ 1 hour after birth. Both of these groups did not receive ECMO treatment. The third group, CDH-3, consisted of 4 term CDH neonates [mean gestational age: 39±2.04 weeks], who received ECMO treatment for an average bypass time of 270 hours. We examined randomly either side of the lung in this study, since on histological screening we did not find significant differences between them. Similarly, others failed to find a difference between the arterial histology of the ipsilateral or contralateral lungs in high-risk group of CDH[22].
Ten other neonates, who died either from acute placental insufficiency, or birth

asphyxia within the first 24 postnatal hours, and who lacked macroscopic or histological features of lung hypoplasia, served as controls. These were divided into two groups of 5 cases each: CON-1 group: neonates with a gestational age below 34 weeks [mean 25.9±0.85 weeks], and CON-2 group: neonates with a gestational age above 34 weeks [mean 38.8±0.61 weeks]. No cases of stillbirth were included in any of the subgroups. All autopsy lung specimens were fixed by immersion in formalin, and embedded in paraffin. Serial 6 μm thick sections were processed for histopathological and immunohistochemical examination.

Clinical Background

Tissue specimens included in this study were obtained from patients with CDH, who were treated in Sophia Children's Hospital, died and parent's consent for autopsy were obtained during the period 1973-1997. All CDH cases suffered from severe respiratory insufficiency, becoming clinically apparent within the first 6 hours after birth, thus fulfilling the criteria of high-risk group of CDH[22-24]. In ten cases, the CDH was right- sided, in 18 cases, it was left-sided and one case had bilateral CDH. Three cases had additional major congenital anomalies: Fallot's tetralogy, tracheo-oesphegeal cleft, and trisomy 21, (one each). All cases were subjected to standard management protocols including delayed surgery from 1986 and ECMO management since the introduction of ECMO in our center in 1992. All CDH patients were subjected to routine cardiac ultrasonography which indicate PH and right-to-left shunting, together with the clinical observation of preductal and postductal transcutaneous O_2 saturation differences of > 10%[23] in all cases. In our institution, for CDH patients, the entry criteria for ECMO were: gestational age of at least 34 weeks; birth weight at least 2000 g; artificial ventilation for less than 7 days; alveolar-arterial oxygen difference (A-aDO_2) > 80 KPa (600 torr); maximal PaO_2 at least 10 KPa[23,24]. The ECMO treated group of CDH has received the treatment for a mean bypass time of 270 hours. Controls were subjected to similar ventilatory therapy for up to 16 hours. No clinical features of PH were reported in any of them.

Histological Staining

Serial sections were mounted on polylysine-coated glass slides, and stained separately with hematoxylin/eosin (HE) stain and with Elastic van Gieson's (EvG) stain using Weigert's solution (resorcinol-fuchsin), which stains elastic fibers dark violet, collagen fibers red, and smooth muscle brownish yellow.

Immunohistochemistry

Paraffin sections of the lung tissues were mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method. Deparaffinized slides were treated with 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity. Slides were placed then in a Sequeza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn, USA). Slides were preincubated for 15 minutes with normal goat serum to block non-specific binding, then incubated for 30 minutes at room temperature with a mouse monoclonal anti-human α-smooth muscle actin (α-SMA) antibodies (clone 1A4:

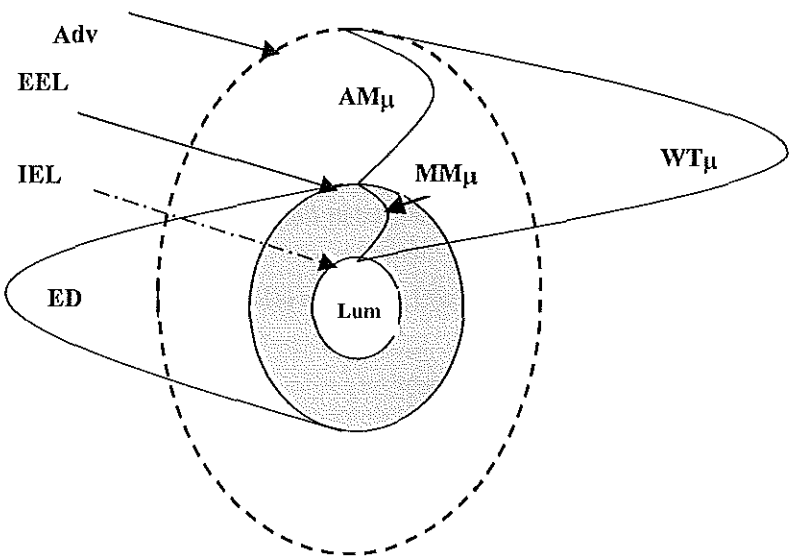
Biogenex, San Ramon, MO, USA) as a primary antiserum in dilution of 1:200. After rinsing with phosphate buffered saline, slides were incubated for 30 minutes with biotinylated secondary antibodies (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). After further rinsing and incubation for 30 minutes with peroxidase conjugated streptavidin (Biogenex, San Ramon, MO, USA) in a dilution of 1:50, slides were labelled with diaminobenzidin as the chromogen. Slides were counter-stained with Mayer's hematoxylin for 20 seconds. Negative controls were prepared by omission of the primary antiserum. EvG and α-SMA immunostaining were compared on serial sections to check that the media is colocalized with α-SMA in pulmonary arteries before starting the morphometric measurements.

Morphometry

Pulmonary arteries were identified on the basis of both position and structure. Pulmonary arteries run mainly along the airways, and have a distinct inner and outer elastic lamina. Only arteries fulfilling these criteria, and with an external diameter (ED) as measured between the external elastic laminae (EEL) of up to 150 μm, and having a complete muscular coat, were measured. Absence or discontinuity of the medial muscle layer of an arterial branch indicates arteriolar morphology[16]. The small pulmonary arteries were grouped into two groups according to ED; the first group of arteries with ED less than 100 μm, and the second group of arteries with ED between 100 and 150 μm. Only arteries that were cut at approximately right angles, so that the maximal ED exceeded the minimal diameter by less than 50%, were analyzed. From each slide, a random area of 12 x 12 mm at the mid-lung area was chosen, and all the arteries fulfilling these criteria were included in the measurements. An average number of 30 arteries from each section were thus assessed. Measurements of the arterial wall layers in microns were done on EvG stained sections using a calibrated eyepiece. Medial thickness in microns (MMμ) was calculated as the distance from the external elastic lamina (EEL) to the internal elastic lamina (IEL) along the shortest diameter of the artery (***Figure 1***). Adventitial thickness in microns (AMμ) was calculated along the shortest arterial diameter. Total wall thickness in microns (WTμ) consisted of [MMμ + AMμ] along the same diameter since the thickness of the intima was minimal in all cases.

Medial thickness (MT) and adventitial thickness (AT) were expressed as a percentage (%) of ED. The measured parameters are demonstrated in ***Figure 1***.

Figure. 1: *Diagram of the morphometric measured parameters*



Where; Adv: adventitial outer limit, ED: external arterial diameter, EEL: external elastic lamina, IEL: internal elastic lamina, Lum: arterial lumen, MMμ: medial thickness in μm, AMμ: adventitial thickness in μm and WTμ: wall thickness in μm.

Statistical Analysis

Values were calculated from different patients' groups and represented as mean ± SEM (standard error of mean) for each variable.

Data from CDH subgroups were compared to their age comparable controls, and to other CDH subgroups. All comparisons were done using the unpaired Student's "*t*" test. Analyses were performed with the Microsoft Excel software package. Differences in results were considered significant where $P \leq 0.05$ value at probability level.

2.4 Results

Histopathological and Morphometrical Results

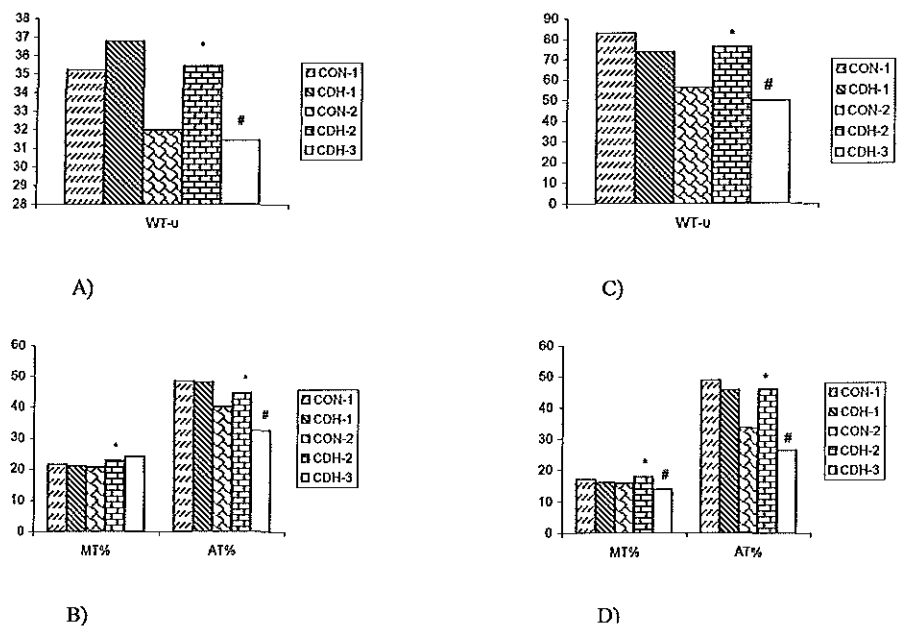
Examination of both the HE and EvG stained sections revealed significant medial and adventitial thickening in small pulmonary arteries of all CDH cases.

The thick arterial adventitias in the small pulmonary arteries of pre-term control cases progressively become thinner when approaching term (***Figure 2A&B***). This

reduction in adventitial thickness did not occur in CDH lungs, (***Figure 2C&D***). However, in ECMO treated CDH patients, thinning of the adventitia was observed, (***Figure 2E***). Also, adventitial thickening was observed in the pulmonary supernumerary arteries in CDH cases.
The smallest completely muscularized artery in CDH cases examined, had an ED of 25 μm. All the morphometry was performed after comparison of sections stained with EvG to immunohistochemical localization of α-SMA (***Figure 2F***).
See picture on page 159

The mean values of measured variables representing total wall thickness and medial and adventitial percentages of the small pulmonary arteries with an ED ≤ 150 μm are schematically represented in ***Figure 3***.

Figure. 3: *The mean of the measured parameters in the different study subgroups*



Where; CON-1: control pre-term, CDH-1: Pre-term congenital diaphragmatic hernia, CON-2: term controls, CDH: term diaphragmatic hernia without ECMO treatment, and CDH-3: term diaphragmatic hernia with ECMO treatment. AND where; WT-u: Total wall thickness in μm, MT%: Medial thickness as percent of the arterial ED and AT%: Adventitial thickness as percent of the arterial ED. A&B representing the values of arteries with ED < 100 μm, while C&D representing the values of arteries with an ED of 100-150 μm. [statistical significance was indicated (*) between CDH and control and (#) following ECMO treatment in CDH group at P value of ≤ 0.05].

Morphometrical analysis for each of the subgroups showed the following results:

Control group:

In the control group, the adventitia of arteries less than 100 μm was significantly thicker with higher AT (*P*: 0.0004) in the pre-term group than in the term neonates (***Figure 2A&B***). This was also reflected in the WTμ thickness (*P*: 0.05). In larger arteries with a ED ≥100 - ≤150 μm, both parameters of the pre-terms were significantly higher with *P*: 0.0002 for AT and 0.0001 for WTμ. The mean values were depicted in table I from the different groups.

CDH group without ECMO treatment:

Term CDH cases had significantly thicker media and adventitia than controls for all the measured variables; WTμ, MT and AT of arteries under 100 μm ED (***Figure 2C&D***) where *P* values were 0.02, 0.005, and 0.01 for AT, MT, and WTμ respectively. Similarly, significant high values were observed the second group of arterial ED in CDH group where *P*: 0.0003 for AT and 0.0001 for WTμ. In this group comparison between both gestational age groups, CDH-1 and CDH-2 revealed only, significant difference for MT in the arteries with an ED under 100 μm, as the term CDH group showed higher value than the pre-term one with a *P* value was 0.05. There were no significant differences in the measured parameters between the 5 patients who died in the first hour postnatally and those who received ventilatory support for longer time period.

Effect of ECMO on vascular morphology of CDH:

ECMO treated CDH cases had the thickest media of arteries under 100 μm ED (12.85 μm±2.39), with *P* value of 0.008 for MMμ when compared to group CDH-2 of non-ECMO treated CDH cases. In pulmonary arteries with an ED less than 100 μm, significant thinning of the adventitia and wall thickness values were observed compared to group CDH-2 (***Figure 2E***) with *P* values of 0.0001 for AT and 0.04 for WTμ. Pulmonary arteries with an ED up to 150 μm showed similar significantly lower values in the ECMO treated group in relation to the non ECMO treated CDH term group regarding all measured parameters in which *P* values were 0.0001, 0.01, and 0.0008 for AT, MT and WTμ respectively.

Table I: *The mean of measurements of arterial wall thicknesses of the patient's subgroups*

Measurement	Gestational age < 34 weeks		Gestational age > 34 weeks		
	CON-1	CDH-1	CON-2	CDH-2	CDH-3
Arterial ED under 100 μm:					
MMμ	10.31	11.03	10.5	11.44	12.85
AMμ	25.0	26.38	21.48	23.83	18.56
WTμ	35.22	36.78	31.98	35.42	31.42
MT	21.5%	21.0%	20.6%	22.7%	24.0%
AT	48.4%	48.0%	40.1%	44.6%	32.5%
Arterial ED 100 -150 μm:					
MMμ	21.5	19.19	18.08	21.78	17.0
AMμ	61.5	54.52	38.08	55.48	32.67
WTμ	83.0	73.71	56.16	76.21	49.67
MT	17.1%	16.1%	15.9%	17.8%	13.9%
AT	48.7%	45.6%	33.5%	45.9%	26.4%

Where: MMμ: Medial thickness in μm, AMμ: Adventitial thickness in μm, WTμ: Wall thickness in μm, MT: Medial thickness as percent of the arterial external diameter and AT: Adventitial thickness as percent of the arterial external diameter (ED).

2.5 Discussion

In this study, we investigated the developmental pattern of small pulmonary arteries in CDH neonates and found a lack of the progressive adventitial thinning seen in normal neonates. This CDH-related persistence of a thick arterial adventitia was, however, abolished partially by ECMO treatment.

Several previous investigators have found that during the final stages of gestation, the pulmonary arteries become progressively more thin-walled[4-6]. So far, attention has largely focused on the arterial media. In this study, control cases with normal lungs exhibited progressively lower values of pulmonary arterial adventitial thickness when their age approached term. Both medial and adventitial thinning probably contribute to a decrease of the pulmonary vascular resistance, necessary for postnatal life. As suggested earlier[14], adventitial thinning near term may be one of the causes of increased compliance in small pulmonary arteries. Beals et al. reported postnatal remodeling of pulmonary vessels in CDH cases, although these authors concentrated more on the media of pulmonary arteries[22]. Our results are not only related to luminal widening, as the adventitial percentage (AT) also, not only the absolute value of adventitial measurement in microns were significantly lower in CON-2 group as compared to either CON-1 or CDH-2 groups. Moreover, we found that in the ECMO group no difference was found in values of cases who received ECMO treatment for less than 30 hours, and died from intracranial hemorrhage, from the case, which received ECMO for 11 days and died after decannulation.

The number of cases in our study is limited, however, in the CDH group we found persistence of the high values of the medial and adventitial thickness of small pulmonary arteries with an ED $\leq$ 150 μm. We have chosen the gestational age of 34 weeks to divide our study groups, since at that time, the saccular stage of lung development is nearly completed[8,25]; while the same time-frame was used earlier in comparable studies[21,26].

Small pulmonary arteries are known to have a major role in regulating the pulmonary resistance and pressure[13,14,17]. We observed increased adventitial thickness around the supernumerary arteries in CDH cases. This adventitial thickening in small pulmonary arteries could reduce their ability to open and/or dilate in order to increase the vascular bed capacity and reduce the pressure in the pulmonary bed after birth. Even a vicious circle of increased intraluminal pressure leading to increased arterial wall thickness and vice versa could be initiated, as reported in an animal model of pulmonary hypertension[27].

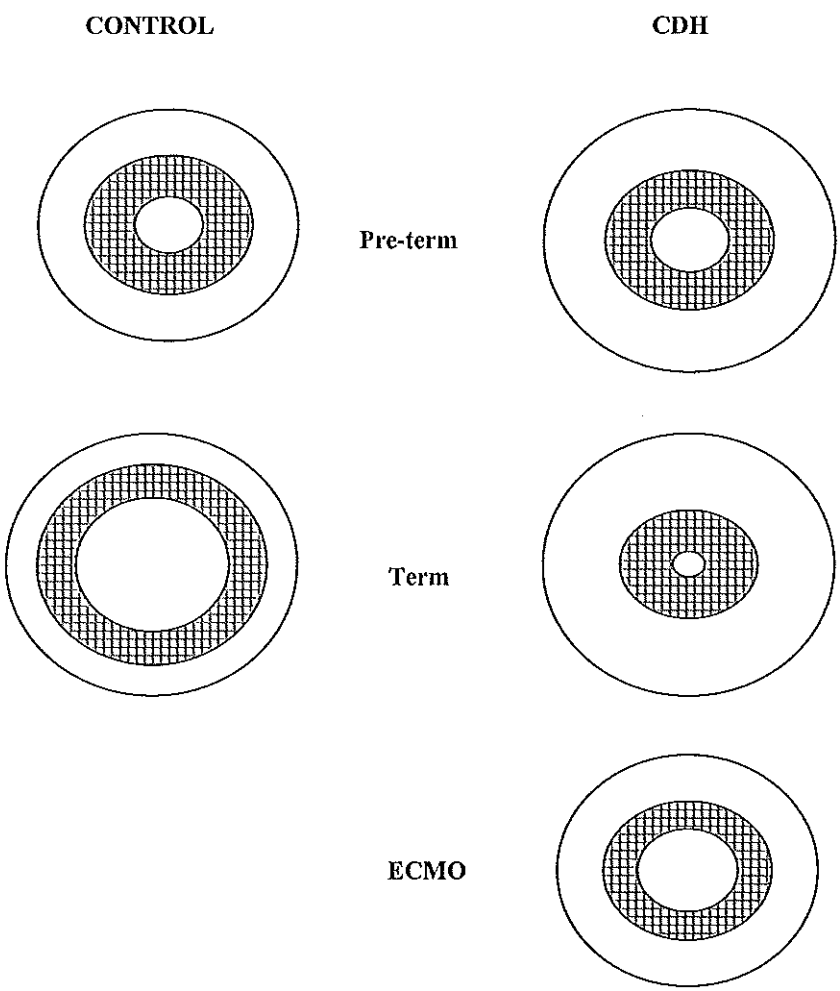
Our knowledge of the pathogenic mechanisms underlying pulmonary hypertension in CDH are still incomplete. Yamataka and Puri mentioned increased adventitial thickness in term CDH neonates that contribute to the persistence of PH[11]. We demonstrate pulmonary arterial structural changes in CDH in relation to gestational age and ECMO treatment. Wigglesworth and Desai reported that infants with lung hypoplasia in CDH born at 34-39 gestational weeks

have a lung cell population comparable to that of a normal fetus at 20-22 weeks[28]. The significant higher MT observed in term CDH compared with the pre-term CDH possibly represent a form of vascular remodeling from a prolonged duration of exposure to persistent high pulmonary pressure. Undoubtedly, the thickening of the adventitia will have affected the mechanical properties of the vessel wall[29] and may cause, or contribute to, the therapy-resistant PH observed in many high-risk patients with CDH. The difference in the physical properties between medial smooth muscle cells and elastic fibers may explain the high failure rate of prostaglandin or inhaled nitric oxide treatment in CDH prior to ECMO institution in some cases of CDH associated PH[24,26]. The interrupted natural process of pulmonary vascular development has been also observed in neonates with so-called idiopathic persistent pulmonary hypertension[30,31].
This is in accordance with our findings because no significant differences were found regarding AT and WT_{μ} between the term CDH group and both pre-term groups either CDH or control.

Meyrick and Reid reported that structural changes in the media and adventitia can be induced by hypoxia[32], and changes in the muscle cell population in an animal model of persistent pulmonary hypertension has been reported[30]. In our study the structural abnormalities are unlikely to be caused by hypoxia, since the patients were subjected to the same immediate ventilatory support, and especially during ECMO the arterial oxygen tension is maintained at a constant and sufficient level. More importantly the CDH cases who died in less than one hour after birth showed the same arterial abnormalities as the cases subjected to ventilatory support for 48 hours.

The state of stunted pulmonary vascular growth in CDH[33,34], and the vascular changes are similar to patients with idiopathic PH[30,35]. These findings point to failure of the naturally occurring structural remodeling of the pulmonary arteries in newborns with CDH. Our findings are summarized schematically in ***Figure 4***.

Figure.4: *Schematic representation of the hypothesis based on our results*



Where the figure demonstrates failed normal structural remodeling in CDH compared to controls in relation to gestational age and ECMO treatment, where the outer white circle represents the arterial adventitia and the stripped circle represents the arterial media.

The mechanism, by which ECMO is beneficial, originally described as "lung rest" [19,20,36-38], is not yet completely cleared. Indeed, Thibeault and Haney recently demonstrated persistence of a thick arterial media in ECMO treated CDH patients[39]. In our study the ECMO treated CDH group exhibited the highest medial thickness (12.85±2.39 μm), reflecting a severe degree of pulmonary vascular abnormality in CDH patients, especially in those subjected to ECMO therapy. There is some evidence from animal models that smooth muscle cells play a critical role in the pathogenesis of vascular changes of pulmonary hypertension, by modifying the phenotype of surrounding adventitial cells[28,30,35]. Although the underlying cause remains incompletely understood, significant reduction in adventitial thickness and total wall thickness following ECMO treatment was observed. In this way, ECMO treatment of CDH neonates can be said to result in reversal of some of the arterial structural changes in hypoplastic lungs of CDH patients. Our finding of adventitial thinning of small pulmonary arteries, points to one of the mechanisms by which ECMO treatment may lead to improvement in pulmonary hypertension associated with congenital diaphragmatic hernia. However, the prediction of this phenomenon for the individual patient is impossible at the present time.

Further studies to unravel the molecular mechanisms underlying these structural changes, would provide useful information in modifying the treatment of high-risk newborns with CDH.

Acknowledgments

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Chapter 3

The Role of Vascular Endothelial Growth Factor in the Vascular Abnormalities of Human CDH

Based on Article:

Enhanced Expression of Vascular Endothelial Growth Factor in Lungs of Newborn Infants with Congenital Diaphragmatic Hernia and Pulmonary Hypertension

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Thorax. 54; 427-431, 1999.

3.1 Summary

Pulmonary hypoplasia accompanied by therapy resistant pulmonary hypertension (PH) is an important feature of congenital diaphragmatic hernia (CDH). The pathogenesis of the pulmonary vascular abnormalities in CDH remains to be elucidated at the molecular level. Vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen, is known to play a role in pulmonary angiogenesis and vascular remodeling, however no data on VEGF expression are available in CDH patients.

Twenty-one lung autopsy specimens of human CDH patients with lung hypoplasia and of seven age-matched control newborns without lung hypoplasia were processed for immunohistochemistry using affinity purified anti-human VEGF antibodies. All CDH cases had pulmonary hypoplasia, as evident from a lung/body weight index ≤ 0.012 and pulmonary hypertension documented by repeated cardiac ultrasound. Cellular localization of VEGF was semiquantitatively analyzed using a staining score ranging from 0 (no staining) to 4 (very strong staining). The results were statistically evaluated with accepted significance at P level of ≤ 0.05.

Significantly elevated levels of VEGF immunoreactivity were observed in CDH lungs as compared to the controls. VEGF was mainly detected in bronchial epithelium and in medial smooth muscle cells (SMC) of large (> 200 μm) and small (< 200 μm) pulmonary arteries, with the most intense staining of the pulmonary vasculature in medial SMC of small pulmonary arteries. In CDH patients, but not in controls, endothelial cells were positive for VEGF staining.

This is the first study on VEGF expression in human CDH neonates. Elevated expression levels of VEGF, especially in the small, pressure-regulating pulmonary arteries, point to a potential role in vascular remodeling. Perhaps this may reflects an unsuccessful attempt of the developing fetus to increase the pulmonary vascular bed in the CDH hypoplastic lungs in order to alleviate the associated pulmonary hypertension.

3.2 Introduction

Congenital diaphragmatic hernia (CDH) remains one of the major challenges in pediatric surgery and neonatology. Despite recent developments in therapeutic modalities such as delayed surgery, exogenous surfactant therapy, nitric oxide (NO) inhalation, extracorporeal membrane oxygenation (ECMO), and partial liquid ventilation, the mortality rate remains around 40% in high-risk cases[1-3]. The main documented pathological findings in CDH lungs are lung hypoplasia and pulmonary vascular abnormalities. The latter consist of: a) reduced total pulmonary vascular bed and decreased number of vessels per volume unit of lung, b) medial hyperplasia of pulmonary arteries together with peripheral extension of the muscle layer into small arterioles[4-6]. The most common cause of the unfavorable outcome in human CDH is persistent pulmonary hypertension (PH)[2-5]. Indeed, follow up of surviving patients with CDH has revealed that the pulmonary perfusion scan does not improve although the ventilation scan improves towards nearly normal level[7].

Recently a developmental study of the lung vasculature showed that in early gestation the pulmonary vasculature develops by a combination of central angiogenic sprouting and the formation of peripheral vasculogenic lakes, which progressively communicate with each other as the gestation advances[8]. A number of growth factors with a proven or potential role in vascular development and remodeling in health and disease conditions have been identified[9,10]. Vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, has been reported to have a narrow target cell specificity to endothelial cells. Moreover, VEGF regulates vasculogenesis and postnatal vascular remodeling[11-13]. VEGF binds to high affinity cell surface receptors, KDR/flk and flt, which are predominantly expressed in endothelial cells[13]. Expression of VEGF is up regulated under a variety of pathophysiological conditions, including pulmonary hypoxia[12-14].
In order to investigate the pathogenesis of the underlying vascular abnormalities in CDH lungs, we investigated the cellular localization of VEGF in pulmonary autopsy specimens obtained from human CDH and age-matched control neonates.

3.3 Materials and Methods

Tissue Specimens:

Lung tissue specimens used in this study were obtained from our archival collection at the department of Pathology. These specimens represent the available material from patients with CDH, who were treated in Sophia Children's Hospital, died and parents' consent for autopsy was obtained during the period 1981-1997. Twenty-one CDH cases were identified. All cases were associated with lung hypoplasia, as evident from a lung/body weight ratio index ≤ 0.012[15]. The control group consisted of 7 age-matched neonates who died in the first 24 hours of extrauterine life because of neonatal asphyxia or placental insufficiency. These control cases did not have lung hypoplasia on histological screening. The CDH group had a gestational age varying from 35 weeks to term, with a mean of 38.4 weeks, while that of the control group was 35.1 weeks. Neither the CDH nor the control group was subjected to ECMO treatment. We examined randomly either side of lung in this study, since on histological screening we did not find significant differences between the two lungs, as all cases represent the high-risk group of CDH. Lung tissue specimens were fixed in formalin by immersion-fixation and embedded in paraffin for histopathological examination and immunohistochemistry.

Immunohistochemistry:

Paraffin sections (6 μm thickness) of the lung tissues were cut and mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method as described earlier[16,17].

In brief, after deparaffinization in xylene and rehydration through graded alcohol, the slides were rinsed with water and phosphate buffered saline (PBS) and placed in a Sequeza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn). Slides were preincubated for 15 minutes with normal goat serum to block non-specific binding, then incubated for 30 minutes at room temperature with affinity-purified rabbit polyclonal antibodies in a dilution of 1:200. The anti-VEGF antiserum used was raised against a 20 amino acid synthetic peptide corresponding to residues 1-20 of the amino

terminus of human VEGF[18] (Santa Cruz Biotechnology, Inc., Santa Cruz, USA).
The optimal dilution was identified by examining the intensity of staining obtained with a series of dilutions of the antiserum from 1:50 to 1:500. The dilution (1:200) resulted in specific and easily visible signals in paraffin sections of a capillary hemangioma. The hemangioma sections served as a positive control in the study. After washing with PBS, the test and control slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). After two washes in PBS, slides were incubated for 30 minutes with alkaline phosphatase conjugated streptavidin (Biogenex) in a dilution of 1:50. Finally, the slides were rinsed with 0.2 M TRIS-HCL pH 8.0, Levamizole (Sigma) was used to block the endogenous alkaline phosphatase activity, and stained for 30 minutes with 0.3% New Fuchsin/TRIS-HCL (Sigma) as color enhancement system. Negative controls were prepared by omission of the primary antiserum. Slides were lightly counterstained with Mayer's hematoxylin for 10 seconds.

Immunolocalization of VEGF in endothelial cells as well as SMC was verified by staining these cells with specific markers. Endothelial cells were identified by CD31 immunostaining[19]. Staining was done by the ABC method, but using 0.025% 3,3-diaminobenzidine (DAB) as chromogen. Slides were incubated for 20 minutes in methanol with 0.3% H_2O_2 to block the endogenous peroxidase activity. Slides were incubated with the primary anti-human CD 31 monoclonal antibody in a dilution of 1:80 (Dako Corporation, Glostrup, Denmark) at room temperature for 30 minutes and subsequently visualized after developing the color using 0.025% 3,3-diaminobenzidine (DAB) (Sigma). Employing DAB based color development method, consecutive tissue sections were stained with anti-human mouse monoclonal alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex) in a dilution of 1:200.
Semiquantitative Analysis:
Prior to screening sections were coded, so that the observers were unaware of the clinical details of the case under study. Expression of VEGF was analyzed semiquantitatively, using a semiquantitative visual scale, ranging from 0-4: grade 0 = no staining, grade 1 = focal staining, grade 2 = diffuse faint, grade 3 = diffuse moderate and grade 4 = diffuse strong staining, respectively[20]. The entire slide of a tissue block, taken from the mid-lung area was investigated and scored at the same magnification by three independent observers. The average of the three scores used for subsequent analysis. Sections were graded from 0-4 for the localization of VEGF in the bronchial epithelium, endothelium and medial SMC in small (50-200 μm external diameter ED) and large (> 200 μm ED) pulmonary arteries[21]. This scoring method has previously been shown to allow the detection of differences in expression level as small as 1.5 times[20,22].
Statistical Analysis:
The VEGF staining score was calculated from the two groups, and the results were expressed as Median. Before ranking score values were rounded to the complete number. Statistical analysis was performed after ranking using one of the non- parametric tests either, Mann–Whitney test or Fisher's exact test according to what is appropriate to the compared groups.

Significance of the results for probability value was accepted at $P \leq 0.05$.

3.4 Results

Clinical and Autopsy Data:

Thirteen of the 21 diaphragmatic hernias were left sided; the remaining 8 were right sided. All patients presented in the first six hours of life and were treated according to a standard protocol including conventional mechanical ventilation, cardiac ultrasound, and delayed surgery[1]. Clinical evidence of right-to-left shunting was obtained by preductal and postductal transcutaneous O_2-saturation differences of > 10% in CDH cases[23]. Echocardiography documented the right to left shunt and pulmonary hypertension. In three cases, associated major congenital anomalies were found: Fallot's tetralogy, tracheo-esophageal cleft, and trisomy 21, respectively (one each). Nineteen of the CDH cases died within 48 hours postnatally, five of these cases died in the first hour. In 3 instances hyaline membrane disease was observed, whereas one patient developed pulmonary bleeding. All seven age-matched controls died in the first 24 hours after birth. Control cases were subjected to ventilatory therapy in settings similar to that of CDH cases, including inspiratory oxygen fraction of 1.0 for variable periods of time up to 16 hours postnatally.

Localization of VEGF:

We detected VEGF in the bronchial epithelium and medial arterial SMC in control cases. Distinct VEGF immunostaining was identified in the bronchial epithelium and in the medial SMC of pulmonary arteries in tissue specimens from the CDH group, as verified with the immunolocalizaton of αSMA. VEGF immunoreactivity in bronchial epithelium and arterial medial SMC was more intense in the CDH cases than in the controls (Fig 1. A& B).

See picture on page 161

In the CDH cases, VEGF staining in pulmonary vasculature was most intense in the medial SMC of small pulmonary arteries, with an ED under 200 μm. High VEGF expression levels were noticed also in the supernumerary arteries of CDH cases.

Furthermore, VEGF expression was detected in the endothelium of pulmonary arteries only in CDH cases (Fig 1.C). This endothelial staining was colocalized with consecutive sections stained with the endothelial cell marker, CD 31 (Fig 1.D). No VEGF immunopositivity was detected in the endothelium of control cases (Fig 1.B). Weak VEGF expression was observed in the medial SMC of large pulmonary veins in CDH cases.

There were no differences in VEGF expression pattern between CDH cases that were artificially ventilated up to 48 hours and the five cases that died in the first hour after birth despite maximal attempted resuscitation, even without receiving vigorous ventilatory support. No VEGF expression was observed in the arterial medial SMC of pulmonary veins in the control group.

Mean graded score values of the semiquantitative analysis for the VEGF expression in CDH cases showed a maximal expression score value of 3.38 in the bronchial epithelial

cells. Endothelial and medial SMC of large pulmonary arteries (ED > 200 μm) depicted low staining values of 0.5 and 1.43, respectively.
Statistical analysis of VEGF expression scores in the two groups; CDH and controls, showed significantly higher levels ($P \leq 0.05$) in the bronchial epithelium, medial SMC of large and small pulmonary arteries where P values were 0.001, 0.027, and 0.002, respectively, using the non-parametric Mann-Whitney U test as shown in Table I.

Since no expression of VEGF in endothelium of pulmonary arteries in control cases was observed and the score was always zero, Fisher's exact test was considered more appropriate for the comparison of endothelial staining scores[24,25]. Significantly higher expression was observed in the endothelial cells of CDH cases using Fisher's exact test, with P values of 0.01 and < 0.001 for large and small pulmonary arteries, respectively, as compared to controls.

Table I: *Table of ranking data for Mann-Whitney U test*

Tissue examined	N	Mean rank	Sum of ranks	P value
Bronchial epithelium				
CDH	21	17.38	365.0	0.001*
control	7	5.86	41.0	
Large artery medial SMC				
CDH	21	16.45	345.0	0.027*
control	7	8.64	60.5	
Small artery medial SMC				
CDH	21	17.12	359.5	0.002*
control	7	6.64	46.5	

Where: CDH = congenital diaphragmatic hernia, SMC = smooth muscle cells, N = number and (*) indicates statistical difference at $p \leq 0.05$.

3.5 Discussion

We have found increased VEGF immunoreactivity in medial SMC and endothelium of pulmonary arteries in CDH cases with pulmonary hypoplasia. The highest levels of expression in the pulmonary vasculature were observed in the medial SMC of arteries with a diameter of less than 200 μm, and especially in the supernumerary arteries, which are known to play an important role in pulmonary blood pressure regulation and vascular resistance[26].

Our results are in agreement with a previous experimental report confirming that, a source of VEGF is the arterial medial SMC[27]. The increased VEGF expression detected in CDH cannot be due to artificial ventilation, since we did not find any difference in VEGF expression between the patients ventilated for short (up to 1 hour) or longer (up to 48 hours) periods. In addition, no differences in the degree of VEGF expression were found in bronchial epithelium in the CDH group, regardless whether or not these lungs were exposed to high levels of inspiratory oxygen or volume trauma and shear forces related to variable periods of artificial ventilation.
Since no significant differences were observed among either lung side in case of high-risk group of CDH regarding lung hypoplasia as descriped previously[28,29], so we have examined randomly either side of lungs from CDH group and also we have found no differences.

VEGF is recognized as an endothelial cell mitogen and angiogenic inducer with activity restricted to the vascular endothelial cells[30,31]. VEGF is expressed in a variety of cells, and a paracrine mechanism of action has been suggested, whereby non-endothelial cells secrete VEGF, which modulates the vasculogenesis and angiogenesis in the adjacent vascular endothelium[27]. This important angiogenic role of VEGF is evidenced from the fact that abnormal vessel development leading to death occurred in embryos lacking a single VEGF allele[32], or followed the experimental inactivation of the VEGF gene by replacing the coding sequence of exon-3 of the VEGF gene in embryonic stem cells[33].
We report endothelial reactivity for VEGF in the pulmonary vasculature of human CDH hypoplastic lungs and not in the control cases, the latter being in accordance with a previous study report that VEGF is not expressed in the normal endothelium of the developing fetus[27]. However, it has been previously documented that in endothelial cells derived from microvessels, VEGF expression can be up regulated in vitro by hypoxia and adenosine[34,35].
There is much similarity between the structural changes in the pulmonary vasculature in CDH hypoplastic lungs and that of another pediatric form of pulmonary hypertension, namely, persistent pulmonary hypertension of neonates[9,36]. It is unclear whether similar growth factors and cytokines contribute to these vascular abnormalities[37]. Furthermore, it is of note that increased VEGF expression has been reported in lungs of patients with primary pulmonary hypertension[37].
Our knowledge of vascular development in congenital diaphragmatic hernia and/or vascular remodeling following postnatal interventions is far from complete, and it is too early to speculate in detail about the pulmonary vascular abnormalities at the molecular level. No doubt, growth factors play an essential role in the pulmonary and vascular development and maturation[38,39]. The increased VEGF expression in small-diameter and supernumerary pulmonary arteries in CDH cases complicated by PH, may reflect an -apparently unsuccessful- attempt of the developing fetus and the neonate to compensate for the stunted lung vessel growth and/or to stimulate the arterial angiogenesis of the pulmonary pressure-regulating arteries caused by a mechanism which remains to be identified.

3.6 References

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Part III

Functional Molecules and Pulmonary Vasculature of Neonatal Lungs in Human Congenital Diaphragmatic Hernia

Chapter 4

Inducible NOS expression in human CDH and age-matched controls

Based on the article:

Altered Pulmonary Expression of Inducible Nitric Oxide Synthase in Human Newborns with Congenital Diaphragmatic Hernia

Shehata SMK, Sharma HS, Van der Staak F, Van de Kaa-Hulsbergen C, Mooi WJ and Tibboel D.

Submitted.

4.1 Summary

Nitric oxide (NO) is a potent vasodilator. Its production is catalyzed by nitric oxide synthase (NOS), the three isoforms of which are endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). The underlying pathogenic mechanisms of pulmonary hypertension (PH) in congenital diaphragmatic hernia (CDH) are not completely understood. Extracorporeal membrane oxygenation (ECMO) has been used to rest these lungs and to diminish the PH. The role of NOS in human CDH is not addressed before, so we investigated its expression in the lungs of CDH neonates and age-matched controls. Also, we investigated the possible effects of ECMO treatment on iNOS expression levels. Thirty-three archival lung specimens of CDH neonates with lung hypoplasia were studied. 23 of these CDH cases were not subjected to ECMO treatment, while the remaining 10 cases had been treated with ECMO for a mean time of 238 hours. Eleven age-matched neonates without lung hypoplasia, who died from neonatal asphyxia or placental insufficiency served as controls. Paraffin-embedded specimens were processed for immunohistochemical localization of iNOS using anti-human iNOS monoclonal antibodies. Computer assisted video image analysis for the endothelial expression of iNOS in the pulmonary vessels with an external diameter (ED) < 200 μm was performed. Statistical analysis was based on the Kruskal-Wallis non-parametric test with significant P value at ≤ 0.05. Inducible NOS was localized in the vascular endothelium, as verified by the endothelial marker [CD-31] staining on consecutive sections. Endothelial expression of iNOS in small pulmonary arteries was reduced in non-ECMO treated CDH cases as compared to controls ($P < 0.041$). However, ECMO treatment was associated with an increase in the endothelial expression of iNOS ($P < 0.034$) as compared to the non-ECMO treated CDH group. Alveolar macrophage showed iNOS staining which was significantly higher in CDH cases than controls, as well as the number of CD 68 positive cells increased in ECMO-treated group. Our results demonstrate that vascular expression of iNOS is down regulated in CDH lungs and ECMO treatment appears to rescue such decrease. This could lead to abnormal vascular reactivity or non-responsiveness to the released NO. Although ECMO has been believed to induce "rest" of the lung and initiates vasodilatation, the increased iNOS in alveolar macrophages points to that the "stress" still present in the lung tissue during ECMO.

4.2 Introduction

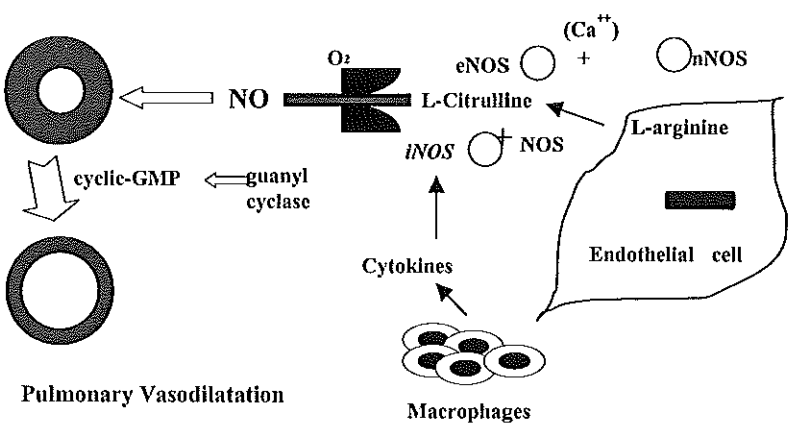
The high mortality and morbidity among infants with congenital diaphragmatic hernia (CDH) are largely determined by the severity of lung hypoplasia and the associated therapy resistant pulmonary hypertension (PH)[1]. Pulmonary vascular abnormalities in CDH consist of a decreased number of pulmonary arteries per unit lung volume and peripheral muscularization of small arteries with medial and adventitial thickening[2,3].

In order to decrease pulmonary blood pressure, a number of therapeutic protocols have been developed, which include nitric oxide (NO) inhalation and

extracorporeal membrane oxygenation (ECMO) with delayed surgery[4]. ECMO, which has been used in an attempt to diminish the pulmonary blood pressure by guaranteed O_2 supply, leads to documented but variable survival rate improvement in high-risk infants with CDH[4,5]. Based on a study of heat shock proteins expression in CDH, recently we postulated that in the pulmonary vasculature of CDH newborns there is a state of stress, which is partially alleviated by ECMO[6]. ECMO decreases the pulmonary arterial pressure, but it remains unclear whether it occurs by inducing vascular remodeling or only by muscle relaxation[5].

NO is a potent vasodilator, produced by conversion of L-arginine to L-citrulline, a reaction catalyzed by a number of enzymes known as NO synthases[7]. NO has been implicated in pulmonary vasodilatation and bronchodilatation[7,8]. cDNA cloning has led to the identification of three subtypes of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS)[9]. Macrophage or iNOS, the expression of which is induced by various cytokines, is the non-constitutive and Ca^{++}-independent form of NOS[10]. Immunohistochemically, iNOS has been identified in normal human pulmonary arteries[9]. The various isoforms of NOS and their role in production of NO is depicted in Figure. 1.
NO has been used in the treatment of various forms of PH, including CDH-related PH[11]. However in CDH, there is a high failure rate of NO therapy[12].

Figure. 1: *The Production of NO from Endothelium and the Role of NOS Enzyme.*



Where: NO = nitric oxide, NOS = nitric oxide synthase, iNOS = inducible, eNOS = endthelial, nNOS = neuronal, empty circles indicates each of the NOS isoforms and Ca++ = indicate the need of calcium ion for the constitutive isoforms.

This may be a direct result of the underdevelopment of the pulmonary vasculature in the hypoplastic lungs of CDH and/or may be due to altered regulation of pulmonary vascular tone as proven in the rat model of CDH[13]. Previously, reduced eNOS in lungs of adult patients with pulmonary hypertension has been demonstrated[14]. Similarly, decreased expression levels of eNOS was shown in a rat model of CDH[15], while no differences in NOS localization between lambs with CDH and controls were found[16]. Immunohistochemically, iNOS has been identified in normal human pulmonary arteries[9]. Based on a study of heat shock proteins expression in CDH, we have recently suggested that in the pulmonary vasculature of CDH newborns there is a state of stress, which is partially alleviated by ECMO[6,11,12]. The anti-stress responses of the lung to injurious stimuli as hypoxia, ventilator therapy or shear forces could be evaluated by investigating iNOS as stress marker. The clinical observation of variable or absent reactivity to NO inhalation illustrates our incomplete knowledge of the regulation of pulmonary vascular tone in CDH[13].

To our knowledge, the role of iNOS in PH or as cytokine-induced stress marker in lungs of human CDH patients under conditions of stress induced by artificial ventilation or ECMO has not been studied before. We therefore investigated the expression pattern of iNOS in human pulmonary vasculature of CDH cases and the effects of ECMO treatment on this expression.

4.3 Materials and Methods

Selection of Specimens:

We studied archival autopsy lung specimens of 33 neonates who died of CDH and lung hypoplasia, as confirmed by lung/body weight ratio index ≤ 0.012[17]. All CDH cases belonged to the high-risk group and presented with respiratory insufficiency within the first 6 hours after birth[6]. After approval of the study design by the research committees, the archival specimens were retrieved. Routine cardiac ultrasonography had been performed in all cases in order to document pulmonary hypertension and right-to-left shunting, together with the clinical observation of preductal and postductal O_2-saturation differences of >10% obtained by pulse oxymetry[6,18].

Twenty-three CDH cases were not subjected to ECMO treatment (CDH group) and treated according to a standard protocol. This group included 5 newborns who died within the first hour after birth. The second group (ECMO group) consisted of 10 CDH neonates, who received ECMO treatment for a mean bypass time of 238 hours (range: 2-21 days). Veno-arterial ECMO, when necessary, was instituted to the neonates who did not have any of the following institutional exclusion criteria: gestational age less than 34 weeks, birth weight less than 2000 g, artificial ventilation more than 7 days, alveolar arterial oxygen difference ($A\text{-}aDO_2$) < 80 KPa [600 Torr], and maximal $Pa\text{-}O_2$ less than 10 KPa[6,18]. Eleven age-matched neonates, who died from acute placental insufficiency or birth asphyxia within the first 24 postnatal hours, served as

controls (CON group). Controls were subjected to similar ventilatory therapy for up to 16 hours. These controls showed no lung abnormalities or hypoplasia on histological examination and had no clinical features of PH. We processed paraffin embedded tissue blocks from either lung randomly for immunohistochemistry.

Immunohistochemistry:

Paraffin sections of lung tissues were cut at 6 μm and mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides. Immunohistochemistry was performed using the peroxidase-anti-peroxidase (PAP) technique. Slides for iNOS were incubated for 20 minutes in methanol with 0.3% H_2O_2 to quench endogenous peroxidase, then cooked for 15 minutes at 100° C in citric acid buffer, rinsed with phosphate buffered saline (PBS) and placed in Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn, UK). After incubation with 10% normal rabbit serum for 15 minutes, slides were incubated overnight at 4° C with mouse monoclonal antibody against iNOS in dilution 1:40. This antibody was raised against an immunogen of mouse iNOS, corresponding to amino acids 961-1144, that has specificity for human iNOS (Transduction Laboratories, Lexington, KY, USA). This antibody shows no cross reactivity to other NOS isoforms using Western blotting techniques. After rinsing with PBS the sections were incubated for 30 minutes with rabbit-anti-mouse serum in a dilution of 1:25 (Dako Corp, Glostrup, Denmark), rinsed with PBS, incubated with mouse PAP-complex 1:300 (Sigma) for another 30 minutes. The chromogen reaction was allowed to take place over 7 minutes in the dark, using 0.025% 3,3-diaminobenzidine [DAB](Sigma). Slides were briefly counterstained with Mayer's hematoxylin.

Staining with Cell Markers:

Consecutive lung sections were stained by alkaline phosphatase using avidin-biotin standard method with the specific macrophage marker (CD68) to identify with certainty the alveolar lung macrophages. Anti-human monoclonal anti-CD68 antibodies (Clone KP1, NeoMarkers, Fremont, CA, USA) were used in dilution of 1: 100 at room temperature for 60 minutes after pretreatment with pronase for 15 minutes.

Immunolocalization of iNOS in endothelial cells was verified by staining of consecutive sections with specific endothelial cell marker CD31(data not shown), using the avidin-biotin complex method. Slides were incubated with the primary anti-human CD31 monoclonal antibody (Dako) in a dilution of 1:80 at room temperature for 30 minutes. Employing the same method, consecutive tissue sections were stained with anti-human mouse monoclonal alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex) in a dilution of 1:200 to identify the medial smooth muscle cells (SMCs) (data not shown). These consecutive sections stained with anti-iNOS, anti-α-SMA and anti-CD31 were compared to localize endothelium and vascular SMC prior to quantification. All slides were lightly counterstained with Mayer's hematoxylin.

Quantification and Statistical Analysis:
Sixteen rectangles were photographed from each slide at the mid-lung area using a 3 chips digital video camera (3CCD Color video Camera, Sony Corporation, Tokyo, Japan) connected to microscope (Leica optical systems, Wetzlar, Germany) and images were analyzed using an image analysis software program (Leica Qwin standard (Y2.2b) package, Cambridge Biotech, UK). Expression of iNOS was analyzed for the intensity of expression signal in the endothelium mainly and medial smooth muscle cells (SMC) of small pulmonary arteries (50-200 μm external diameter ED). Furthermore, all CD68 positive alveolar macrophages were counted manually in an equal area from each slide and compared with consecutive sections stained with iNOS using Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany).
Data of iNOS localization quantified by video-image analysis as well as alveolar macrophage cell counting were averaged from each slide and eventually from each of the three groups were subjected to statistical analysis. Values are expressed as mean±SEM from the 44 neonates of the three groups. Statistical analysis was performed using the non-parametric Kruskal-Wallis ANOVA test[19], using SPSS packet (SPSS. Inc, Chicago. USA). P values ≤ 0.05 were considered significant.

4.4 Results

In control specimens, inducible NOS was detected in decreasing order of intensity in the vascular endothelium, bronchial epithelium and vascular SMC as seen in Figure. 2A. In CDH cases, distinct iNOS immunostaining was identified in the endothelial cells of small pulmonary arteries with an ED less than 200 μm, which was significantly lower than the expression in controls (P = 0.04; Figure. 2A& B).
See picture on page 169

Consecutive sections stained with the endothelial cell marker, CD 31, confirmed that the iNOS positive cells were endothelial cells. Although we observed differences in the endothelial expression levels in CDH cases, ranging from a faint staining to amounts comparable to that of controls, the overall iNOS expression was significantly lower than that of controls.
This was not related to the duration of ventilation, since there were no differences in iNOS expression pattern between CDH cases who were artificially ventilated up to 48 hours and the five cases who died in the first hour after birth. ECMO treatment was associated with a significant increase in iNOS expression as compared to the non-ECMO treated cases in the vascular endothelium of small pulmonary arteries (P = 0.034; Figure. 2D). The mean score was higher in ECMO cases as compared to controls, but this did not reach statistical significance (Table. I).

Table I: *P Values of iNOS from Different Groups Using Kruskal-Wallis test*

Groups tested	N	Inducible NOS	
		SAE	Macrophage
CDH Control	23 11	0.041*	0.027*
CDH ECMO	23 10	0.034*	0.003*
Control ECMO	11 10	0.62	0.011*

Where: CDH = congenital diaphragmatic hernia, ECMO = extracorporeal membrane oxygenation, N = number of cases, SAE = small arteries endothelium, LAE = large arteries endothelium and (*) indicates statistical significance at P value < 0.05.

Large pulmonary arteries endothelium showed similar differences among groups but again, these differences were not significant statistically. In the vascular SMC, a faint staining without differences in the expression among the studied three groups was seen.

Furthermore, iNOS expression in alveolar macrophages was also observed in all specimens (Figure. 2C) [9,10]. Alveolar macrophages were verified by positive staining for CD 68, which is known as a specific marker of macrophages (Figure .3). The parenchymal expression of iNOS in alveolar macrophages was increased in CDH cases as compared to controls (P = 0.027). ECMO-treated cases of CDH exhibited the highest expression of iNOS in alveolar macrophages as compared to non-ECMO cases (P = 0.003) or controls where P = 0.011 (Table. I). The intensity of expression of iNOS did not alter in alveolar macrophages by ECMO whereas, we observed that in ECMO-treated cases these cells are stretched and edematous (Figure. 3).
See picture on page 165

4.5 Discussion

In the present study, we found a significant decrease in the expression levels of iNOS in the pulmonary arterial endothelium of hypoplastic lungs of newborns

with CDH as compared to age-matched controls. ECMO therapy resulted in increased iNOS expression in CDH cases.

The decrease in the NOS expression of human CDH cases, which is in accordance with a report of the rat model of CDH[14], may indicate decreased NO production, which may play a role in the development of PH associated with CDH. Recently, decreased eNOS expression was reported in human newborns with persistent PH[20], as well as in adults with PH[14].

Our concern is mainly directed to the small diameter pulmonary arteries, which are the most important in the regulation of pulmonary flow and considered as resistance arteries[3,18]. The increased expression of iNOS in the vascular endothelium after ECMO therapy in CDH indicates that somehow, ECMO enhances the production of NO that would result in vasodilatation of the pulmonary bed, with decreased pressure as reported earlier[5].

Unfortunately, we have not been able to study whether this contribute to increased survival or not, since all our study material was derived from autopsies. However, the result of up regulation of iNOS by ECMO could provide an explanation of the underlying mechanism for the clinical observation of the unpredictable response to NO and ECMO therapies reported earlier[11,12].

Another underlying mechanism could be the altered reactivity of pulmonary arterial SMC to NO and/or might indicate that the vasoconstrictor, endothelin-1 has gained the upper hand in balancing the vascular tone, as we have previously reported in the rat model of CDH[13].

Hecker and co-workers reported that high levels of iNOS and subsequent increased NO production could lead to actual down regulation or desensitization of the enzyme guanyl-cyclase, resulting in muscle contraction rather than relaxation in case of vascular smooth muscle cell in vitro[21]. The results of the current study with that of Hecker's and co-workers[21], with the findings of increased expression of iNOS after ECMO therapy support the hypothesis of abnormal reactivity or non-responsiveness to the released NO leading to altered pulmonary vascular response with development and/or persistence of PH in CDH newborns.

The role of iNOS as stress marker has been proposed[7]. We observed increased iNOS positive alveolar macrophages in CDH sections, from non-ECMO as well as ECMO treated groups, compared to controls ($P = 0.27$ and 0.011 respectively). This increased expression points to parenchymal lung injury[7,22] or cytoprotection against this injury after longer periods of exposure to mechanical ventilation. This is supported by our results of significant higher numbers of positive alveolar macrophages in ECMO-treated cases of CDH as compared to non-ECMO cases ($P = 0.003$). Endogenous NO has been reported to promote leukocyte adhesion to post-capillary venules mediating inflammatory response[23], iNOS apparently participates in this protective function. Epithelial expression of iNOS indicates its role in airway homeostasis in different pathophysiological conditions[9,24]. We reported that ECMO decreases the cellular stress in CDH cases as represented by decreased expression of heat shock proteins[6]. Since ECMO cases are the worst

CDH cases with highest risk of lung injury, hence the enhanced iNOS expression in ECMO cases points to its maximal protective attempt at molecular level in lung parenchyma. This again supports our notion ECMO decreases stress and has a potential role in protection against injury. Inducible NOS has been reported to increases capillary permeability in inflammatory processes[7,21] and the resting hemodynamic effects of ECMO has been reported also[5,25]. Our observation of stretch and edema observed in alveolar macrophage in ECMO-treated cases support the state of parenchymal stress in cases of ECMO despite the decreased stress in the vascular level, demonstrating another factor in difficulty of outcome prediction[26,27]. Increased oxidant stress may contributes to the impaired NO-mediated response in PH of CDH, as it has been shown experimentally in rat skeletal muscle[28].

Further studies are needed for a complete elucidation of the roles of functional molecules "including eNOS isoform" in human CDH in order to unravel the mechanisms leading to PH in CDH and its resistance to vasodilator therapy.

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Chapter 5

Endothelial NOS expression in human CDH and age-matched controls

Based on the article:

Pulmonary Expression of Endothelial Nitric Oxide Synthase in Human Newborns with Congenital Diaphragmatic Hernia and Pulmonary Hypertension
Shehata SMK, Tibboel D, Sharma HS, Mooi WJ.

Submitted.

5.1 Summary

The pathophysiology of pulmonary hypertension (PH) associated with congenital diaphragmatic hernia (CDH) in humans is not yet fully elucidated. Particularly the role of nitric oxide synthase (NOS) needs to be clarified. NOS catalyzes the formation of NO, which has a vasodilating effect and a potential cytoprotective role. Its most important isoform is the endothelial one (eNOS) that stimulates NO production, and which is known as the endothelium-derived relaxing factor. Furthermore, the molecular basis of unpredicted outcome of CDH therapies such as extracorporeal membrane oxygenation (ECMO) has to be explored.

Autopsy lung specimens from 33 cases of CDH associated with PH were examined immunohistochemically for expression of endothelial (eNOS).We distinguished into non-ECMO (n =23) and ECMO-treated (n =10) CDH patients, and added age-matched controls (n =11). No significant differences in eNOS immunolocalization were found among groups except for endothelium of large pulmonary arteries. There is a state of imbalance between vasodilating and vasoconstricting molecules in the hypoplastic pulmonary vasculature of CDH. Decreased pulmonary expression of eNOS could explain the altered response of pulmonary vasculature to inhaled nitric oxide (NO) resulting in defective vasodilatation, and points to a mechanism that brings about PH in CDH cases.

5.2 Introduction

Lung hypoplasia and therapy-resistant pulmonary hypertension (PH) largely determine the high mortality and morbidity associated with congenital diaphragmatic hernia (CDH). Pulmonary vascular abnormalities in CDH include reduced quantities of pulmonary arteries per unit lung volume, increased peripheral muscularization of small arteries, and medial and adventitial thickening of pulmonary arteries[2,3] . Several therapies have been developed to decrease pulmonary blood pressure; these include nitric oxide (NO) inhalation and extracorporeal membrane oxygenation (ECMO) with delayed surgery[4]. ECMO, which is used in an attempt to "rest" the lung with consequent diminished pulmonary blood flow, may have a possitive effect on survival rate in high-risk infants with CDH[4,5]. ECMO decreases the pulmonary arterial blood flow, but it is not clear whether this results from pulmonary vascular remodeling or muscle relaxation[5].

Endothelium contributes to local regulation of vascular smooth muscle tone by synthesizing and releasing various vasoactive products, including nitric oxide (NO) [6]. NO is a potent vasodilator, produced by conversion of L-arginine to L-citrulline, with the reaction catalyzed by a number of enzymes known as NO synthases (NOSs) [7]. NO diffuses rapidly to adjacent vascular smooth muscle cells (VSMC), where it binds to guanylate cyclase to produce cGMP, resulting in relaxation of the VSMC[8-10]. NO is a key factor in maintaining the low, normal pulmonary vascular resistance (PVR) in children and adults[11]. NO has

also been implicated in bronchodilatation[7,12]. cDNA cloning has led to the identification of three subtypes of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [13]. The two isoforms of importance in the vasculature are eNOS and iNOS.

Endothelial NOS is constitutively produced by endothelial cells mainly and by airway epithelial cells in small amounts in response to some stimuli such as shear forces and hypoxia[6,10]. Recently it was shown that eNOS-deficient mice have systemic and pulmonary hypertension[10], and that iNOS expression was induced by various cytokines. Endothelial NOS as well as nNOS are constitutive and Ca^{++}-dependent forms of NOS[14]. The clinical observations of variable or absent reactivity to NO inhalation illustrate our incomplete knowledge of the regulation of pulmonary vascular tone in CDH[15].

Reduced eNOS expression in lungs of adult patients with pulmonary hypertension has been demonstrated[16]. Similarly, decreased expression levels of eNOS have been shown in a rat model of CDH[17], whereas no differences in the localization of eNOS between CDH and control lambs have been reported[21]. Interestingly, NO inhalation does not reverse the pulmonary vascular tone in-vivo under standard conditions[10,19]. In a rat model eNOS expression remained unaltered during pulmonary vascular remodeling due to increased pulmonary blood flow[20]. In a previous study in normal human newborns, we found that the pulmonary arteries became progressively more thin-walled towards term[21]. This normal perinatal remodeling process is lacking in CDH newborns, indicating impaired development of the pulmonary vasculature. The persistent thick adventitia of small pulmonary arteries in CDH, however, partially reversed under ECMO[24].

To our knowledge, the role of eNOS in the pulmonary vasculature of human CDH cases has not been investigated before. In this study we investigated expression patterns of eNOS in the lungs of human CDH patients, and the way in wich ECMO treatment effects these expression patterns.

5.3 Materials and Methods

Selection of Specimens

We studied archival autopsy lung tissue specimens of 33 neonates who died from CDH and lung hypoplasia, as confirmed by a lung/body weight (LW/BW) ratio index ≤ 0.012[21,22]. All belonged to the high-risk group and presented with respiratory insufficiency within 6 hours after birth[21,23]. These specimens represent all the available material from patients with CDH, who died and of whom parents' consent for autopsy was obtained during the period 1978-1998. The ethical and research committee of the Erasmus University Medical Center, Rotterdam, approved this study. In this period, over 350 CDH neonates were treated in Rotterdam and Nijmegen [the two pediatric surgery centers with ECMO in Netherlands], with an overall survival rate of about 60%.

All cases had been subjected to standard management protocols including delayed surgery from 1986 and, if eligible, ECMO treatment since 1991. Routine

cardiac ultrasonography was performed in all cases in order to document pulmonary hypertension and right-to-left shunting, together with the clinical observation of preductal and postductal O_2-saturation differences of >10% obtained by pulse oxymetry[23].

Twenty-three CDH cases had not been subjected to ECMO treatment (CDH group); these included 5 newborns who died within one hour after birth. Patients from the non-ECMO treated CDH group were subjected to ventilator therapy till death. The second group consisted of 10 term CDH neonates (ECMO group), who received ECMO treatment for a period ranging between 2 and 21 days, with a mean bypass time of 237.4 hours. Venoarterial ECMO, when necessary, was instituted to those who did not meet any of the exclusion criteria: gestational age less than 34 weeks, birth weight less than 2000 g, artificial ventilation for more than 7 days, alveolar arterial oxygen difference < 580 mm Hg, and maximal Pa-O_2 less than 72 mm Hg. Patients who received ECMO were all successfully decannulated and remained on conventional ventilator support till death. Three of these ECMO patients received NO therapy prior to ECMO institution for less than 48 hours in a dose of 2-5 PPM. One was treated with NO post-ECMO for recurrent PH. All patients were early post-ECMO deaths, defined as death within 30 days after decannulation. Eleven age-matched neonates, who had died from acute placental insufficiency or birth asphyxia within 24 hours after birth, served as controls (CON group). They had been subjected to similar ventilator therapy for up to 16 hours, and controls showed no lung abnormalities or hypoplasia on histological examination, and no clinical features of PH. Because we did not identify significant differences between both lungs on histological examination[5,21,24], we randomly processed tissues from either side of the lung for histopathological and immunohistochemical examination.

Immunohistochemistry

Paraffin-embedded lung tissue sections were cut at 6 µm at the mid-lung area and mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides and preserved for immunohistochemistry.

eNOS Staining: Immunohistochemistry was performed using the avidin biotin complex method. Slides for eNOS were cooked for 15 minutes at 100° C in citric acid buffer, rinsed with phosphate buffered saline (PBS) and placed in Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn, UK). After incubation with 10% normal goat serum for 15 minutes, slides were incubated for 2 hours at room temperature with mouse monoclonal antibody against eNOS in dilution 1:40 (Transduction Laboratories, Lexington, KY, USA). This antibody shows no cross reactivity to other NOS isoforms using Western blotting techniques. After washing with PBS, the test and control slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). After two washes in PBS, slides were incubated for 30 minutes with alkaline phosphatase conjugated streptavidin

(Biogenex) in a dilution of 1:50. Finally, the slides were rinsed with 0.2 M TRIS-hydrochloride pH 8.0, Levamisole (Sigma) was used to block the endogenous alkaline phosphatase activity, and stained for 30 minutes with 0.3% new fuchsin/TRIS-HCL (Sigma) as color enhancement system.

Staining with Cell Markers: Immunolocalization of eNOS and iNOS in endothelial cells was verified by staining of consecutive sections with specific endothelial cell marker CD31, using the avidin-biotin complex method. Slides were incubated with the primary anti-human CD31 monoclonal antibody (Dako) in a dilution of 1:80 at room temperature for 30 minutes. The chromogen reaction was allowed to take place over 7 minutes in the dark, using 0.025% 3,3-diaminobenzidine [DAB](Sigma). Employing the same method, consecutive tissue sections were stained with anti-human mouse monoclonal alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex) in a dilution of 1:200 to identify the medial smooth muscle cells (SMCs). These consecutive sections stained with anti-eNOS, anti-α-SMA and anti-CD31 were compared to localize endothelium and vascular SMC prior to quantification as seen in Figure 1. All slides were lightly counterstained with Mayer's hematoxylin.

Quantitative Analysis

All tissues were analyzed in a blinded fashion in random order. Prior to screening sections stained for eNOS were coded by two independent observers, who were unaware of the clinical data of the case under study. Due to the faint staining of eNOS, immunoreactivity was analyzed semi-quantitatively, using an arbitrary visual scale with score ranging from 0 to 4: grade 0 represents no staining, grade 1 represents focal staining, grades 2, 3 and 4 represent diffuse weak, moderate and strong staining, respectively[25,26]. Sections were graded from 0-4 for the intensity of expression signal of eNOS in the endothelium of small (50-200 μm external diameter [ED]) and large (> 200 μm ED) pulmonary arteries.

Statistical Analysis

The scores from each slide were averaged and subjected to inter-group statistical analysis. Values were expressed as mean±SEM. Statistical comparisons between the three groups were performed by the non-parametric Kruskal-Wallis ANOVA test[27], using the SPSS software packet (SPSS Incorporation, Chicago, USA). Values of $P < 0.05$ were considered significant.

5.4 Results

Clinical Results

The patients' characteristics, and ventilator settings including: inspiratory oxygen fraction (FIO_2), peak inspiratory pressure (PIP), positive end expiratory pressure (PEEP) and frequency, are listed in Table I.

Table I: *Patients characteristics*

Parameter	CDH non ECMO	ECMO	Control
Number of cases	23	10	11
Gestational age (wk)	38.1±0.9	39.0±0.8	36.2±1.9
Birth weight (g)	2760±264.0	3160±134.0	2785±432.0
LW/BW ratio	0.0079±0.0016	0.0075±0.0015	0.0167±0.0051
Age at death (h)	32.9±7.2	333.6±65.0	10.6±6.4
Pre-ECMO ventilator settings:			
FIO_2	1.0	1.0	1.0
PEEP (cm H_2O)	5.1	5.0	5.0
PIP (cm H_2O)	35.2	34.6	32.0
Frequency (cycle per minute)	65	85	60
Time of pre-ECMO ventilation (h)	NA	15	NA
ECMO duration (h)	NA	237.4±47.8	NA
Post-ECMO ventilator settings:			
FIO_2	NA	1.0	NA
PEEP	NA	5.0	NA
PIP	NA	25	NA
Frequency	NA	50	NA

CDH = congenital diaphragmatic hernia, ECMO = extracorporeal membrane oxygenation, LW = lung weight, BW = body weight, FIO_2 = flow of inspired oxygen, PEEP = positive end expiratory pressure, PIP = peak inspiratory pressure, and NA = non-applicable.
Parameters represented as mean or mean±SEM from the number of cases indicated in each group.

As to the ventilator settings in the CDH groups, FIO2 was 1.0 in all cases, PEEP ranged between 4-6 cm H_2O, PIP ranged between 28-40 cm H_2O, and frequency ranged from 40-80 cycles per minute in non-ECMO treated cases, reaching 100 in cases subjected to ECMO therapy. ECMO cases were ventilated prior to ECMO institution for 11-24 hours and all died within 30 days after decannulation. Three

cases were subjected to NO inhalational therapy pre-ECMO, one case received NO post-ECMO therapy.

Immunohistochemical Localization of NOS

Endothelial NOS was detected in the arterial endothelium, as verified with staining of CD31 on consecutive sections (Figure 1).

See picture on page 167

The mean expression score of eNOS in the endothelium of pulmonary arteries with an ED < 200 μm was highest in CDH without ECMO treatment, followed by ECMO treated cases. The lowest values were observed in controls, but the difference did not reach statistical significance (Table II). In large pulmonary arteries, endothelial expression score was highest in the ECMO-treated cases, followed by CDH cases without ECMO therapy; the lowest mean score was observed in control cases. Statistical significance was observed only between ECMO-treated cases of CDH and controls ($P = 0.027$) as shown in Table II.

Table . II: *P Values of eNOS in the Different Groups established by the Kruskal-Wallis Test.*

Groups tested	N	Endothelial NOS	
		SAE	LAE
CDH Control	23 11	0.27	0.23
CDH ECMO	23 10	0.86	0.39
Control ECMO	11 10	0.22	0.027*

CDH = congenital diaphragmatic hernia, ECMO = extracorporeal membrane oxygenation, N = number of cases, SAE = small arteries endothelium, LAE = large arteries endothelium, and (*) indicates statistical significance at P value < 0.05.

Consecutive sections stained with the endothelial cell marker CD 31 confirmed that the eNOS positive cells were endothelial cells (Figure1). Although we observed differences in the endothelial expression levels in CDH cases, ranging from a faint staining to amounts comparable to that of controls, the overall eNOS expression difference did not reach statistical significance.

5.5 Discussion

This is the first study investigating the role of eNOS in human CDH cases. We found non-significant differences in the expression levels of eNOS in the pulmonary arterial endothelium of hypoplastic lungs of newborns with CDH as compared to age-matched controls, either subjected to ECMO treatment or not. The decrease in NOS expression in human CDH, which is in accordance with a previous report in a rat model of CDH[17], may indicate that subsequent decreased NO production could play a role in the development of PH associated with CDH. Recently, decreased eNOS expression was reported in human newborns with persistent PH[27,28]. These data are in accordance with a recent report on decreased eNOS expression in a rat model of CDH at pre-term, however, without significant differences at term[29]. Previous reports of the role of eNOS in different conditions of PH, human and experimental, showed widely-ranging outcomes[6,16-18,20,26, 28-30]. Even more, variability has been reported in the same pathology[16,30]. Our findings in PH associated high-risk cases of CDH clearly show that the role of eNOS is probably minimal, as we did not find any significant difference in eNOS expression in the small, pressure-regulating pulmonary arteries, regardless of ECMO treatment. This is in agreement with a report by Le Cras and coworkers[6]. We were mainly concerned with the small diameter pulmonary arteries including the intraacinar arteries with ED ranging from 50-200 μm-[3,31], which are the most important in the regulation of pulmonary blood flow[3,21].

NO-therapy has been used in the treatment of various forms of PH, including CDH-related PH[32], but shows a high failure rate in CDH patients[33]. This may be a direct consequence of the underdevelopment of the pulmonary vasculature in the hypoplastic lungs in CDH, or be due to altered regulation of the pulmonary vascular tone, as proven in the rat model of CDH[15]. Both mechanism might also exert a combined effect.

The increased expression of iNOS in the vascular endothelium after ECMO therapy in CDH may indicate that ECMO enhances the production of NO resulting in vasodilatation of the pulmonary bed[34]. We were not able to investigate whether this vasodilatory event might contribute to increased survival, as all our study material was derived from autopsies. However, the results on up-regulation of iNOS, and the unchanged eNOS by ECMO, may provide an explanation of the underlying mechanism for the variable clinical responses to NO and ECMO therapies[32,33]. Another underlying mechanism could be altered reactivity of pulmonary arterial SMC to NO or, alternatively a vasoconstrictor, such as endothelin-1 may have gained the upper hand in balancing the vascular tone and regulating the pulmonary vascular tone. Our findings on eNOS expression provide another argument to this upper hand role exerted by endothelin, documented previously in the CDH rat model developed by our group[15]. Indeed, the finding of an increase in the plasma endothelin levels in human newborns with CDH and PH as compared to their age-matched controls supports this notion[35]. Additionally, impaired eNOS expression as a

result of intrauterine PH in CDH cases, has been shown in an ovine model with failed postnatal adaptation[36]. This could be due to failed maturation in CDH lungs as we have reported at a structural level[21] and in regard to vascular endothelial growth factor (VEGF), as these lungs have increased expression levels[37], similar to those reported in earlier normal fetal life.

Hecker and coworkers reported that increased NO production could lead to down regulation or desensitization of the enzyme guanyl-cyclase, resulting in muscle contraction rather than relaxation in cases of vascular smooth muscle cell in vitro[38]. Increased expression of eNOS in fetal lambs after ventilation and oxygenation with increased NO production has been reported[39]. In human cases we did not notice any specific expression in these subjected to NO therapy. This could be attributed to the scarcity of the available human material and to the differences between the actual pathogenic mechanism in diseased humans and in the animal experimental model. Our findings support the hypothesis of abnormal reactivity or non-responsiveness to the released NO without involvement of eNOS, which may lead to altered pulmonary vascular responses together with the development and/or persistence of PH in CDH newborns. The current study supports the notion of the minimal role of eNOS in the production of PH in human CDH, both in the large and small–pressure regulating-pulmonary arteries. This notion was reported at a gene expression level in a rat model of CDH[17] and at the level of the main pulmonary trunks in a lamb model of CDH[18]. Although these studies contributed to our knowledge on the roles of NO and NOS further studies are needed for a complete elucidation of the roles of functional molecules in the pulmonary vascular bed of human CDH. These need to unravel the mechanisms leading to PH in CDH, and its resistance to the currently used vasodilator therapy.

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Part IV

Lung Response to Stress in Human Neonates

Based on Article:

Expression Patterns of Heat Shock Proteins in Lungs of Neonates with Congenital Diaphragmatic Hernia

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Chapter 6

Expression Patterns of HSPs in Lungs of CDH Human Newborns as a Natural Model of Stress

6.1 Summary

Congenital diaphragmatic hernia (CDH) is associated in many cases with pulmonary hypertension (PH). Currently, extracorporeal membrane oxygenation (ECMO) is one of the possible modalities of treatment of PH and prevention of parenchymal lung injury in neonates with CDH. To investigate the expression pattern of stress genes (heat shock proteins, HSP 27 & 70) in lungs of CDH patients with PH, and to evaluate the influence of ECMO on the expression levels of these genes in order to understand the underlying molecular mechanisms.

Paraffin-embedded lung autopsy specimens with CDH and lung hypoplasia, either received ECMO treatment or not and age-matched controls were immunostained using monoclonal anti-human antibodies against HSP-70 and HSP-27, employing the streptavidin-biotin complex (ABC) method. Expression levels of both HSP 27 and 70 were semiquantitatively evaluated in bronchial epithelium, as well as in medial smooth muscle cells (SMC) and endothelium of large and small pulmonary arteries, using a score ranging from 0 to 4. Statistical analysis of the data was performed using the non-parametric Mann-Whitney test, with significant probability value at ≤ 0.05. For HSP-70, the most pronounced immunoreactivity was observed in the bronchial epithelium, followed by the medial SMC of small arteries (of external diameter < 200 μm). The overall expression was significantly higher in CDH cases than controls in bronchi as well as in pulmonary arteries. For HSP-27, intense expression was found in medial SMC followed by the bronchial epithelium in controls, with significantly increased expression in medial SMC of large and small arteries in CDH cases. ECMO treatment was associated with significantly reduced expression levels of HSP-70 in medial SMC of both large and small arteries. Whereas, HSP-27 expression levels were decreased only in small arteries. In addition, the expression levels of both HSPs were significantly lower in endothelium of small arteries.

This is the first study of the expression of HSPs in lungs of CDH patients. We found increased expression of HSPs in CDH, which points to a condition of pulmonary stress. This pulmonary stress appears to be partially ameliorated under ECMO treatment. This may probably point to one of the mechanisms by which ECMO alleviates PH associating CDH.

6.2 Introduction

The high mortality and morbidity in infants with congenital diaphragmatic hernia (CDH) are largely determined by the severity of lung hypoplasia and therapy resistant pulmonary hypertension (PH)[1]. Pulmonary vascular abnormalities in CDH, which consist of decreased number of pulmonary arteries per unit lung volume and peripheral muscularization of small arteries with medial and adventitial thickening[2,3]. In order to decrease the PH, a number of management protocols have been developed, including extracorporeal membrane oxygenation (ECMO) with delayed surgery after patient's stabilization[4,5]. ECMO has been used in an attempt to diminish the abnormal pulmonary vascular tone by guaranteed O_2 supply and to reverse the pulmonary structural abnormalities with documented variable improvement of the survival rate in the high-risk infants with CDH[5,6]. It is unclear whether ECMO may impose the possible pulmonary stress responses in CDH infants.

A number of genes are expressed immediately when cells are subjected to stress, stretch and in case of acute and chronic lung injury[7,8]. Among these are the heat shock proteins (HSPs), a group of highly conserved proteins that can be induced by exposure to heat, as well as a variety of pathophysiological conditions including hypoxia, oxidative and metabolic stresses[9,10]. Heat shock proteins represent one of the lung defense mechanisms against injury, as the anti-oxidant enzyme system[7]. The HSP-70 family of proteins binds to adenosine tri-phosphate (ATP) and plays a role in the transport of proteins into the endoplasmic reticulum and mitochondria[10-12]. The small heat shock protein HSP-27 is expressed in developing organs and under conditions of cellular stress. It is localized in the cellular cytoplasm and migrates to the nucleus upon stress. HSP-27 acts as a molecular chaperone and plays an important role in signal transduction and drug resistance[12-14]. HSPs have been reported to participate in embryonic and fetal development[15].

To our knowledge, no study of HSPs expression in CDH has been presented before. Although the mechanism by which ECMO treatment is of benefit in CDH, originally referred to as "lung rest", the molecular basis remain unknown. We hypothesize that vigorous ventilatory support in CDH may results in a state of severe pulmonary stress.

The present study was carried out in order to investigate the pulmonary expression of the stress genes HSP-27 and HSP-70 in human CDH. In addition to study whether, the institution of ECMO alters their expression levels in CDH human lungs, aiming to understand the molecular mechanisms of the hypoplastic lungs following injury

6.3 Materials and Methods

Tissue specimens

We studied archival autopsy lung tissue specimens of 24 neonates who died of CDH and lung hypoplasia, as confirmed by a lung/body weight ratio index ≤ 0.012[16]. All CDH cases belong to the high-risk group. After approval of the study design by the departmental research committee, the specimens were retrieved. These specimens represent the available material from patients with CDH, who were treated in Sophia Children's Hospital, Erasmus University, Rotterdam, died and parents' consent for autopsy were obtained during the period 1981-1997. At the same period more than 120 CDH neonates were treated in our hospital with an overall survival rate of 65%. All cases were subjected to standard management protocols including delayed surgery from 1986 and ECMO management since the introduction of ECMO in our center at 1992. All cases were subjected to routine cardiac ultrasonography which indicate PH and right-to-left shunting, together with the clinical observation of preductal and postductal transcutaneous O_2-saturation differences of > 10%[17].

Cases were divided into two groups. Group A consisted of 20 CDH neonates who did not receive ECMO treatment (mean gestational age: 38.6±0.4 weeks), all died in the first two days of life, including 4 cases who died in the first postnatal hour. The primary cause of death in group A was extreme lung hypoplasia in 11 cases, persistent fetal circulation in 8 cases, and interstitial pulmonary hemorrhage in one case. Group B consisted of 4 CDH neonates (mean age: 39±2 weeks) who were treated with ECMO for a period ranging between 3 and 21 days with a mean bypass time of 270 hours. Venoarterial ECMO, when instituted, was offered to the neonates who did not have any of the following institutional exclusion criteria. These criteria are: gestational age less than 34 weeks, birth weight less than 2000 g, artificial ventilation more than 7 days, alveolar arterial oxygen difference (A-aDO_2) < 80 KPa [600 Torr], and maximal Pa-O_2 less than 10 KPa. Patients who received ECMO were all successfully decannulated and remained on conventional ventilatory support till death, in 3 instances from persistent fetal circulation and multiple organ failure in the fourth patient. In the CDH cases hernia was left-sided in 16 cases and right-sided in 8 cases. None of the patients received surfactant treatment. Five age matched neonates (mean age: 37.8±1.2 weeks), who died within the first 24 hours due to birth asphyxia and were subjected to ventilation periods up to 16 hours, served as controls constituting group C. All these controls showed no lung abnormalities on histological examination and had no clinical features of PH. Patients from non-ECMO groups were subjected to ventilation therapy till time of death. Table I represent the patients' demographic criteria and data of ventilator settings including: inspiratory oxygen fraction (FIO_2), peak inspiratory pressure (PIP), positive end expiratory pressure (PEEP) and frequency from all groups. No case of stillbirth was included in any of the studied groups.

Table I: *Demographic Criteria of Patients in Various Study Groups*

Parameter	CDH non ECMO	ECMO	Control
Number of cases	20	4	5
Gestational age (weeks)	38.6±0.4	39.0±2.0	37.8±1.2
Birth Weight (grams)	2655±138.0	3045±256.0	3180±244.0
Age at Death (h)	29.0±2.8	339.0±123.7	11.8±4.0
Pre-ECMO Ventilator Settings:			
FIO_2	1.0	1.0	1.0
PEEP (cm H_2O)	5	5	5
PIP (cm H_2O)	34	34	31.4
Frequency (cycle per minute)	60	80	60
Pre-ECMO ventilation (h)		18	
ECMO duration (h)	ND	270	ND
Post-ECMO Ventilator Settings:			
FIO_2		1.0	
PEEP		5	
PIP		20	
Frequency		50	

Where values of parameters presented as mean or mean±SEM from the number of cases indicated in each group, where: FIO_2: Flow of inspired oxygen, PEEP: Positive end expiratory pressure, PIP: Peak inspiratory pressure, h: hour, and ND: not done.

Autopsy was performed in the first 24 hours following death in all cases. Since we did not find significant differences between both ipsilateral and contralateral lung sides on screening by histological examination, we processed tissues from either side of the lung randomly for immunohistochemistry in this study.

Immunohistochemistry

Paraffin sections of lung tissues were cut at 6 μm and mounted on coated glass slides. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method. Slides for HSP-27 were incubated for 20 minutes in methanol with 0.3% H_2O_2 to block endogenous peroxidase, then cooked for 15 minutes at 100 C°, rinsed with phosphate buffered saline (PBS) and placed in Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn). After preincubation with 10% normal goat serum for 15 minutes, slides were incubated at room temperature for 45 minutes with mouse monoclonal antibody to HSP-27 (dilution 1:750) and for 1 hour with mouse monoclonal antibody to HSP-70 (dilution 1:25), both supplied from NeoMarkers, Fremont, USA.

After rinsing with PBS, the slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). Slides were rinsed again, incubated for 30 minutes with peroxidase conjugated streptavidin for HSP-27 and with alkaline phosphatase conjugated streptavidin for HSP-70, using a dilution of 1:50 for both (Biogenex). HSP-27 slides were colored using 0.025% 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) in 0.01 mol/L PBS, containing 0.03% H_2O_2. HSP-70 slides were rinsed with 0.2 mol/L TRIS-HCL pH 8.0, incubated with levamizole in order to block the endogenous alkaline phosphatase activity, then stained with 0.3% New Fuchsin/TRIS-HCL (Sigma) and briefly counterstained with Mayer's hematoxylin. Positive controls consisted of breast carcinoma tissue specimens. Negative controls consisted of omission of the primary antibody.

Semiquantitative analysis

Prior to screening by two independent observers, sections were coded so that both were unaware of the clinical group of the case under study. Expression of HSP-27 and HSP-70 was analyzed semi-quantitatively, using an arbitrary visual scale with score ranging from 0 to 4: grade 0 represents no staining, grade 1 represents focal staining, grades 2, 3 and 4 represent diffuse weak, moderate and strong staining, respectively[18]. Sections were graded from 0-4 for the intensity of expression signal of HSPs in the bronchial epithelium, as well as in the endothelium and medial smooth muscle cells (SMC) of small (50-200 μm external diameter ED) and large (> 200 μm ED) pulmonary arteries. Intraacinar arteries representing the group of resistance arteries in the pulmonary vascular bed have ED ranged from 50-200 μm, while preacinar arteries have lower ED of 200 μm[3,19].

Statistical analysis

The expression of HSPs immunostaining score was calculated from the three groups, and the results were expressed as Mean±SEM. Statistical analysis was performed after ranking using the non-parametric Mann-Whitney test, which is appropriate to the compared groups of the study. Significance of the results for probability value was accepted at $P \leq 0.05$.

6.4 Results

Immunolocalization of HSP-70:

In control cases, HSP-70 was localized in the bronchial epithelium and medial arterial SMC. In the CDH group, the most pronounced immunoreactivity was observed in the bronchial epithelium with maximal expression score value of 2.98±0.14, followed by medial SMC of small arteries (2.13±0.15), medial SMC of large arteries (1.15±0.15) and endothelium of small (0.55±0.16) and large (0.2±0.11) arteries. The overall pulmonary expression of HSP-70 in CDH was higher than in controls (Fig 1 A&B).

Statistical analysis of HSP-70 expression scores between control and non-ECMO treated CDH groups showed significantly higher levels of expression ($P \leq 0.05$) in the bronchial epithelium, large arteries medial SMC, small arteries medial SMC and small artery endothelium where P values were 0.001, 0.008, 0.001 and 0.02, respectively, using the non-parametric Mann-Whitney U test. In both control and ECMO treated CDH groups, no HSP-70 expression was observed in pulmonary arterial endothelium.

ECMO treatment was associated with a significantly lower HSP-70 expression in medial SMC of large (P: 0.008) and small (P: 0.004) arteries and small artery endothelium (P: 0.04) in CDH patients as compared to the non-ECMO treated CDH group (Fig 1 C&D).

There were no statistical differences in expression levels of HSP-70 in pulmonary arteries between the controls and ECMO treated CDH infants.

See picture on page 169

In control cases, HSP-27 was localized in the bronchial epithelium and medial arterial SMC. In the CDH group, the highest expression score value was observed in the medial SMC of small arteries (2.2±0.14) followed by the bronchial epithelium (2.0±0.15), medial SMC of large arteries (1.83±0.14) and endothelium of large (1.2±0.18) and small (1.03±0.11) arteries. The CDH group showed higher levels of expression of HSP-27 than controls (Fig 2 A&B).

See picture on page 171

Statistical analysis of HSP-27 expression scores between controls and non-ECMO treated CDH groups showed significantly higher levels of expression ($P \leq 0.05$) in the medial SMC of large (P: 0.02) and small (P: 0.01) arteries using the non-parametric Mann-Whitney U test. Also, endothelium showed significantly higher expression in both large (P: 0.003) and small (P: 0.001) arteries. Although the mean value of expression in bronchial epithelium was higher in the CDH group, this difference did not reach statistical significance. No HSP-27 expression was found in the endothelium of arteries of the control group and of the large arteries of the ECMO treated group.

ECMO treatment was associated with a significant decrease in levels of HSP-27 expression in medial SMC of small arteries (P: 0.03) and in endothelium of both large and small arteries with P values of 0.007 and 0.008 respectively, as compared to the non-ECMO treated CDH group (Fig 2 C&D). There were no statistical differences in expression levels of HSP-27 in pulmonary arteries between the controls and ECMO treated CDH infants.

For both HSP-27 and 70 we did not find differences in the expression levels between the 4 cases who died in the first hour of life and the other cases of CDH neonates who did not receive ECMO treatment and were subjected to ventilatory therapy for longer time periods till death. In the meanwhile, we did not observe such differences when compared the CDH cases that died from pulmonary hypertension as a primary cause of death and those of extreme lung hypoplasia. The same holds true for the post-ECMO cases, regardless of the duration of ECMO treatment and who died at a mean age of 339 hours following successful decannulation from ECMO.

6.5 Discussion

In the present study, we found increased expression of HSP-70 and HSP-27 in hypoplastic lungs of infants with CDH. HSP-70 expression was highest in the bronchial epithelium, followed by the small arterial medial SMC, while HSP-27 expression was more pronounced in medial SMC of pulmonary arteries than in bronchial epithelium. Immunoreactivity for both HSPs was mainly cytoplasmic, with a minor degree of nuclear positivity, as has been reported previously[13,20].
HSPs expression could not be attributed to ventilatory trauma only, since the controls were subjected to a similar ventilatory treatment and duration with regards to FIO_2, PIP, and volume changes. More importantly, no difference in the expression levels was found in the 4 CDH cases, who died in the first hour after birth due to unsuccessful resuscitation in any of the examined tissues and that of other cases of CDH neonates who did not receive ECMO treatment. Also, the significant difference in expression was found mainly in the pulmonary arteries, not in the bronchial epithelium that might reflect the direct effects of ventilation and volutrauma. The enhanced pulmonary expression of HSPs in CDH indicates a state of stress, and HSPs may be regarded as molecular markers of pulmonary stress.
Although, the number of control cases is small, we believe that the birth asphyxia neonates are good controls. They were screened on histological examination and showed normal lung architecture except for some congestion and edema, and a normal vascular pattern. Moreover, these patients were used in comparable studies for the same reason[21].

Several reports confirmed the notion that the expression of HSPs is up regulated in a wide variety of stress associated conditions[7,8,20,22]. Possibly, the enhanced expression of HSPs in the CDH group reflects a neonatal attempt to establish a

protective mechanism against stress as shown earlier for antioxidant enzyme activity in a rat CDH model[23].

It has been well documented that the small diameter pulmonary arteries are the most important vessels in the regulation of pulmonary blood flow and pressure[2,3,19]. Structural changes have been described in pulmonary arteries of CDH cases complicated by PH[16,24]. Increased expression of HSP-27 – which is reported to be enhanced in an earlier developmental stage[15]- in CDH cases reported here may even confirm the stunted growth condition of the pulmonary vasculature because our cases were nearly term.

Our results of increased HSPs expression in small-diameter pulmonary arteries indicates that the cellular stress may act especially in the pulmonary pressure-regulating arteries. This partially answer the question raised by DeMello and Reid:" . An intriguing question is how ECMO in some cases allows resolution of pulmonary hypertension of the newborn and whether this occurs by allowing growth or by resting the microcirculation and the small resistance arteries to avoid exposure to blood pressure is not clear"[25].

In our series, ECMO treatment was associated with a significant reduction in the expression levels of HSPs, especially in the medial SMC of pulmonary arteries. Although the ECMO group was small, ECMO probably has a role in alleviating the CDH associated PH[26], as reported in a recent review[27]. It was reported that HSP-27 has been related to smooth muscle cell contraction as it constitutes the main phosphoprotein[28], and has been enhanced in vessel walls in response to varying types of stress[29]. Our findings of increased expression in small arteries SMC of pulmonary vasculature in CDH cases point to the same. We could not measure mRNA levels in this study, since our archival materials were formalin fixed and paraffin embedded, but interestingly data of ongoing studies in our laboratory showed similar significant decrease of HSPs after partial liquid ventilation both at mRNA and protein levels in a rat CDH model (Okazaki T, et al, submitted). So, our current results probably present one of the molecular mechanisms involved in the documented beneficial effect of ECMO in the management of the pulmonary hypertension in CDH cases[5,6,21,25-27]. However, it is unpredictable for the individual patient to determine whether it is possible to achieve complete resolution of the pulmonary vascular abnormalities following ECMO institution. Our findings indicate that a state of pulmonary stress in CDH appears to be ameliorated under ECMO treatment. Decreased pulmonary arterial stress may be a factor by which ECMO results in improvement of the PH associating cases of CDH.

Further multi-center studies with increased patients' numbers including other members of stress genes are needed for complete understanding of the underlying molecular mechanisms.

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PART V

Morphological Changes of the Lung in Congenital Diaphragmatic Hernia and ECMO Therapy

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Based on Article:

Remodeling of Pulmonary Arteries in Human Congenital Diaphragmatic Hernia With or Without Extracorporeal Membrane Oxygenation

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Chapter 7

Pathological-Clinical Correlation in CDH Cases with or without ECMO Treatment

7.1 Summary

To describe in-detail the perinatal developmental profile of the pulmonary vasculature in congenital diaphragmatic hernia (CDH) and to examine the potential beneficial effects of extracorporeal membrane oxygenation (ECMO) on the vascular morphology. Additionally, to identify the differences in pulmonary vascular morphology among CDH cases according to the primary cause of death either: extreme lung hypoplasia (LH) or persistent pulmonary hypertension (PPH). We studied autopsy sections from 30 high-risk CDH cases with respect to the pulmonary arteries, in relation to gestational age (GA) and ECMO treatment. They were grouped into CDH-I: 20 cases with GA >34 weeks (w) who were not subjected to ECMO and CDH-II: 10 cases with GA >34 w, who were subjected to ECMO for an average time of 237 hours. Five age-matched neonates who died from placental insufficiency or birth asphyxia without evidence of lung hypoplasia served as controls (CON). Medial and adventitial thicknesses of pulmonary arteries were measured in lung sections stained with Elastic van Gieson by two investigators blinded for the clinical data. Immunohistological staining with anti-α-smooth muscle actin (α-SMA) was performed in order to confirm the precise localization of the arterial media, prior to morphometry. CDH cases were subgrouped and compared according to the primary cause of death. Unpaired Student "*t*" test was used for statistics, with significant p value ≤ 0.05.

In CDH newborns, a significant increase in medial, adventitial and total wall thickness was found in pulmonary arteries with an external diameter < 200 μm as compared to age-matched controls ($p < 0.004$, 0.0001 and 0.0009 respectively). ECMO-treated CDH newborns showed a significantly thinner arterial adventitia than CDH cases who did not receive this treatment ($p < 0.0001$), approaching normal values. However, the medial thickness remained increased. Morphometrically, no significant differences in CDH cases between cases dying from PPH or severe LH could be determined.

We conclude that: 1) In CDH, there is failure of the normal arterial remodeling processes occurring in the perinatal period. 2) Pulmonary vascular morphology in CDH does not differ between the groups with lung hypoplasia or persistent pulmonary hypertension as primary cause of death.3) Adventitial thinning of these arteries might be one of the mechanisms by which ECMO alters PPH in CDH cases.

6.2 Introduction

Congenital diaphragmatic hernia (CDH) remains a major therapeutic challenge in neonatology. Despite the emergence of new therapeutic modalities such as exogenous surfactant therapy, nitric oxide (NO) inhalation, extracorporeal membrane oxygenation (ECMO), partial liquid ventilation, and delayed surgery, the mortality rate remains around 50% in high-risk cases[1-5].

The severity of lung hypoplasia (LH) and/or the presence of therapy-resistant pulmonary hypertension (PPH) are the major determinants of the mortality rate in infants with CDH[6]. Pulmonary vascular abnormalities in CDH consist of: decreased number of pulmonary arteries per unit lung volume, and peripheral muscularization of small arteries with medial and adventitial thickening[7,8].

The smaller arteries, with an external diameter (ED) under 200 μm, are predominantly responsible for pulmonary vascular resistance[8,9]. These small arteries rapidly increase in number during the last trimester of gestation and dilate at term to accommodate the postnatal demands[10,11]. In the fetus, pulmonary vascular resistance is high. At the time of birth with the onset of extrauterine respiration, pulmonary vascular resistance falls abruptly, and pulmonary blood flow rapidly increases to approximately tenfold as much as that reported during intrauterine life[12,13].

In high-risk newborns with CDH, ECMO has been used in an attempt to diminish the abnormal pulmonary vascular tone by guaranteed O_2 supply and lung rest to prevent the ongoing volutrauma and shear forces and has been shown to result in improved survival rates[4,5]. Our knowledge of the cause for the stunted development of the pulmonary vasculature in CDH is far from complete. The exact effects of ECMO on CDH infants are not completely understood and only few studies are available on the possible effects of ECMO on the pulmonary vascular architecture[14-16]. Recently, Taira and coworkers have reported the presence of abnormally thick pulmonary arteries in term CDH newborns either; born alive or stillbirth without significant difference between them in the amount of alpha smooth muscle actin (α-SMA) of vascular smooth muscle cells[17].

Nagaya and coworkers speculated on the clinical data of ECMO-treated CDH cases, differences regarding pre-ECMO hypercapneic and hypoxemic conditions that necessitate its institution preoperatively, in relation to the major underlying pathology either PPH or LH[18].

In a study of the molecular changes in the pulmonary vasculature of CDH cases, we have recently shown that the expression of the angiogenic polypeptide, vascular endothelial growth factor (VEGF) is significantly enhanced in CDH human newborns associated with PPH when compared to age-matched controls[19]. In addition, we have reported the presence of structural abnormalities of the pulmonary vasculature in CDH cases, which simulate those of the normal neonates during earlier gestational periods[16,19]. Correlation of the structural changes and molecular changes in the pulmonary vasculature is important for

complete understanding of the underlying pathogenic processes, especially those involved in vascular growth and/or remodeling.
In order to go a step further in understanding the developmental profile of lung vessels in CDH and normal neonates in the perinatal period, a detailed study on the different underlying pathologic conditions and their correlation to clinical data and morphometric measurements of pulmonary vasculature is essential.
The present study was carried out in an attempt to correlate the primary cause of death with the structural changes of pulmonary arteries, and to study the possible arterial structural changes following ECMO treatment for CDH.

6.3 Materials and Methods

Selection of Patients' Specimens

We studied archival autopsy lung tissue specimens of 30 neonates who died of CDH and lung hypoplasia, as confirmed by a lung/body weight (LW/BW) ratio index ≤ 0.012[20-22]. All CDH cases belonged to the high-risk group and presented with respiratory insufficiency within the first 6 hours after birth[22]. Extreme lung hypoplasia was defined as inability to reach normocapnia with persistent hypoxemia and acidosis[18]. After approval of the study design by the university research committees, the specimens were retrieved. These specimens represent all the available material from patients with CDH, who died and of whom parents' consent for autopsy was obtained during the period 1978-1998. In this period, over 350 CDH neonates were treated in the two participating institutions, with an overall survival rate of about 60%.
All cases were subjected to standard management protocols including delayed surgery from 1986 and ECMO management since 1991. Routine cardiac ultrasonography was performed in all cases in order to document PH and right-to-left shunting, together with the clinical observation of preductal and postductal transcutaneous O_2-saturation differences of >10%[21].
Twenty CDH cases with a gestational age above 34 weeks were not subjected to ECMO treatment (CDH-I group); these included 4 newborns who died within the first hour after birth. The primary cause of death in group CDH-I was extreme lung hypoplasia (LH) in 11 cases, persistent pulmonary hypertension (PPH) in 8 cases, and interstitial pulmonary hemorrhage with LH in one case. Non-ECMO treated CDH patients were subjected to ventilation therapy till death. The second group consisted of 10 term CDH neonates (CDH-II), who received ECMO treatment for a period ranging between 2 and 21 days, with a mean bypass time of 237.4 hours. Venoarterial ECMO, when necessary, was instituted to the neonates who did not have any of the following institutional exclusion criteria: gestational age less than 34 weeks, birth weight less than 2000 g, artificial ventilation more than 7 days, alveolar arterial oxygen difference ($A\text{-}aDO_2$) < 80 KPa [600 Torr], and maximal $Pa\text{-}O_2$ less than 10 KPa. Patients who received ECMO were all successfully decannulated and remained on conventional ventilatory support till death due to recurrent or therapy-resistant pulmonary hypertension (n=5),

inability to oxygenate (n=3) and multiple organ failure (n=2). All above-mentioned instances were early post-ECMO deaths.

Five age-matched neonates, who died from acute placental insufficiency or birth asphyxia within the first 24 postnatal hours, served as controls (CON). Controls were subjected to similar ventilatory therapy for up to 16 hours. These controls showed no lung abnormalities or lung hypoplasia on histological examination and had not any of the clinical features of pulmonary hypertension.

Autopsy was performed in the first 24 hours after death. Since we did not find significant differences between both lungs on screening by histological examination[16,22], we processed tissues from either side of the lung randomly for histopathological and immunohistochemical examination.

Histology

Six μm thick serial sections were mounted on polylysine-coated glass slides, and stained with Elastic van Gieson (EvG) stain using Weigert's solution (resorcinol-fuchsin), which stains elastic fibers dark violet, collagen fibers red, and smooth muscle brownish yellow[7,17].

Morphometry

All the small pulmonary arteries with external diameter (ED) less than 200 μm and with a complete muscular coat with distinct inner and outer elastic laminae were measured. Only arteries that were cut at approximately right angles, so that the maximal ED exceeded the minimal diameter by less than 50% were analyzed. All the arteries fulfilling the previous criteria in the mid-lung area for each section were included in the measurements. An average number of 32 arteries from each section were thus assessed. Measurements of the arterial wall layers were performed with a calibrated eyepiece on EvG stained sections.

Medial thickness in microns [MMμ] was calculated as the distance from the external elastic lamina [EEL] to the internal elastic lamina [IEL] along the shortest axis of the artery. (Figure 1.A). Adventitial thickness in microns [AMμ] was calculated along the shortest arterial axis. (Figure 1.A). Total wall thickness in microns [WTμ] was 2 X {MMμ + AMμ} along the same axis. Medial thickness [MT] and adventitial thickness [AT] was expressed as a percentage [%] of the vascular ED to nullify the effect of vasodilatation or vasoconstriction on the measurements as described earlier[14,23]. The morphometric assessment was performed by two of the authors blinded for the clinical data of the cases under investigation.

Immunohistochemistry

Paraffin-embedded lung tissues were cut at 6 μm and mounted on propyl-trioxysilane coated glass slides (Sigma, St Louis, MO, USA). The avidin-biotin complex (ABC) method was employed for immunohistology as described earlier[19]. Briefly, α-SMA slides were incubated for 20 minutes in methanol with 0.3% H_2O_2 to block endogenous peroxidase, then rinsed with phosphate buffered saline (PBS) and placed in Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn, UK). Slides were preincubated with 10%

normal goat serum for 15 minutes, then incubated at room temperature for 30 minutes with a mouse monoclonal anti-human α-SMA antibodies (clone 1A4: Biogenex, San Ramon, MO) in dilution of 1:200. After rinsing with PBS, slides were incubated for 30 minutes with biotinylated secondary antibody (Biogenex) in a 1:75 dilution. Slides were rinsed again, incubated for 30 minutes with peroxidase conjugated streptavidin using a dilution of 1:50 (Biogenex). Slides were colored using 0.025% 3,3-diaminobenzidine (Sigma, St Louis, MO) in 0.01 mol/L PBS, containing 0.03% H_2O_2 and subsequently, slides were lightly counterstained with Mayer's hematoxylin. Negative controls consisted of omission of the primary antibody. α-SMA immunostaining were compared with EvG staining on serial sections to check that the arterial media is colocalized with α-SMA in pulmonary arteries before starting the morphometric measurements.

Statistics

Morphometric values were calculated from different patients' groups and represented as mean ± SEM (standard error of mean) for each variable. Data from CDH groups were compared to their age-matched controls as well as comparison of the data in CDH subgroups were assessed in accordance to the primary cause of death. All comparisons were done using the unpaired Student's "*t*" test. Analyses were performed with the Microsoft Excel software package. Significant results were considered where $p \leq 0.05$ values at probability level.

6.4 Results

Clinical Results

The patients' demographic data and ventilator settings from CDH patients and controls including: inspiratory oxygen fraction (FIO_2), peak inspiratory pressure (PIP), positive end expiratory pressure (PEEP) and frequency are represented in Table I. In the thirty cases of CDH, hernia was left-sided in 22 cases, right-sided in 7 cases, and bilateral in one case.

When comparing the ventilatory settings of CDH groups, we found that FIO2 was 1.0 in all cases, PEEP ranged between 4-6 cm H_2O, PIP ranged between 28- 40 cm H_2O and frequency ranged from 40-80 cycles per minute in non-ECMO treated cases and reached 100 in cases subjected to ECMO therapy.

Table I: *Patients' Criteria of Various Study Groups*

Parameter	CDH non-ECMO		ECMO	Control
	PPH	LH		
Number of cases	8	12	10	5
Gestational age (weeks)	38.8±0.6	37.6±1.1	39.0±0.8	37.8±1.2
Birth Weight (grams)	3150±203	2535±250	3160±134	3180±244
LW/BW ratio	0.0095±0.0016	0.006±0.0015	0.0075±0.0015	0.0156±0.0048
Age at Death (hours)	56.1±19.9[$]	26.4±4.9[*]	333.6±65.0	11.8±4.0
Pre-ECMO Ventilator Settings:				
FIO_2	1.0	1.0	1.0	1.0
PEEP (cm H_2O)	5.2	5.0	5.0	5.0
PIP (cm H_2O)	35.4	35.0	34.6	31.4
Frequency (cycle per minute)	70.0	70.0	85.0	60.0
Time of pre-ECMO ventilation			15 (h)	
ECMO duration (hours)	ND	ND	237.4±47.8	ND

Values of demographic parameters presented as mean±SEM from the term cases indicated in each Group. Where: PPH: Persistent pulmonary hypertension and LH: Lung hypoplasia, LW/BW: Lung weight/body weight, FIO_2: Flow of inspired oxygen, PEEP: Positive end expiratory pressure, PIP: Peak inspiratory pressure, h: hour, and ND: not done. [$]After exclusion of the 1 case that died in ≤ 1 h. [*]After exclusion of the 3 cases that died in ≤ 1 h.

There were no specific differences observed in the demographic data of the CDH cases who died in the first hour of life except that the major cause in 3 of them was extreme lung hypoplasia in whom the LW/BW ratio was 0.004, 0.003 and 0.002 respectively. We observed less body weight; LW/BW ratio and survival in the lung hypoplasia (LH) subgroup of non-ECMO treated CDH cases as compared to PPH subgroup. These values were 2535 g vs 3150 g for body weight, 0.006 vs 0.0095 for LW/BW and 26.4 h vs 56.1 h for survival time, but without statistical significance. (Table I)

ECMO cases were ventilated prior to ECMO institution for a time period ranged between 11-24 hours and all died within < 30 days after decannulation (early deaths).

Morphometric Results

CDH group without ECMO treatment: Pulmonary arteries in CDH cases had significantly thicker media and adventitia as compared to controls, for all the measured variables of arteries under 200 μm ED, with p values: < 0.0001, 0.004, 0.001, 0.02 and 0.0009 for AT, MT, AMμ, MMμ and WTμ, respectively. (Figure 1. A& C).

See picture on page 173 (1)

When comparing the clinical and morphomteric data from the PPH and LH subgroups of CDH-I, no statistically significant differences were found in the measured values except for the MT ($p < 0.03$). The pulmonary arteries of the PPH subgroup had thinner media, but the mean±SEM values of other parameters were higher in the PPH than the LH subgroup without statistical differences. (Figure 1. C& D and Table II). We did not observe any differences in the morphometric values in the 4 cases who died in the first postnatal hour as compared with other cases irrespective of the primary cause of death.

Table II: *Morphometric Values from the Non-ECMO and ECMO-treated CDH subgroups.*

Measured	Non-ECMO CDH		ECMO-treated CDH	
Value	PPH	LH	PPH	LH
MMμ	14 ± 2.3	13.1 ± 1.7	13.6 ± 2.2	14.1 ± 3.1
AMμ	34.9 ± 6.3	30.9 ± 4.9	*20.9 ± 6.5*	*22.5 ± 7.2*
WTμ	97.2 ± 16	87.7 ± 12.4	*68.9 ± 16.4*	*73.4 ± 19.1*
MT	0.198 ± 0.02	0.205 ± 0.02*	0.226 ± 0.03	0.23 ± 0.03
AT	0.461 ± 0.06	0.444 ± 0.05	*0.317 ± 0.07*	*0.337 ± 0.07*

Values of the measured morphometric parameters presented as mean±SEM from the subgroups of CDH cases where: PPH: Persistent pulmonary hypertension, LH: Lung hypoplasia, MMμ: Medial thickness in microns, AMμ: Adventitial thickness in microns, WTμ: Total wall thickness in microns, MT: Percentage of medial thickness to arterial external diameter (ED), and AT: Percentage of adventitial thickness to arterial ED.

Values in (Italic) indicate statistical significance at p value of ≤ 0.05 using unpaired Student "t" test when comparing ECMO-treated and non-ECMO treated CDH main groups collectively without subgrouping any of both groups, while (*) Indicate statistical significance at p value of ≤ 0.05 using unpaired Student "t" test when comparing subgroups: PPH or LH related to one main group only: ECMO treated CDH or non-ECMO treated CDH.

Effect of ECMO on adventitial remodeling in CDH: The pulmonary arteries of ECMO treated CDH cases had the thickest media (13.86 μm±1.79), with p value of 0.05 for MMμ when compared to group CDH-I of non-ECMO treated CDH cases. In small pulmonary arteries, significant thinning of the adventitia and wall thickness were observed in CDH-II group when compared with group CDH-I, with p values of < 0.0001 for the three parameters: AT, AMμ and WTμ values. (Figure 1B).

The ECMO treated group of CDH newborns was divided into 2 subgroups according to the primary cause of death: subgroup LH (5 cases) where the cause of death is inability to oxygenate or multiple organ failure and subgroup PPH (5 cases) where the cause of death was severe therapy-resistant pulmonary hypertension. When comparing both subgroups, the mean±SEM values for each of the measured morphometric parameters were higher in cases of LH subgroup as compared with those of PPH subgroup (Table II), but these results did not reach statistical significance for any of the measured parameters.

Immunohistological Results

α-SMA staining revealed increased amount of vascular smooth muscle actin in CDH cases as compared to controls. (Figure 2. A&C).

See picture on page 173 (2)

No difference regarding the immunostaining of α-SMA among CDH cases either subjected to ECMO therapy or not. (Figure 2. C&D).

6.5 Discussion

In this study we have found that CDH is associated with failure of the normal structural remodeling of pressure-regulating pulmonary arteries after birth, and a partial reversal of this abnormality by ECMO. The primary cause of death among CDH subgroups did not directly influence the tested morphometric parameters.

Previous studies of normal fetal development have shown that the pulmonary arteries become progressively more thin-walled by approaching term[24,25]. In studies concerning CDH, investigators have largely focused on the arterial media as the site of the main structural remodeling[22], but we have reported recently, a lack of progressive adventitial thinning in CDH newborns as seen in normal neonates[16]. The persistence of a thick adventitia of small pulmonary arteries in CDH was partially reversed in our 10 ECMO treated CDH cases. However, a possible correlation with survival could not be evaluated since all cases studied were autopsy cases.

Intraacinar arteries represent the group of resistance arteries in the pulmonary vascular bed and generally have an ED ranging from 50-200 μm, while most

preacinar arteries have ED higher than 200 μm[8,9], hence we measured the arteries with ED ≤ 200 μm only. Although many morphological changes in the developing pulmonary vasculature have been documented in-detail, the molecular bases underlying these changes are not known yet[26]. Indeed, the role of the vascular smooth muscle cells (SMC) in the remodeling process appears to be crucial[26]. But also, the role of adventitia has to be evaluated in-detail. Medial and adventitial thinning observed in control cases with normal lungs probably contributes to the decrease of the pulmonary vascular resistance, necessary for postnatal life. As suggested earlier[9], adventitial thinning near term may be one of the causes of increased compliance in small pulmonary arteries. In the CDH group, there were no differences in the morphometric parameters of the 4 CDH cases, who died in the first hour after birth following unsuccessful resuscitation and other CDH cases irrespective of the primary cause of death. So, the differential structural changes cannot be attributed to ventilator trauma. In addition, controls were subjected to a similar ventilatory treatment with regards to FIO_2, PIP, and volume changes up to 16 hours. Moreover, in the ECMO group, the measured values did not correlate with the duration of ECMO therapy.
In CDH cases, adventitial thickening of small pulmonary arteries could reduce their ability to open and/or dilate in order to increase the vascular bed capacity and to reduce the pressure in the pulmonary bed after birth. Yamataka and Puri mentioned increased adventitial thickness in term CDH neonates and postulated that it contributes to the persistence of pulmonary hypertension[7]. We demonstrated pulmonary arterial structural changes in CDH in relation to gestational age and ECMO treatment[16]. The results of the present series with increased number of CDH cases confirm our previous report and may point to immaturity or underdevelopment of the pulmonary vasculature in CDH. This is supporting our findings of the absence of significant differences regarding AT and WTμ between the term CDH group and either pre-term CDH or pre-term control groups[16]. Furthermore, we looked for correlation between clinical, morphometric and pathologic findings in our subgroups of CDH cases.
Thickening of the adventitia will hamper the distensibility of the vessel wall[27], thus contributing to the production of PPH in CDH cases[28]. These histological changes could be added to the altered arterial functions in CDH as reported in the CDH rat model by our group[29]. These findings could explain the high failure rate of prostaglandin or inhaled nitric oxide treatment in CDH prior to ECMO institution in CDH newborns[30]. Similar abnormal structural pulmonary vascular development has also been observed in neonates with so-called idiopathic persistent pulmonary hypertension[31].

Comparing the data from non-ECMO treated CDH subgroups revealed, no statistical differences between the demographic data, although patients from LH subgroup had a lower mean body weight (2535 g vs 3150 g), LW/BW ratio (0.006 vs 0.0095) and survival time (26.4 h vs 56.1 h, after exclusion of the cases those

died in < 1 h from its related group) than the PPH subgroup. The morphometric measurements from both subgroups of non-ECMO treated newborns revealed higher mean values in the PPH subgroup of the different variables than the LH subgroup except for MT. The significantly lower medial percentage value in PPH subgroup as compared to LH may reflect either a wider lumen or the presence of less muscle in the arterial wall. These may explain the observation of the longer survival time in this subgroup.
Lung rest has been postulated to constitute the main mechanism by which ECMO institution is beneficial in selected CDH patients[4,14,32,33]. A recent report demonstrated the persistence of a thick arterial media in ECMO treated CDH patients[34]. Also, in our study the ECMO treated CDH group exhibited significant high value of MMμ (13.86±1.79 μm) as compared to the non-ECMO treated group of CDH, which may be related to selection bias, the more severe degrees of pulmonary vascular abnormality necessitating ECMO institution. Our data of α-SMA staining, showed significant increased amounts of medial smooth muscle actin in CDH cases whether subjected to ECMO or not as compared to controls, in accordance to previous studies[7,17,22,34].

Although the underlying cause remains incompletely understood[35], significant reduction of adventitial thickness and total wall thickness following ECMO treatment was observed in CDH newborns with $p < 0.0001$ for both AT and AMμ. Hereby, we can provide an explanation to the observation reported by Taira and coworkers that no significant differences were noticed between live-born or stillbirth cases of CDH regarding the thick arterial walls[17]. In CDH, the underlying vascular pathology results from an early fetal event. Interestingly, comparing the values of the ECMO subgroups, the LH subgroup exhibited higher values as compared to PPH subgroup, but these values did not reach statistical significance yet. The degree of LH can not be predicted with any degree of precision prior to ECMO institution during the immediate postnatal period, which is in agreement to previous literature reports[18,36,37].
In our study, lung hypoplasia is present in all cases of study but with variable degrees of severity and all are considered high-risk newborns, based on clinical definition, so selection remains a confounding variable. Investigating the pathology in neonatal cases subjected to ECMO is rarely reported in the literature[38,39]. Our study is the first regarding CDH cases in respect to ECMO treatment and underlying pathology/clinical correlation.

In conclusion, we found that ECMO treatment of CDH neonates resulted in reversal of the adventitial changes in the hypoplastic lungs as a part of pulmonary arterial wall remodeling. Adventitial thinning of the pressure-regulating small pulmonary arteries is possibly one of the underlying mechanisms by which pulmonary hypertension in CDH is ameliorated after ECMO institution by influencing the pulmonary vascular tone. However, the set-up of this study is not suitable to investigate or predict survival benefit, because our observations are all

based on autopsy material. Our data are in agreement to those of Meyrick and Reid who described different underlying histological features of different pathologic conditions that all result in pulmonary hypertension in infants[40]. Consequently, a better understanding of the molecular mechanisms underlying these structural changes resulting in vascular remodeling remains a challenge for future studies.

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PART VI

General Discussion and Summary

Chapter 8

General Discussion and Concluding Remarks:

8.1 Introduction

CDH remains a major therapeutic challenge in neonatology and pediatric surgery. Despite new therapeutic modalities such as exogenous surfactant therapy, NO inhalation, ECMO, partial liquid ventilation, and delayed surgery, the mortality rate remains around 50% in high-risk cases[1-5]. The severity of lung hypoplasia (LH) and the presence of therapy-resistant pulmonary hypertension (PPH) are major determinants of the mortality rate in infants with CDH[6]. The pulmonary vascular abnormalities in CDH consist of decreased number of pulmonary arteries per unit lung volume, and peripheral muscularization of small arteries with medial and adventitial thickening[7,8].

We studied pulmonary vascular development in human CDH patients and age-matched controls. Gestational age 34 weeks served as a caesura to subdivide our study groups, as the saccular stage of lung development is nearly completed at this age [9,10]. The same time frame was investigated earlier in comparable studies[11-13]. This thesis demonstrates pulmonary arterial structural and molecular changes in patients with CDH in relation to gestational age and ECMO treatment.

The specific aims of the studies presented in this thesis are:

1. to study the perinatal developmental remodeling of the pulmonary vasculature in normal and CDH neonates;
2. to investigate the role of VEGF, an angiogenic growth factor, in the pulmonary vasculature of CDH patients;
3. to examine the role of the functional vasodilator NO in CDH lungs by investigating the expression pattern of the enzyme nitric oxide synthase (NOS);
4. to assess molecular lung injury in CDH both at a parenchymal and a vascular level by examining the expression patterns of heat shock proteins (HSPs) as stress markers;
5. to understand the structural and molecular mechanisms underlying the potential beneficial effects of ECMO therapy that lead to the improvement of the associated PPH.

Pulmonary morphological vascular abnormalities in CDH are well documented but complete understanding of the underlying structural and molecular mechanisms is lacking. As knowledge of the developmental pattern of the pulmonary vasculature in the normal human fetus is lacking as well, a reference scale is not available.

8.2 Vascular Remodeling and Morphometric Changes

Several investigators have found that during the final stages of gestation the pulmonary arteries become progressively more thin-walled[14-16]. This represents an important perinatal normal remodeling process to prepare the fetus for extrauterine circulation. At the time of birth, with the onset of extrauterine respiration, pulmonary vascular resistance falls abruptly, and pulmonary blood flow rapidly increases up to tenfold[17]. So far, attention has largely focused on the arterial media and its morphological changes related to differences in cell population, extracellular matrix composition in animal models of neonatal hypoxia, and resulting pulmonary hypertension[18].

In our studies, lung hypoplasia was present in all cases but with variable degrees of severity, and all patients were clinically considered high-risk newborns. We conducted morphological studies in CDH cases, including 10 cases who had been treated with ECMO for an average bypass time of 237 hours, to document the developmental profile of the pulmonary arteries. In a control group of age-matched newborns who had no macroscopic or microscopic histological features of lung hypoplasia or pulmonary hypertension, the adventitia of arteries with < 150 μm external diameter (ED) -pressure regulating arteries- was significantly thicker in pre-term neonates than it was in term neonates. This was also reflected in the total wall thickness[19]. In CDH, there are persistent significant high values for both medial and adventitial thickness. As was suggested earlier[20], adventitial thinning near term may be one of the causes of increased compliance in small pulmonary arteries. The decrease in the adventitial and arterial wall thickness near term constitutes a natural mechanism to prepare the fetus to the extrauterine low-pressure pulmonary circulation. We have also reported significant decrease of adventitial thickness to the values similar to controls with persistent thick media in ECMO-treated CDH cases[19]. Beals et al concentrating rather on the media alone, reported postnatal remodeling of pulmonary vessels in CDH cases[21]. The study of Thibeault and Haney documenting decreased pulmonary pressure after two weeks of ECMO, with persistence of a thick arterial media, in ECMO treated CDH patients with decreased wall thickness, supports our data[22]. Our data demonstrated that the effect of ECMO is based on vascular remodeling rather than muscle relaxation, as was presumed[22], documenting the role of vascular remodeling in different developmental and pathophysiological conditions.

On the basis of clinical data of ECMO-treated CDH cases, Nagaya and coworkers speculated on the existence of differences regarding the pre-ECMO hypercapneic and hypoxemic conditions that necessitate ECMO institution preoperatively, in relation to the underlying pathology, either PPH or LH[23]. To describe the developmental profile of lung vessels in CDH as well as in normal neonates during the perinatal period, we conducted a study by which we could correlate the clinical data with the morphometric measurements of the pulmonary vasculature in CDH subgroups in accordance to the different underlying pathologic conditions.

We found that in ECMO-treated cases of CDH the mean values for each of the measured morphometric parameters were higher in the lung hypoplasia subgroup than in PPH subgroup. In contrast, in CDH cases without ECMO, the morphometric parameters in the PPH subgroup were higher than in the LH subgroup. However, these differences did not reach statistical significance except for the percentage of medial thickness as compared among the subgroups of the non-ECMO group of CDH. Hence it can be inferred that the primary cause of death among CDH subgroups could not directly be correlated with the morphometric parameters regardless of the ECMO treatment[24]. The precise degree of LH can not be predicted prior to ECMO institution during the immediate postnatal period, which is in agreement with earlier reports[23,25]. Even prenatal Doppler flow measurements used in prenatally diagnosed cases of CDH failed to predict the degree of lung hypoplasia [Laudy J, personal communication].

8.3 Angiogenic and "Functional" Molecules in Pulmonary Vasculature

Some molecules, like VEGF, FGF, and the isoforms of PDGF, have been reported to have potential importance in angiogenesis and vascular remodeling. VEGF is an endothelial mitogen, which is regulated at the receptor level[26,27]. Fms-like tyrosine kinase (Flt-1) and Flk-1 are receptors for VEGF and are expressed during early vascular development in human embryos. VEGF has been shown to play a role in fetal angiogenesis; its expression increases at mid gestation to enhance angiogenesis and formation of vascular beds, and decreases towards term[28,29]. Studies performed in different types of PH showed that the expression of VEGF was up-regulated in persistent pulmonary hypertension in neonates (PPHN)[30]. We reported enhanced expression of VEGF at the level of the SMC and endothelium in small pulmonary vessels of CDH cases complicated with pulmonary hypertension as compared to age-matched controls [31]. Our data point at persistent lung vessel growth stunt in CDH cases, as VEGF was increased at mid-gestation in human fetuses[28]. This is in accordance with the findings of Wigglesworth and Desai who reported that infants with lung hypoplasia in CDH born at 34-39 gestational weeks have a lung cell population comparable to that of a normal fetus at 20-22 weeks[13]. In our study, the increased expression possibly represents a fetal attempt to stimulate angiogenesis of the stunted bed in case of CDH. Interestingly, we did not observe differences in VEGF expression between ECMO-treated and non-ECMO treated CDH cases. Our data on PDGF-BB expression revealed a lower expression in CDH cases as compared to controls but without reaching statistical significance[32]. Furthermore, this expression pattern of PDGF-BB does not differ between ECMO-treated and non-ECMO-treated cases. Thus, the role of PDGF-BB in the VEGF-stimulated pathway of pulmonary angiogenesis (This thesis, *Chapter 1, figure 1*) in case of CDH appears to be minimal, in contrast to that reported in the developing rat lung[33].

Furthermore, the functional reactivity of pulmonary arteries needed to be verified. We investigated the effect of the endothelium-derived relaxing factor nitric oxide (NO) by assessing the expression pattern of NO synthase (NOS) enzymes involved in the synthesis of NO. Two isoforms, endothelial (eNOS) and inducible (iNOS), have a major role in regulating the vascular tone through facilitating vasorelaxation (This thesis, *Chapter 4, figure 1*)[34-36]. We could not detect any significant differences in the immunolocalization of eNOS in the endothelium of small pulmonary arteries in CDH cases either subjected to ECMO therapy or not, as compared to age-matched controls [This thesis, *Chapters 4,5*]. Comparatively we reported significantly decreased expression of iNOS in CDH cases not subjected to ECMO therapy. In addition, ECMO cases showed increased expression values of iNOS -towards basal expression- in the small pulmonary arteries[37]. Our data indicate that impaired NO-mediated response in CDH cases may be attributed to decreased expression of eNOS. Enhanced expression of iNOS in CDH cases in the vascular endothelium after ECMO therapy may eventually contribute to the production of NO, which would result in vasodilatation of the pulmonary bed. However, unchanged levels of eNOS in the pulmonary arteries in CDH cases either subjected to ECMO therapy or not may provide an explanation for the underlying mechanism of the clinically observated unpredictable responses to NO and ECMO therapies reported earlier[12,38]. Another underlying mechanism could be the altered reactivity of pulmonary arterial smooth muscle cells to NO in CDH cases. Also, the vasoconstrictor ET-1 might have gained the upper hand in balancing the pulmonary vascular tone. These observations are conform those of Hecker and coworkers in a VSMC cell culture model in rats[39], and also conform our findings in the rat model of CDH[40]. This provides another mechanism by which PH persists or resists vasodilator and/or NO therapy in CDH cases[12], one which certainly warrants further investigation.

8.4 Pulmonary Vasculature under Stress

A number of genes are expressed immediately when cells are subjected to stress, stretch, and in case of acute and chronic lung injury. HSPs are groups of highly conserved proteins that can be induced by exposure to heat, as well as under a variety of pathophysiological conditions including hypoxia, oxidative and metabolic stress[41-43]. The effects of "protective molecules" such as HSPs or iNOS have not been studied before in CDH patients. HSPs represent one of the defense mechanisms against injury and have been reported to participate in embryonic and fetal development[44]. We studied the expression patterns of HSP-27 and HSP-70 in CDH cases with and without ECMO, quantifying their expression in the lungs. Sections were graded from 0-4 for the intensity of expression signal of HSPs in the endothelium and medial SMC of small (50-200 μm external diameter [ED]) and large (> 200 μm ED) pulmonary arteries. Our data on HSP-70 showed significantly increased expression in CDH cases, which was down regulated after ECMO treatment in medial SMC of large and small

arteries and endothelium of small arteries[45]. Also, HSP-27 expression was significantly increased in the pulmonary vascular SMCs and endothelium. ECMO treatment was associated with a significant decrease in levels of HSP-27 in medial SMC of small arteries and in endothelium of both large and small arteries, as compared to the non-ECMO treated CDH group[45]. HSP-27 is a stress protein that has been shown to translocate from the cytoplasm to the nucleus upon stress, acting as a molecular chaperone with a vital role in signal transduction and drug resistance[43,44]. HSP-27 is related to smooth muscle cell contraction as it constitutes the main phosphoprotein, and was found to be enhanced in vessel walls in response to various types of stress[46]. Our findings of increased expression in SMC of small pulmonary arteries in CDH cases point to the presence of stress mainly at the vascular level. Stressed SMC possibly react abnormally, thus intensifying the existent PH. ECMO treatment was associated with significant reduced expression levels of HSPs, especially in the medial SMC of pulmonary arteries[45]. ECMO probably helps in alleviating CDH-associated PH, as reported in a recent review[47]. Our findings indicate that this state of pulmonary stress in CDH appears to be ameliorated under ECMO treatment, thus improving the PH associated cases of CDH. Our current results therefore reflect probably one of the molecular mechanisms involved in the documented beneficial effect of ECMO in the management of pulmonary hypertension in CDH cases[4,5,22,47-49].

Furthermore, in another study, vascular stress was evaluated using iNOS as a stress marker. Distinct iNOS immunostaining was identified in the endothelial cells of small pulmonary arteries with a ED less than 200 μm, which was significantly lower than the expression in controls[37]. Consecutive sections stained with CD 31 confirmed that the positive cells were endothelial. At the same time, ECMO treatment was associated with significant increase in iNOS expression in the vascular endothelium of small pulmonary arteries as compared to non-ECMO treated cases of CDH. However, differences with controls were not statistically significant using the non-parametric Kruskal-Wallis test [This thesis, *Chapter 5*]. Large pulmonary arteries endothelium showed similar differences among groups without reaching statistical significance either. Enhanced iNOS in ECMO cases towards control levels again support our recent report, on the role of ECMO in decreasing cellular stress in CDH cases, as reflected by decreased expression of HSPs[45]. In that respect, ECMO not only induces hemodynamic rest of the pulmonary circulation as reported in humans[22] or experimentally in goats[50], but also alleviates the stress in the pulmonary resistance arteries at the molecular level. This could answer partially the question raised by DeMello and Reid [51], on the mechanisms contributing to the beneficial effects of ECMO in CDH newborns. We have to realize that an as yet unsolved problem is the fact that all the patients we studied after ECMO therapy and successful decannulation died. This raises the need to extend the studies to include survivors, which will only be possible by longitudinal follow up of individual patients using lung biopsies.

8.5 Parenchymal Lung Stress

The lung defense mechanisms against injury involve multiple enzyme systems, such as anti-oxidant enzymes and HSPs, capable of protecting the lung tissue against different injurious or noxious stimuli[41]. The efficacy of these systems varies according to age and has specific roles in different pathophysiological conditions. We have reported the presence of lung parenchymal stress in CDH, both in humans[45] and in our rat model of CDH [Okazaki et al, submitted]. The stress could be related to hypoxia, hyperoxia or ventilation trauma and shear forces. We found significantly higher expression of HSP-70 in the bronchial epithelium of CDH cases, which was down regulated after ECMO[45]. However, in the same study, HSP-27 expression revealed non-significant differences in the bronchial epithelium irrespective of ECMO treatment[45].

Furthermore, we have shown significantly increased expression of iNOS in alveolar macrophages in CDH lungs as compared to controls as verified by staining with anti-CD 68, which is a known macrophage marker [This thesis, *Chapter 4*]. ECMO-treated CDH cases did not exhibit decreased iNOS in alveolar macrophages. Increased expression of iNOS points at potential involvement of parenchymal lung injury or cytoprotection against this injury after longer periods of exposure to artificial ventilation[52,53]. Epithelial expression of iNOS confirms its role in airway homeostasis after injury or during different pathophysiological conditions[54]. It seems, therefore, that ventilation techniques need to be adapted in order to minimize the parenchymal lung injury, although preliminary data on high frequency oscillation or partial liquid ventilation in human cases could not provide the required option[55-57].

8.6 ECMO Therapy: Underlying Mechanisms

ECMO has been used in an attempt to diminish the abnormal pulmonary vascular tone by guaranteed O_2 supply, and to reverse the pulmonary structural abnormalities[1,4]. It is unclear whether ECMO imposes pulmonary stress responses in CDH infants. Our data showed that ECMO results in adventitial thinning with decreased percentages of adventitial thickness, which probably reduces resistance from thick stiff collagen and facilitates wall distensability[58]. Although decreased pulmonary pressure following ECMO in CDH cases has been reported[22], a standard or predictable response to this therapy is not known. Other mechanisms presumably play a role. Our data on enhanced iNOS in ECMO cases support the role of ECMO in decreasing the cellular stress in the pulmonary vasculature in CDH lungs [37,45], as well as in lung parenchyma[37,45] as reflected by decreased expression of heat shock proteins[45]. One of the limiting factors of our studdies was the fact that ECMO cases of CDH represent the most severe cases in which ECMO was used after failure of other therapeutic tools. Our findings show that the current trend of changing the ECMO criteria to include smaller or more immature babies should not be advocated in CDH cases. These patients have severely hypoplastic lungs incompatible with life in more than 90% of

cases[24]. A recent study showed remodeling of airway muscles beside the vascular changes after ECMO in CDH cases[59].
At a microscopic level, ECMO is beneficial in decreasing the pulmonary vascular stress and in inducing vascular remodeling by adventitial wall thinning of pulmonary arteries, as potential mechanisms in alleviating pulmonary hypertension associated CDH. Another important mechanism is the production of NO via induction of iNOS enzyme expression, which eventually alters the pulmonary vascular reactivity towards the vasodilatation side. Also at the parenchymal level, ECMO could enhance cytoprotection and decrease cellular stress as it brings down the high level of HSP-70 and HSP-27. Our data do not enable to predict the response to ECMO therapy in individual patients, as all our studied ECMO patients had died.

The role of ECMO in CDH is still controversial[55,60-63], with regard to protocols, additional therapies, and short or long-term follow up[55,63-70]. Our data provide further evidence for the benefits of ECMO in high-risk cases of CDH in well-trained hands at the molecular and cellular levels[19,24,37,45]. The conclusion of many reports that the overall survival of CDH has not changed over 20 years, is not true, as ECMO centers with having gained experience started to include more high-risk cases, to prolonge ECMO duration, and to withdraw some of the exclusion criteria. Our studies regarding ECMO therapy in CDH cases aiming at confirming the pathological changes at the microscopic level expanded our knowledge of the mechanism(s) of ECMO.
Table I summarizes the structural changes and the related angiogeneic and functional molecules in CDH cases with or without ECMO therapy.

Table I: *Comparative Structural and Molecular Changes Following Conventional and ECMO Therapy in Human CDH Cases.*

Parameters	Control	CDH without ECMO	ECMO-treated CDH
Vascular Remodeling:			
Adventitia	N	↑ (7,19)	↓ (19)
Media	N	↑ (7,60)	↑ (19,21,22)
Wall Thickness	N	↑ (7,19,22)	↓ (19,22)
α-SMA	N	↑ (7,19)	↑ (19)
Airway Remodeling:			
α-SMA	N	↑ (59)	↓ (59)
Growth and Functional Molecules:			
VEGF	↓ (29,31)	↑ (31)	↑ (#)
PDGF-BB	↑ (32)	↓ (32)	↓ (#)
ENOS	= (*)	= (*)	= (*)
INOS	↑ (37)	↓ (37)	↑ (37)
Stress Molecules:			
Vascular stress			
HSP-70	↓ (45)	↑ (45)	↓ (45)
HSP-27	↓ (45)	↑ (45)	↓ (45)
INOS	↑ (37)	↓ (37)	↑ (37)
Parenchymal stress			
HSP-70	↓ (45)	↑ (45)	↓ (45)
HSP-27	= (45)	= (45)	= (45)
INOS	= (*)	= (*)	= (*)
Alveolar Macrophages	↓ (*)	↑ (*)	↑ (*)

CDH = congenital diaphragmatic hernia, ECMO = extracorporeal membrane oxygenation, N = normal, ↑ = increase, ↓ = decrease (both ↑ and ↓ are in relation to normal), α-SMA = alpha smooth muscle actin, VEGF = vascular endothelial growth factor, PDGF-BB = platelet derived growth factor-BB, eNOS = endothelial nitric oxide synthase, iNOS = inducible NOS, (=) = non-different and HSP = heat shock protein. Number in brackets indicates reference, number (*) indicates data in the chapters of this thesis, and (#) indicates unpublished data.

Multicenter randomized studies with increased numbers of patients are essential to fill our lack of knowledge of the mechanisms of ECMO at the structural and molecular levels. Such studies could be conducted via the CDH collaborative study group. Having gained understanding, we will be able to change our management strategies and improve the outcome in infants with CDH.

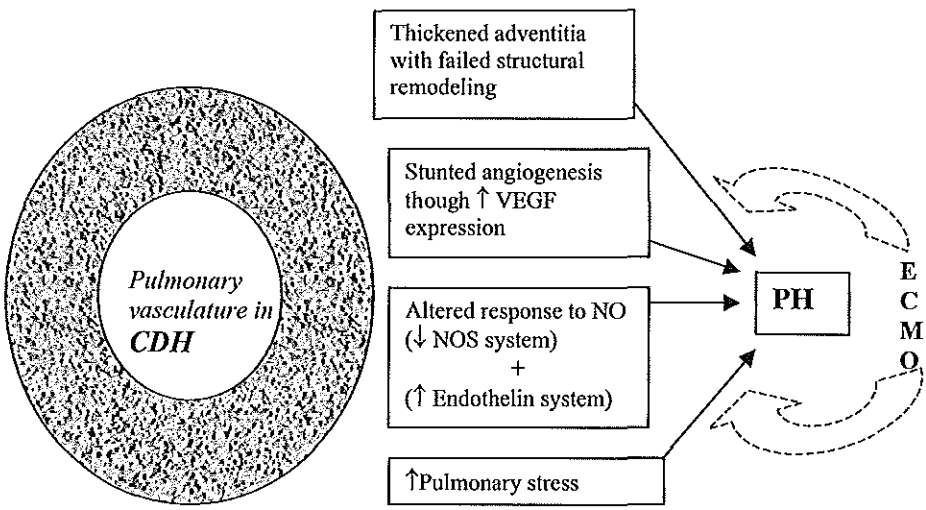
8.7 Concluding Remarks

Various chapters included in this thesis have established the fact that the pulmonary vasculature in term CDH infants with lung hypoplasia is premature, resembles earlier fetal stages at structural and molecular levels. The previous finding that CDH infants with lung hypoplasia born at 34-39 gestational weeks have a lung cell population comparable to that of a normal fetus at 20-22 weeks [13], is in agreement with our findings.

At the level of the pulmonary vasculature, especially in the smaller arteries, we found structural immaturity[19], as well as the angiogenic molecule: VEGF[31]. Similar interrupted natural processes of pulmonary vascular development have been observed in PPHN[69,70]. Additionally, altered NO-dependent vasodilatatory function points to abnormal or immature functional reactivity of the pulmonary vasculature in CDH cases. The previous three elements participate in failed accommodation to normal postnatal demands in the pulmonary bed in a number of patients. The presence of thickened arterial walls with decreased numbers and eventually abnormal reactivity points to complex mechanisms involved in the production of PH in CDH cases. Another contributing factor could be the state of stress at the vascular as well as at the parenchymal level indicated by our data[37,45].

ECMO therapy could be beneficial in reversing some of the above-described abnormalities. The data empoided in this thesis provide for the first time some information on the molecular and structural changes that underlie the beneficial effect of ECMO in the treatment of CDH cases. Moreover, the anti-stress effect of ECMO shown in our studies is another mechanism by which ECMO seems to ameliorate PH in some CDH cases. Figure 1 schematically represents the structural and molecular mechanisms leading to the development of PH in CDH, and the possible influences of ECMO.

Figure. 1: *Structural and Molecular Abnormalities in the Pulmonary Vasculature of CDH: Possible Role of ECMO in Ameliorating the PH in Some Cases.*



CDH = congenital diaphragmatic hernia, VEGF = vascular endothelial growth factor, NO = nitric oxide, NOS = nitric oxide synthase, PH = pulmonary hypertension and ECMO = extracorporeal membrane oxygenation.

The data presented in the thesis create a foundation for the understanding of the pathophysiology of CDH. However, further studies are warranted to improve our knowledge of the pulmonary vascular abnormalities in CDH, with a view to providing more efficient therapeutic modalities in these infants. Future research is suggested to be along one of the following lines:

- Search for other molecules of importance in angiogenesis or remodeling such as the FGF family of proteins.
- Define the exact time point during prenatal development for molecules like VEGF, eNOS enabling future therapies like anti-VEGF and anti-ET that have been developed in a few adult conditions like PH and ischemic heart disease with VEGF or anti-ET-1 antibodies.
- Search for specific genes which may lead to the development of PH in CDH or other related neonatal conditions of importance, as this may open the way for the implication of gene therapy when available.
- Modulate the current strategies of ventilation and ECMO management to keep lung injury and stress to a minimum.

Certainly, there are still many gaps to be filled and CDH remains a challenge for pediatric surgeons, neonatologists and pediatric intensivists. Step by step evaluation of the normal and abnormal development of the pulmonary vasculature using techniques ranging from standard morphology to radiological assessments will lead us to differentiate between different pathogenetic mechanisms of PH in different neonatal conditions. In addition, understanding the developmental biological events will help us to evidence-guided modification at the therapeutic level. In this way we will be able to change our policy from a wait and see attitude towards pre-emptive management.

8.8 References

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Chapter 9

9.1 Summary

In spite of modern therapeutic modalities, congenital diaphragmatic hernia (CDH) remains a major problem, since the overall mortality rate could be up to 60% in high-risk cases. The pulmonary vascular abnormalities in CDH constitute a major determinant factor to such high mortality. However, the pulmonary vasculature has not been studied in detail from a structural and molecular point of view in order to assess the anatomical and functional correlation in infants with CDH. As a new therapeutic tool, extracorporeal membrane oxygenation (ECMO) has accomplished its role in the management protocols of CDH cases associated with pulmonary hypertension (PH). The major beneficial mechanism attributing to ECMO effect in some CDH cases is described as "lung rest" and diminishing pulmonary blood pressure.
This thesis composes of five parts: *Part I*: general introduction (Chapter 1); *Part II*: describes studies focusing on the structural and molecular aspects in CDH cases and controls (Chapters 2 and 3); *Part III*: focuses on studies concerning the role of nitric oxide (NO) as a vasodilator molecule in the pulmonary vascular bed in CDH lungs (Chapters 4 and 5); *Part IV*: describes the human lung response to stress in case of CDH (Chapter 6); *Part V*: describes the pathological changes resulting from ECMO therapy in CDH cases in correlation with the clinical data (Chapter 7), and *Part VI*: includes the general discussion and summary (Chapters 8 and 9).

Chapter 1 contains the subject of the thesis with a detailed literature review and the rationale of the studies included in this thesis. The problem of CDH is discussed with the emphasis on the role of the associated pulmonary vascular abnormalities. A detailed description of normal and abnormal pulmonary and vascular development is followed by the use of different animal models in an attempt to understand the human situation. The lack of knowledge of the normal and abnormal pulmonary vascular development in the human is addressed. The description of the use of ECMO in the management of CDH cases which so far lacks an appropriate mechanistic explanation and consequently possible improper way of management of these high-risk infants is addressed also. This chapter ends by the aims of the studies included in the thesis.

Chapter 2 describes histological and morphometric data from investigating the structural abnormalities in the pulmonary vascular bed of CDH cases and age-matched controls. We studied the histology of the lungs of twenty-nine CDH autopsy cases, with special attention to the pulmonary arteries, and related our findings with gestational age and the application of ECMO treatment. Formalin-

fixed and paraffin-embedded specimens were stained with hematoxylin & eosin (HE) and elastic van Gieson (EvG) stains followed by morphometric measurements of the arterial media and adventitia. A significant decrease was observed in adventitial percentage and total wall thickness of small pulmonary arteries with an external diameter ≤ 150 μm at term in control newborns as compared to pre-term controls. In CDH newborns, all the measured values of the arterial wall remained significantly higher as compared to their respective controls. In CDH neonates, the increase of adventitial thickness also affected the supernumerary arteries. CDH newborns subjected to ECMO treatment showed a significantly thinner arterial adventitia than CDH cases who did not receive ECMO, the latter approaching normal values. Comparatively, there was no change in the medial thickness in CDH cases treated with ECMO as compared to the non-ECMO group. These results indicate that in CDH, there is a failure of the normal arterial remodeling process occurring during the perinatal period as manifested by persistent thick adventitia at term in CDH patients. This appears to be partially reversed by ECMO treatment, thus constituting one of the mechanisms by which ECMO treatment aids in alleviating the associated PH in CDH newborns.

In *Chapter 3* we describe the role of the important angiogenic growth factor: vascular endothelial growth factor (VEGF) in the stunted pulmonary vascular bed of CDH cases. Twenty-one lung autopsy specimens of human CDH patients with lung hypoplasia and seven specimens of age-matched control newborns without lung hypoplasia were processed for immunohistochemistry. All CDH cases had pulmonary hypoplasia, as evident from a lung/body weight index ≤ 0.012 and pulmonary hypertension. Cellular localization of VEGF was analyzed semiquantitatively using a staining score ranging from 0 (no staining) to 4 (very strong staining). Statistically significant elevated levels of VEGF immunoreactivity were observed in CDH lungs as compared to controls. VEGF was mainly detected in bronchial epithelium and in medial smooth muscle cells (SMC) of large (> 200 μm) and small (< 200 μm) pulmonary arteries, with the most intense staining of the pulmonary vasculature in medial SMC of small pulmonary arteries. In CDH patients, but not in controls, endothelial cells were positive for VEGF staining. The elevated expression levels of VEGF, especially in the small, pressure-regulating pulmonary arteries, point to a potential role in vascular remodeling. It appears that in the developing fetus, an unsuccessful attempt to increase the pulmonary vascular bed occurs in the CDH hypoplastic lungs in order to alleviate the associated pulmonary hypertension.

Chapter 4 demonstrates for the first time, the role of NO synthase (NOS) in human CDH. We investigated the expression of the inducible form of NOS in the vascular bed as well as its role as a stress molecule in the lungs of CDH neonates and age-matched controls. Also, we investigated the effects of ECMO treatment on iNOS expression levels. Thirty-three archival lung specimens of CDH neonates with lung hypoplasia were studied. Twenty-three of these CDH cases were not subjected to ECMO treatment, while the remaining 10 cases had been treated with ECMO for a mean time of 238 hours. Eleven age-matched

neonates without lung hypoplasia, who had died from neonatal asphyxia or placental insufficiency, served as controls. Paraffin-embedded specimens were processed for immunohistochemical localization of iNOS. Computer assisted video image analysis for the endothelial expression of iNOS in the pulmonary vessels with an external diameter (ED) < 200 μm was performed. Parenchymal lung injury was evaluated through assessment of iNOS expression in lung macrophages as verified by staining with the macrophage marker: CD 68.

Inducible NOS was localized in the vascular endothelium, as verified by staining with an endothelial cell marker: CD-31, on consecutive sections. Endothelial expression of iNOS in small pulmonary arteries was reduced in non-ECMO treated CDH cases as compared to controls. However, ECMO treatment was associated with an increase in the endothelial expression of iNOS as compared to the non-ECMO treated CDH group. Alveolar macrophages expressed iNOS in significantly higher levels in CDH cases as compared to controls, while the highest number of macrophages was observed in the ECMO-treated group. We conclude that vascular expression of iNOS is down regulated in CDH lungs and ECMO treatment appears to compensate for such decrease. This could lead to abnormal vascular reactivity or non-responsiveness to the released NO. Consequently, altered production of NO via the altered NOS system could be one of the molecular mechanisms underlying the development of PH and non-response to NO therapy in selected CDH cases. Although ECMO induces "rest" at the level of the vascular bed, it appears that lung parenchyma exhibit a more stressful condition as demonstrated by increased iNOS in alveolar macrophages.

Chapter 5 describes the role of endothelial NOS (eNOS) in the production of NO as endothelium-derived relaxing factor in the pulmonary vascular bed of CDH cases in the same groups as described in *Chapter 4*. No significant differences in eNOS immunolocalization were found among groups except for the endothelial localization in large pulmonary arteries. We conclude that a state of imbalance is present between vasodilating and vasoconstricting molecules in the immature pulmonary vasculature in lungs of CDH newborns. Altered pulmonary expression of NOS isoforms could explain the altered response of the pulmonary vasculature to inhaled nitric oxide (NO) and points to a mechanism leading to the production of PH in CDH cases. This indicates a functional immaturity or abnormal reactivity of the vascular bed in CDH. ECMO appears to induce rest and to initiate vasodilatation at the level of the vascular bed. Since this is not reflected into increased survival among patients' groups, further studies are needed to investigate this effect in more detail.

Chapter 6 describes the expression pattern of stress genes (heat shock proteins, HSP 27 & 70) in lungs of CDH patients with PH, and the influence of ECMO on the expression levels of these genes in order to understand the underlying molecular mechanisms. Paraffin-embedded lung autopsy specimens with CDH and lung hypoplasia, either having received ECMO treatment or not, and age-matched controls were immunostained using monoclonal anti-human

antibodies against HSP-70 and HSP-27. Expression levels of both HSP 27 and 70 were semiquantitatively evaluated in bronchial epithelium, as well as in medial smooth muscle cells (SMC) and endothelium of large and small pulmonary arteries, using a score ranging from 0 to 4. For HSP-70, the most pronounced immunoreactivity was observed in the bronchial epithelium, followed by the medial SMC of small arteries (of external diameter < 200 μm). The overall expression was significantly higher in CDH cases than in controls, in bronchi as well as in pulmonary arteries. For HSP-27, intense staining was found in medial SMC followed by the bronchial epithelium in controls, with significantly increased expression in medial SMC of large and small arteries in CDH cases. ECMO treatment was associated with significantly reduced expression levels of HSP-70 in medial SMC of both large and small arteries, whereas HSP-27 expression levels were decreased only in small arteries. In addition, the expression levels of both HSPs were significantly lower in endothelium of small arteries. Our data on the expression of stress genes showed increased expression of HSPs in CDH, which points to a condition of pulmonary stress. This pulmonary stress appears to be partially ameliorated under ECMO treatment.

In *Chapter 7* we attempted to correlate the underlying pathologic features with the clinical data among ECMO-treated and non-ECMO treated CDH groups. Also, we tried to identify the differences in pulmonary vascular morphology among CDH cases according to the primary cause of death: either extreme lung hypoplasia (LH) or persistent pulmonary hypertension (PPH). We studied autopsy sections from 30 high-risk CDH cases with respect to the pulmonary arteries, in relation to gestational age (GA) and ECMO treatment. They were grouped into CDH-I: 20 cases with GA >34 weeks (w) who were not subjected to ECMO and CDH-II: 10 cases with GA >34 w, who were subjected to ECMO for an average time of 237 hours. Five age-matched neonates without evidence of lung hypoplasia served as controls. We used the same methodology as described in *Chapter 2* with increased numbers of cases in this study. CDH cases were subgrouped and compared according to the primary cause of death. In CDH newborns, a significant increase in medial, adventitial and total wall thickness was found in pulmonary arteries with an external diameter < 200 μm as compared to age-matched controls. ECMO-treated CDH newborns showed a significantly thinner arterial adventitia than CDH cases who did not receive this treatment. In the former of which, values approached normal values, however the medial thickness remained increased. Morphometrically, no significant differences in CDH cases between cases dying from PPH or severe LH could be determined. From the data included in this chapter, we concluded that: 1) Pulmonary vascular morphology in CDH does not differ between the groups with lung hypoplasia or persistent pulmonary hypertension as a primary cause of death.2) Adventitial thinning of these arteries might be one of the mechanisms by which ECMO alters PPH in CDH cases.

Chapters 8 and 9 give a detailed discussion of the entire work included in this thesis and summaries in English and Dutch languages.

In conclusion, our findings demonstrate that in CDH cases, the pulmonary vasculature is in an immature state structurally and morphometrically as shown by failed adventitial remodeling. These findings can not be attributed to the underlying primary cause of death. At the molecular level, the expression (pattern) of VEGF at term in CDH cases is high as comparable with what reported in pre-term normal cases at earlier stages of angiogenesis. Functionally, there is a state of abnormal reactivity while the role of NO as vasodilator is of minor importance, which points to a possible pivotal role of vasoconstricting molecules such as endothelin-1. A state of molecular stress in CDH lungs is present both at the level of the vascular bed and the lung parenchyma, although it is more obvious in the vascular bed. Many mechanisms that participate in the production of PH in CDH could be inferred from our studies, such as persistent thick vascular wall layers, decreased expression of eNOS with altered expression of iNOS and increased expression of the stress markers; HSPs. Our results show that all previous observations are more documented in the pressure-regulating small diameter pulmonary arteries. The beneficial effects of ECMO in some cases of CDH are partly attributed to these mechanisms as shown by adventitial thinning, decreased pulmonary stress, and restoration of the expression of iNOS, and not just due to the subjective term "lung rest".

9.2 Samenvatting

Congenitale hernia diafragmatica is nog steeds een groot probleem, met een sterfte tot 60% in zeer ernstige gevallen, ondanks de huidige geavanceerde behandelingsmogelijkheden. Pulmonale vaatafwijkingen zijn sterk bepalend voor deze hoge sterfte. Het pulmonale vaatstelsel is structureel en moleculair nog niet in detail bestudeerd, zodat een anatomische en functionele correlatie in kinderen met CDH nog niet is vastgesteld. Extracorporele membraanoxygenatie (ECMO) wordt als nieuwe behandelmethode toegepast in die patiënten die therapie-resistente pulmonale hypertensie vertonen. Als belangrijkste voordeel van ECMO in bepaalde gevallen wordt rust voor de longen en verlaging van de pulmonale bloeddruk gezien. Dit proefschrift gaat in op onderzoek naar de structurele en moleculaire aspecten van het pulmonale vaatstelsel in CDH-patiënten en controlepatiënten, de vaatverwijdende rol van stikstofoxide (NO) in het pulmonale vaatbed bij CDH, de respons van de long bij stress in patiënten met CDH, en de pathologische veranderingen ten gevolge van de ECMO behandeling bij CDH, gecorreleerd aan de klinische gegevens.

Hoofdstuk 1 is een algemene inleiding met een gedetailleerd literatuuronderzoek. CDH wordt besproken met de nadruk op de rol van de bijkomende pulmonale vaatafwijkingen. De normale en abnormale ontwikkeling van de longen en de vaten worden beschreven, alsmede de toepassing van verschillende diermodellen om de omstandigheden in de mens te kunnen begrijpen. Ook wordt aandacht besteed aan het feit dat onze kennis over de normale of abnormale pulmonale vaatontwikkeling in de mens incompleet is. Voorts wordt gesteld dat de werking van ECMO in CDH gevallen nog niet afdoende is verklaard, en dat ECMO-behandeling derhalve wellicht niet geschikt is bij zeer ernstige gevallen van CDH.

Hoofdstuk 2 beschrijft histologische en morfometrische bevindingen uit onderzoek naar de structurele afwijkingen van het pulmonale vaatbed van CDH-patiënten en controlepatiënten. Deze geven aan dat bij CDH het arteriële hermodeleringsproces dat normaal plaats vindt in de prenatale periode, uitblijft, getuige de persisterende dikke adventitia die bij CDH-patiënten a term werden aangetroffen. Dit blijkt gedeeltelijk ondervangen te worden door ECMO-behandeling, als een van de mechanismen die pulmonale hypertensie in pasgeborenen met CDH omlaag brengen.

Hoofdstuk 3 gaat over de rol van het vasculaire endotheel groeifactor (VEGF) in het onvolgroeide pulmonale vaatbed in CDH-patiënten. Met behulp van semi-kwantitatieve kleuringanalyse werd sterk verhoogde VEGF immunoreactiviteit aangetoond in CDH-longen vergeleken met controlelongen. VEGF bevond zich met name in bronchiaal epitheel en in mediale gladde spiercellen van zowel grote als kleine longvaten, met de hoogste concentratie in mediale gladde spiercellen van kleine longvaten. Deze bevindingen wijzen op een mogelijke rol van VEGF bij de vasculaire hermodelering.

Hoofdstuk 4 toont voor het eerst aan dat NO synthase (NOS) een rol speelt bij CDH in de mens. We onderzochten de expressie van de induceerbare

vorm van NOS (iNOS) in het vaatbed en de rol daarvan als stressmolecuul in de longen van pasgeborenen met CDH en controlepatiënten. We onderzochten ook de effecten van ECMO-behandeling op de expressie van iNOS. We concluderen dat de vasculaire expressie van iNOS lager is in CDH-longen en dat ECMO-behandeling dit weer gedeeltelijk goedmaakt. Aangepaste productie van NO d.m.v. het aangepaste NOS-systeem zou een van de moleculaire mechanismen kunnen zijn die aan pulmonale hypertensie en het niet-reageren op NO-behandeling ten grondslag liggen.

Hoofdstuk 5 beschrijft de rol van endotheliaal NOS (eNOS) bij de productie van NO als endotheelafkomstige ontspanningsfactor in het pulmonale vaatbed bij CDH. We concluderen dat de vaatverwijdende en vaatvernauwende moleculen in het onvolgroeide longvatenstelsel bij CDH niet in evenwicht lijken te zijn. ECMO-behandeling lijkt rust te induceren en initieert vaatverwijding in het vaatbed. Aangezien dit nog niet tot grotere overleving heeft geleid, zijn verdere studies nodig om dit effect gedetailleerder te onderzoeken.

Hoofdstuk 6 beschrijft de expressie van stressgenen ('heat shock proteins', HSP 27 & 70) in de longen van CDH-patiënten met pulmonale hypertensie, en de invloed van ECMO op de expressieniveaus van deze genen. De gevonden verhoogde expressie van HSPs in CDH wijzen op een staat van pulmonale stress, die gedeeltelijk verlicht lijkt te worden door ECMO-behandeling.

In *hoofdstuk 7* wordt nagegaan of de onderliggende pathologische kenmerken gecorreleerd kunnen worden aan de klinische gegevens van CDH-patiënten die wel of niet ECMO-behandeling hebben ondergaan. Ook werd gepoogd de verschillen in de pulmonale vasculaire morfologie van CDH-patiënten te identificeren naar de primaire doodsoorzaak, hetzij extreme longhypoplasie of persisterende pulmonale hypertensie. Pasgeborenen met CDH die ECMO-behandeling hadden ondergaan hadden significant dunnere arteriële adventitia dan zij die geen ECMO hadden ondergaan. Dit zou een van de mechanismen kunnen zijn waarmee ECMO persisterende pulmonale hypertensie beïnvloedt. Morfometrisch konden geen significante verschillen tussen de twee vormen van doodsoorzaak worden vastgesteld.

De conclusie in *hoofdstuk 8* luidt dat bij CDH het pulmonale vaatstelsel structureel en morfometrisch in onvolgroeide staat verkeert, zoals blijkt uit het falen van de adventitiële hermodelering. Op moleculair niveau is het a term expressiepatroon van VEGF hoog bij CDH, zoals is gerapporteerd bij premature normale patiënten in vroegere stadia van het ontstaan van de vaten. Functioneel bestaat er een toestand van abnormale reactiviteit waarbij NO een geringe vaatverwijdende rol speelt, hetgeen wijst op een mogelijke grote rol van vaatvernauwende moleculen zoals endothelin-1. Longen van CDH-patiënten vertonen moleculaire stress, zowel op het niveau van het vaatbed en het longparenchym, maar meer uitgesproken in het vaatbed. Uit de besproken onderzoeken kunnen diverse mechanismen die bijdragen aan het ontstaan van pulmonale hypertensie bij CDH worden afgeleid, zoals: persisterende dikke

vaatwandlagen, verlaagde expressie van eNOS met gewijzigde expressie van iNOS en verhoogde expressie van de stressmoleculen, de HSPs. Deze effecten zijn het nadrukkelijkst in kleinere drukregulerende pulmonale vaten. De gunstige effecten van ECMO in bepaalde gevallen van CDH kunnen gedeeltelijk worden toegeschreven aan deze mechanismen, getuige de dunnere adventitia, verlaagde pulmonale stress, en herstel van de expressie van iNOS, en niet alleen maar aan de subjectieve constatering "rust voor de longen".

Abbreviations

$AaDO_2$	alveolar-arterial oxygen difference
ABC	avidin-biotin complex method
α-SMA	alpha smooth muscle actin
ANG II	angiotensin II
AT	adventitial percentage
ATμ	adventitial thickness in microns
CD31	endothelial cell marker
CDH	congenital diaphragmatic hernia
DAB	diaminobenzidine
DH/TL	diaphragmatic hernia + tracheal ligation
ECMO	extracorporeal membrane oxygenation
ED	external diameter
EEL	external elastic lamina
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ET-1	endothelin 1
EvG	Elastic von Gieson stain
FGF	fibroblast growth factor
FiO_2	fraction of inspired oxygen
HE	hematoxylin eosin stain
HSP	heat shock protein
IEL	internal elastic lamina
I(L)GF	insulin (like) growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
LH	lung hypoplasia
LW/BW	lung weight/ body weight ratio

MHC	myosin heavy chain
MTμ	medial thickness in microns
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PAF	platelet activating factor
PAP	peroxidase-anti-peroxidase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEEP	peak end expiratory pressure
PHMP	posthepatic mesenchymal plate
PIP	peak inspiratory pressure
PKC	protein kinase
PH	pulmonary hypertension
PPH	persistent pulmonary hypertension
PPHN	persistent pulmonary hypertension of the neonate
PVR	pulmonary vascular resistance
SMC	smooth muscle cell
TGF β	transforming growth factor β
TL	tracheal ligation
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
WTμ	total wall thickness in microns

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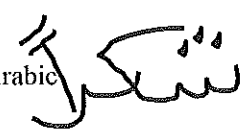

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PICTURES

Figure. 2: Micrograph of the adventitial staining of the intraacinar arteries with EvG stain in the studied human lungs (page 40)

Where: (A) Thickened adventitia in control pre-term lung, (B) Thinning of the adventitia in control term lung as part of the natural remodeling, (C) Thick adventitia in CDH pre-term lung, (D) Persistent thick adventitia in CDH term lung without ECMO treatment, (E) Adventitial thinning in CDH lung after ECMO treatment, and (F) CDH lung section stained immunohistochemically with α-SMA using peroxidase method for localization of pulmonary arterial media.

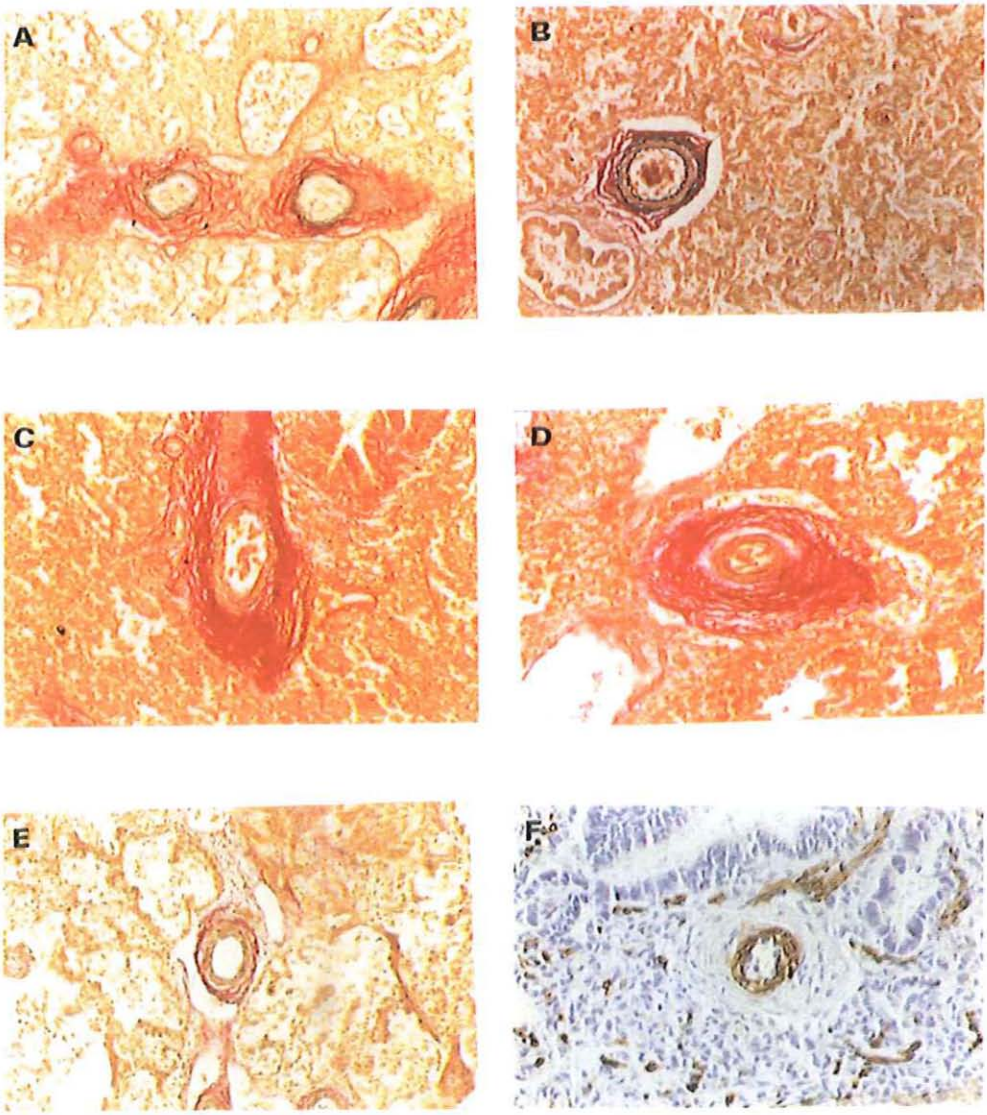
A
B
C
D
E
F

Figure. 1: Immunohistochemical localization of VEGF in lung tissue. (Page 55)

Where: A; intense expression of VEGF in the medial smooth muscle cells of small diameter pulmonary arteries and in the bronchial epithelium of CDH lung tissue, B; expression of VEGF in the bronchial epithelium and faintly in the smooth muscle cells of the pulmonary arteries of control non-hypoplastic lung tissue, C; expression of VEGF in the arterial endothelium (arrow) and medial smooth muscle cells of pulmonary arteries in CDH hypoplastic lung tissue, and D; arterial endothelium identified (arrow) by CD31 staining using peroxidase technique.[Calibration bars = 50 μm in A and B and 25 μm in C and D].

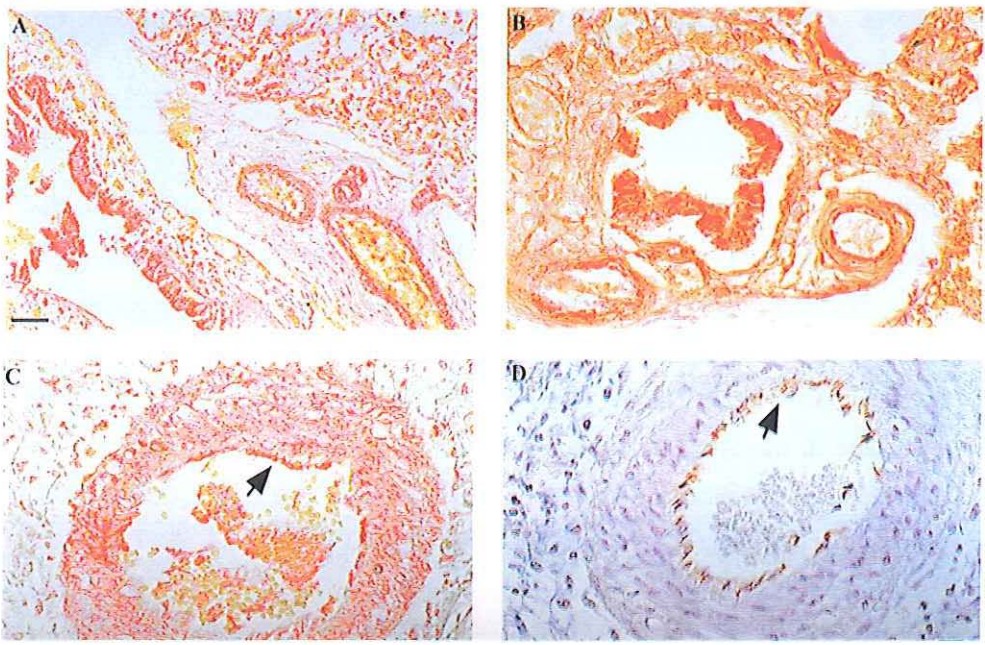
A
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Figure.2: The iNOS Expression in the Pulmonary Vascular Endothelium and Alveolar Macrophages (page 69)

Micrograph showing the immunohistochemical localization of iNOS in the pulmonary vascular endothelium (arrow heads) of the studied human lungs employing the peroxidase-anti-peroxidase (PAP) method (brown color): A) Intense endothelial expression of the small pulmonary arteries in control lung, B) Reduced endothelial expression in CDH lung who was not subjected to ECMO, C) iNOS expression in the cytoplasm of alveolar macrophages (arrow), and D) Enhanced endothelial expression of the small pulmonary arteries in lung section from ECMO-treated CDH newborn. [Bar for all = 25 μm]

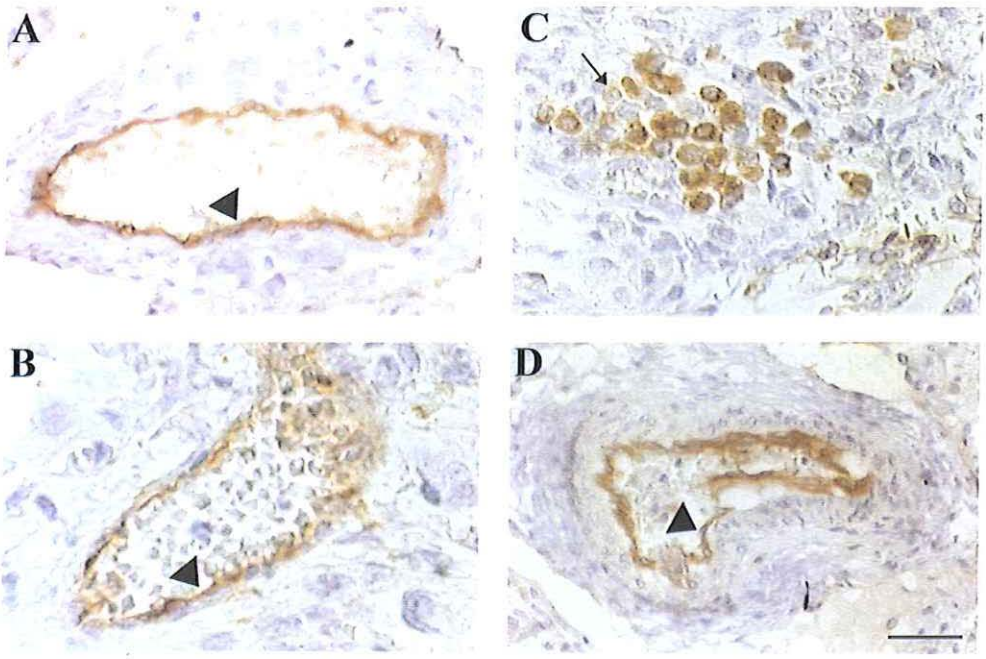

A
C
B
D

Figure. 3: Micrograph of the Immunohistochemical Localization of Alveolar Macrophages Stained with Anti-CD 68 Antibodies Employing Alkaline Phosphatase Method (Red) (page 70)

Where: A) Few positive stained macrophages (indicated by the arrow) in control lung, B) Negative control section after omission of the primary antibody, C) Increased number of macrophages in section of lung from non-ECMO treated CDH newborn, and D) Much increase in the alveolar macrophage number which appear stretched and edematous (arrow head) in lung section from ECMO-treated CDH newborn. [Bar for all = 25 μm]

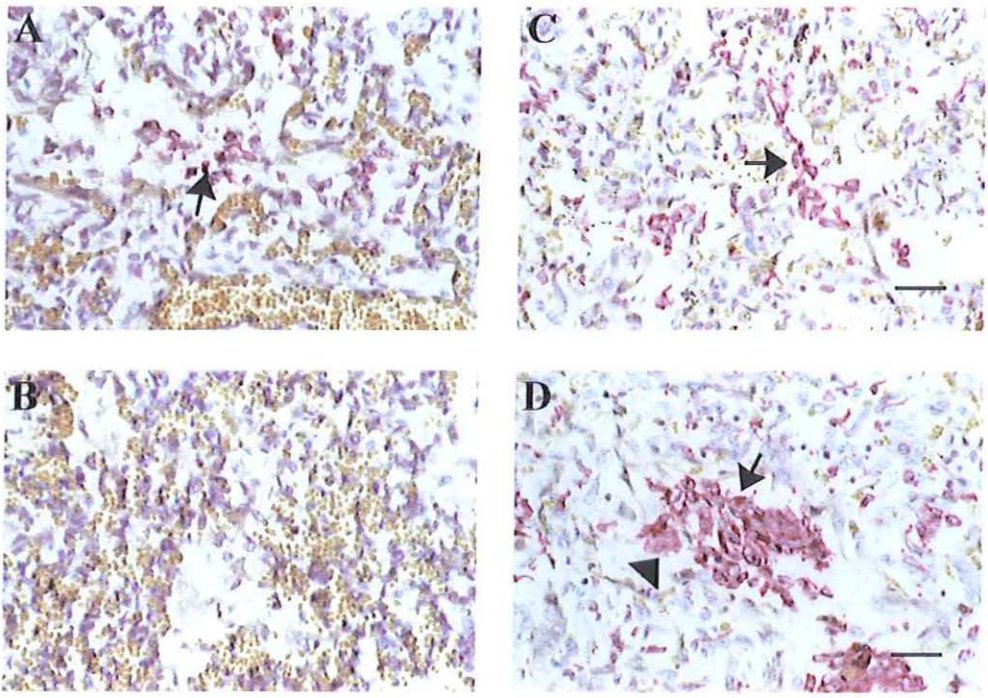
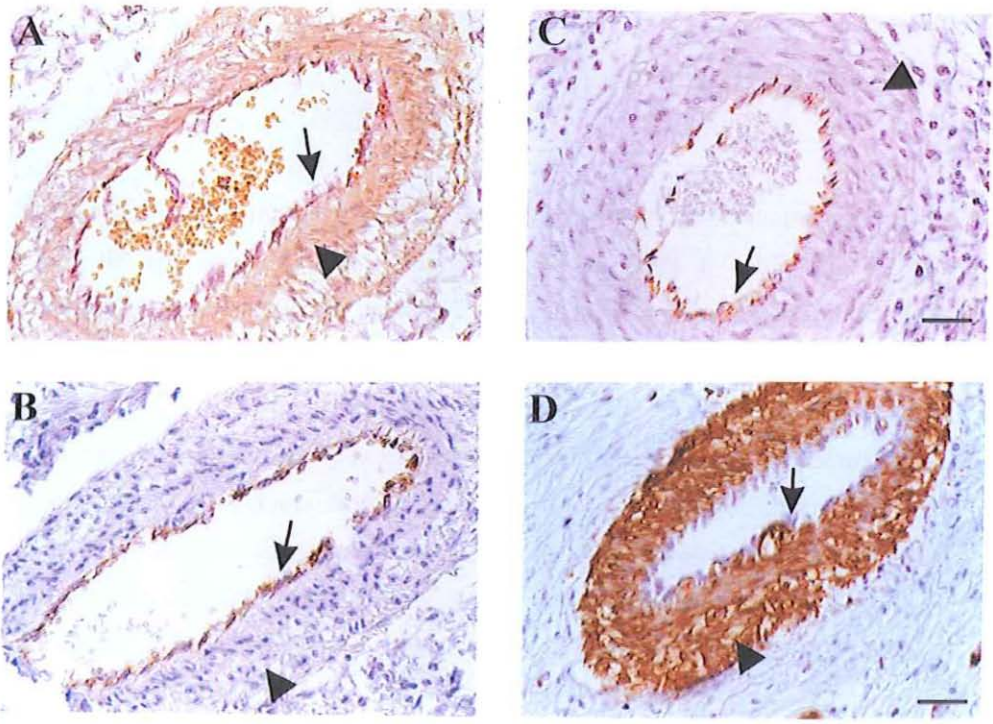

A
C
B
D

Figure. 1: Micrograph Showing Immunostaining with eNOS and iNOS in the Pulmonary Vascular Endothelium as Colocalized with CD 31 and α-SMA Staining on Consecutive Sections of the Studied Human Lungs. (page 83)

Where: A) Endothelial expression (red color) of eNOS in pulmonary artery using alkaline phosphatase method, B) Endothelial expression (brown color) of iNOS in pulmonary artery using peroxidase-anti-peroxidase (PAP) method, C) Endothelium (brown color) as verified with anti-CD 31 staining using peroxidase method, and D) Vascular SMC (brown color) in media as verified with anti-α-SMA staining using peroxidase method with negative endothelium. Endothelium indicated by the arrow and vascular SMC by the arrowhead in all sections. All from lung of CDH newborn. [Bar for all = 50 μm]


A
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D

Figure. 1: Immunohistochemistry of HSP-70 in Lung Sections Using Alkaline Phosphatase Method (page 97-1)

Note the immunoreactivity for HSP-70 in (A). Bronchial epithelium (thick arrow) and medial SMC of small artery (small arrow) in CDH case. (B). Bronchial epithelium (thick arrow) and medial SMC of small artery (thin arrow) in control case. (C). Bronchial epithelium (thick arrow) and medial SMC of small artery (thin arrow) in ECMO treated CDH case. [Bar = 50 μm].

(D). Histogram showing the difference in expression grade of HSP-70 according to the semiquantitative scale for CDH (stripped), control (empty) and ECMO treated (filled) groups, in bronchial epithelium (Bronch.Epi), medial SMC (L.A.Med) and endothelium (L.A.End) of large arteries and medial SMC (S.A.Med) and endothelium of (S.A.End) of small arteries. Statistical significance was accepted at $P \leq 0.05$ for CDH vs controls (*) and for CDH vs ECMO (#) using the Mann-Whitney U test.

Immunolocalization of HSP-27:

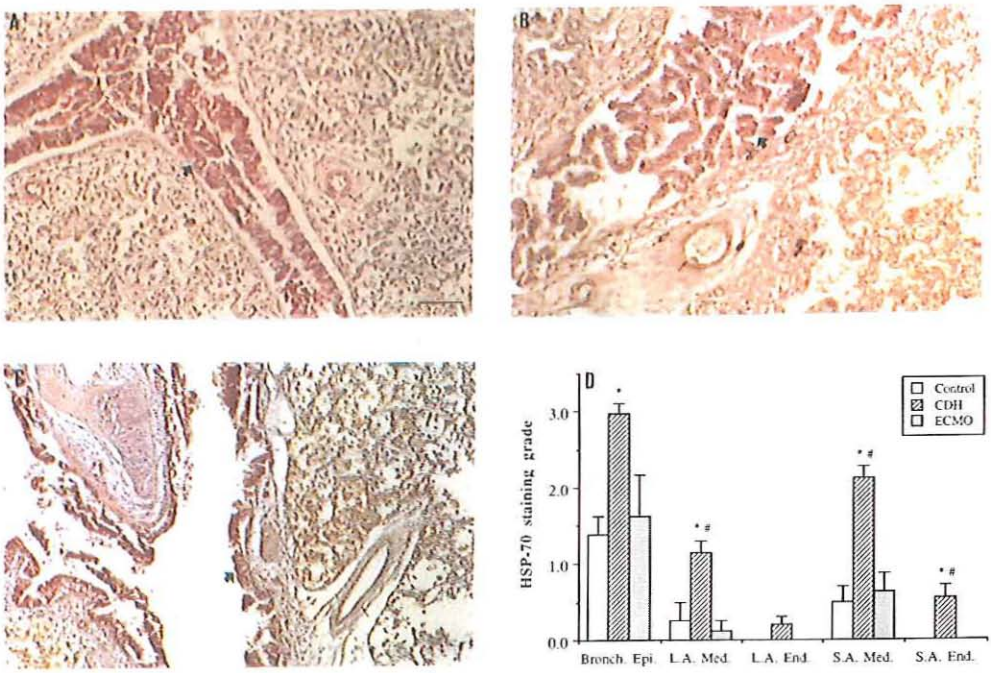
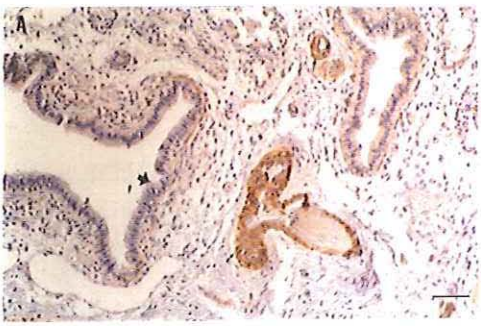
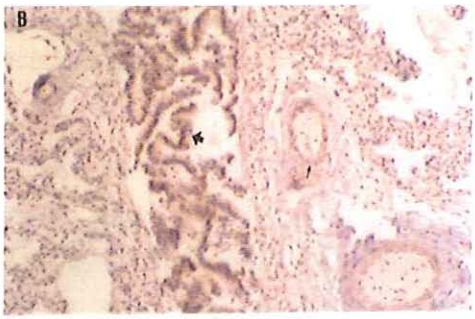

A
B
C
D
HSP-70 staining grade
3.0
2.0
1.0
0.0
Control
CDH
ECMO
Bronch. Epi.
L.A. Med.
L.A. End.
S.A. Med.
S.A. End.

Figure. 2: Immunohistochemistry of HSP-27 in Lung Sections Using Peroxidase Method (page 97-2)

Note the immunoreactivity for HSP-27 in (A). Bronchial epithelium (thick arrow) and medial SMC of small artery (small arrow) in CDH case. (B). Bronchial epithelium (thick arrow) and medial SMC of small artery (thin arrow) in control case. (C). Bronchial epithelium (thick arrow) and medial SMC of small artery (thin arrow) in ECMO treated CDH case. [Bar = 50 μm].

(D). Histogram showing the difference in expression grade of HSP-27 according to the semiquantitative scale for CDH (stripped), control (empty) and ECMO treated (filled) groups in bronchial epithelium (Bronch.Epi), medial SMC (L.A.Med) and endothelium (L.A.End) of large arteries and medial SMC (S.A.Med) and endothelium of (S.A.End) of small arteries. Statistical significance was accepted at $P \leq 0.05$ for CDH vs controls (*) and for CDH vs ECMO (#) using the Mann-Whitney U test.


A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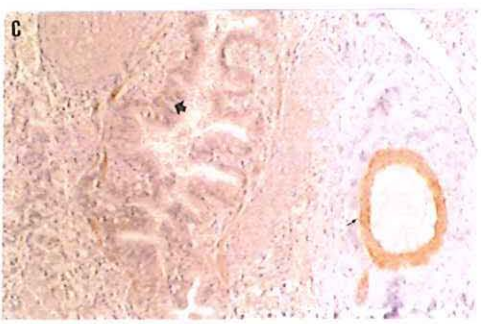

C

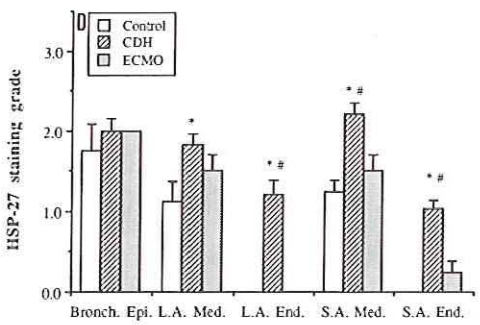
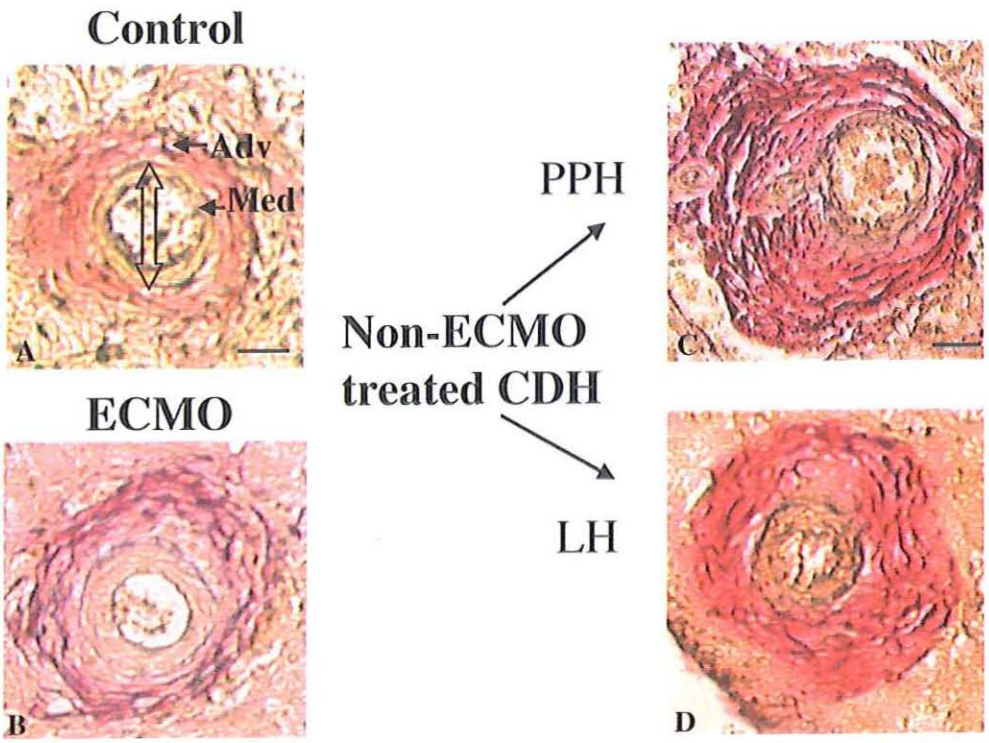

D
Control
CDH
ECMO
HSP-27 staining grade
3.0
2.0
1.0
0.0
Bronch. Epi.
L.A. Med.
L.A. End.
S.A. Med.
S.A. End.

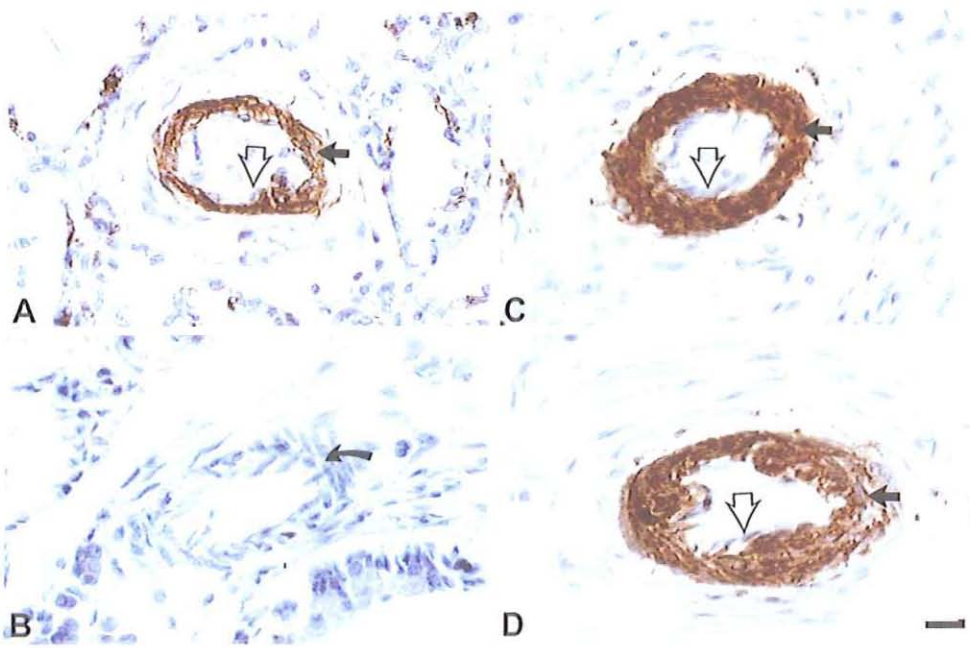
Figure 1: Micrograph of adventitia of intraacinar small pulmonary artery stained with EvG (page 112)

Where: A) Thin adventitia in control lung section where Adv: adventitial thickness in microns [AMμ] and Med: medial thickness in microns [MMμ] along the shortest diameter of the artery while the empty arrow passes along the external diameter [ED], this diameter extends from external elastic lamina [EEL] at one side [outer black circle {arrow head tip}] to that on the other side, B) Thin adventitia with thick media in ECMO-treated CDH lung section, C) Thick adventitia with relatively wide lumen in lung section from persistent pulmonary hypertension (PPH) subgroup of non-ECMO treated CDH group, and D) Thick adventitia with relatively narrow lumen in lung section from lung hypoplasia (LH) subgroup of non-ECMO treated CDH group. [Bar = 50 μm]

Figure 2: Micrograph of the Media of Intraacinar Pulmonary Arteries Stained with α-SMA Using Peroxidase Method (page 113)

Where: sections were immunostained using peroxidase technique (brown color) to localize the media (straight filled arrow) on the consecutive sections stained with Elastic van Gieson: A) Thin muscle layer (media) in control lung section, B) negative control with absent staining (media indicated here by curved filled arrow), C) Thick media non-ECMO treated CDH, and D) Persistent thick media in lung section from ECMO-treated CDH group. In all sections, the straight empty arrow indicates the negative endothelial layer (blue color). [Bar = 25 μm]


Control
Adv
Med
A
ECMO
B
Non-ECMO
treated CDH
PPH
C
LH
D


A
C
B
D