Congenital Diaphragmatic Hernia

The Importance of Genetic and Environmental Factors

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Congenital Diaphragmatic Hernia

The Importance of Genetic and Environmental Factors

Congenitale Hernia Diafragmatica
Het belang van genetische en omgevingsfactoren

Proefschrift

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Promotoren:	Prof.dr. D. Tibboel Prof.dr. B.A. Oostra
Overige leden:	Prof.dr. F.W.J. Hazebroek Prof.dr. R.C.M. Hennekam Prof.dr. M. Post

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General aspects of Congenital Diaphragmatic Hernia

Part I

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Congenital anomalies are observed in 1 in 40 newborns (2,5%). Structural birth defects are usually classified according to organ systems, such as the central nervous system, cardiovascular system, urogenital system, etc., according to coding systems such as the one developed by the British Pediatric Association. These anomalies occur as single defects in 50% of cases and in the remaining 50%, they occur as multiple anomalies (Winter 1996). 20-30% of all infant deaths and 30-50% of deaths occurring after the neonatal period are attributed to congenital abnormalities (Berry et al. 1987; Hoekelman and Pless 1988). Congenital heart defects are the major cause of infant mortality.

In 6% of cases, birth defects are associated with a recognizable chromosomal abnormality. In 7.5%, they are thought to be of monogenic origin. In 20% they are regarded as having a multifactorial origin, and in 6-7% they are caused by known environmental factors such as maternal diseases, infections, and teratogens (Winter 1996). However, the etiology of the majority of birth defects remains still unknown.
One of the most severe birth defects is Congenital Diaphragmatic Hernia (CDH). It consists of a defect in the formation of the diaphragm, a variable amount of pulmonary hypoplasia and abnormal pulmonary vascular resistance. In contrast to many congenital anomalies the mortality is still considerable especially in patients with respiratory insufficiency within 6-12 hours after birth.

HISTORY

The literature on CDH goes as far back as the late 16th century (Irving and Booker 1990). Originally, it was believed to be a single anatomical defect. In 1761, Giovanni Battista Morgagni described the association of CDH with pulmonary hypoplasia (Morgagni 1769). In 1848, a Hungarian anatomist named Bochdalek was the first to describe the hole in the diaphragm that characterizes the posterolateral diaphragmatic hernia now called the Bochdalek type hernia (Bochdalek 1848).
It is generally accepted that there are three main anatomical types of CDH: the posterolateral defect or Bochdalek hernia, the Morgagni hernia (through the sternocostal hiatus), and the hernia of the pars sternalis, which represent respectively about 96%, 2% and 2% of all cases (Torfs et al., 1992). Eventration of the diaphragm can simulate CDH but is not really a herniation, but a superior displacement of the intra-abdominal organs, into a sac-like structure. Because these anatomical types are thought to have different etiologies, we will evaluate only the Bochdalek hernia in this study.

What is CDH?

Various specialists, involved in the care and cure of patients with CDH, will answer the question differently, according to their specific interest. Approaches of basic specialists, such as embryologists, toxicologists, molecular geneticists, epidemiologists and clinical geneticists, will be described and also the point of view of the pediatric pathologists and ultrasonographers. This short review is completed by a description by the attending physicians such as neonatologists, pediatricians, and pediatric surgeons involved in the postnatal care of the CDH-patient.

THE EMBRYOLOGIST'S / DEVELOPMENTAL BIOLOGIST'S POINT OF VIEW

In humans, the development of the diaphragm starts in the fourth gestational week. The septum transversum lies opposite the third, fourth, and fifth cervical somites. During the fifth week, myoblasts from the myotomes of these somites migrate into the developing diaphragm together with nerve bundles from the cervical region. Consequently the phrenic nerves that supply the diaphragm come from cervical roots 3, 4 and 5. The phrenic nerves pass to the septum transversum via the pleuropericardial membranes. The diaphragm then forms a continuous sheet, which completely separates the thoracic and abdominal cavities prior to the major period of internal organ enlargement. For pictures of the embryological stages of human development, and specifically that of the diaphragm, see the reports on the Carnegie collection (Raymond 1975; O'Rahilly and Müller 1987).

However, in the case of a Bochdalek-type CDH, a significant proportion of the diaphragmatic musculature – typically the left postero-lateral region – is 'absent'. Consequently the developing abdominal viscera invade the thoracic cavity, in particular the liver in the early stages of development, thus occupying space normally reserved to accommodate the growing lungs. While the mechanisms underlying the etiology of CDH remain obscure, several theories on the pathogenesis of the condition have been proposed. Currently it is not feasible to critically assess these theories by examining human infants diagnosed with CDH as the pathogenetic events occur before clinical detection is feasible.

However, several animal models, either knock-out mice models or teratogenic models have added to our knowledge of the embryology and pathogenesis of the diaphragm and will be discussed in the following sections of this chapter.

THE TOXICOLOGIST'S POINT OF VIEW

Teratogenic causes of birth defects in humans are very difficult to unravel. The adverse effects of drugs can only be recognized when a drug has already received marketing

approval, and, even more important, after it has been used by pregnant women. In human, the teratogenic effects of a class of drugs cannot be reliably predicted based on current knowledge about its pharmacology and toxicology. Usually pregnant women are excluded from participation in trials (Mitchell 2000). Therefore, drugs have often been described for a long time before their adverse effects become available to the medical profession. A well known example is that of thalidomide which caused severe limb reduction defects in the fetuses of women who used the drug during pregnancy (Lenz 1962). Animal models for studying adverse effects of drugs are limited in their ability to predict human teratogenesis. However, they can lead us to possible mechanisms. As such, CDH could be the result of a teratogenic factor in individual cases.
Several teratogenic agents are known to cause CDH in animals.

1. Nitrofen

An animal model of CDH was developed by administration of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen) to pregnant rodents. Abnormal organogenesis of the diaphragm, lungs, heart and kidney occurred in the offspring (Ambrose et al. 1971). This model has been extensively studied in order to gain more insight in the pathogenesis of CDH. However, the exact mechanism of action of nitrofen is not completely understood. Nitrofen might function through alteration of the thyroid status of the fetus (Manson et al. 1984). The significance of thyroid hormones on lung development in this CDH model, is based on the observations that nitrofen has a high stereochemical similarity to thyroid hormones. Manson *et al* demonstrated that following the concomitant administration of thyroxin and nitrofen to pregnant rats, a drastic reduction (70%) in the number of malformations in the offspring was observed (Manson et al. 1984). However, there is no published evidence that demonstrates the direct interaction of nitrofen with the thyroid hormone metabolism. Interestingly, antenatal vitamin A treatment of nitrofen-induced CDH rats, reduces the incidence and severity of CDH, and improves pulmonary growth and maturation (Thebaud et al. 1999). Despite the proven usefulness of the nitrofen model over the years, in particular to characterize abnormal lung growth and pulmonary vascular abnormalities, no correlation between nitrofen and thyroid hormone status has been shown to play a pivotal role in the etiology of CDH in humans. Hence, the results obtained with the nitrofen model should be extrapolated to the human situation with a fair amount of reservation.

Based partly on the nitrofen model of CDH, Keijzer *et al* suggested the dual hit hypothesis, in which the lung hypoplasia causes the diaphragmatic defect, as proposed earlier by Iritani (Iritani 1984; Keijzer et al. 2000). However, Greer *et al* suggested that the diaphragm does not form correctly as a consequence of the improper embryogenesis of the primordial diaphragmatic anlage (Greer et al. 2000a; Greer et al. 2000b). However, no definitive answer is accepted at this time for the pathogenesis of CDH.

2. Other teratogenic agents

CDH associated with extensive cardiovascular malformations, such as abnormalities in the aortic arch, pulmonary trunk and ventricular septal defects is observed in Sprague-Dawley rats exposed to SB-210661, a benzofuranyl urea derivate (Solomon et al. 2000). Other compounds that induce diaphragmatic defects are 4-biphenyl carboxylic acid (BPCA), a metabolite of a thromboxane-A_2 receptor antagonist (Sutherland et al. 1989), cadmium (Barr 1973), and bisdiamine, which is a spermatogenesis inhibitor (Taleporos et al. 1978).
All these CDH-inducing compounds share a common mechanism by inhibiting RALDH2, an observation which supports the idea that the retinoic acid system is involved in the etiology of CDH (Mey et al. 2003).

3. Vitamin A deficiency

Historically, it has been known for a long time that hypovitaminosis A can cause CDH (Andersen 1941; Wilson et al. 1953). Also, hypervitaminosis A can cause diaphragmatic defects (Geelen 1979). Although isotretinoin treatment during pregnancy causes severe malformations in the human fetuses, it does not cause diaphragmatic hernia (Lammer et al. 1985). Further evidence suggesting that abnormalities linked with the retinoid signaling pathway early in gestation may contribute to the etiology of CDH comes from recent studies by the group of Greer. They found reduction in the incidence of nitrofen-induced CDH by vitamin A and retinoic acid (Babiuk et al. 2004) and they described also that RALDH2 is inhibited by compounds, such as nitrofen, that induce CDH in rodents (Mey et al. 2003).

4. Surgically induced CDH in lambs

This CDH model, originally described by de Lorimier (De Lorimier et al. 1967) has been extensively studied by the Harrison's group (Harrison et al. 1980). Although this model is valuable in studying several aspects of late-gestational and perinatal pathophysiology, it can not be used for studies on the etiology of CDH because the diaphragm has originally normally been developed.

THE MOLECULAR BIOLOGIST'S / MOLECULAR GENETICIST'S POINT OF VIEW

Many genes have been proposed to play a role in the embryogenesis of the diaphragm, either through gene mutation or by genetic-environmental interaction. Some of those genes have been studied in knockout mouse models of diaphragmatic defects.
The knockout mouse for the compound null mutations of the genes coding for the Retinoic Acid Receptors (RAR) α and β, the receptors through which vitamin A exerts its effects, has variable foregut malformations. Some knockout mice displayed a CDH-like phenotype, whereas others suffered from an absent tracheoesophageal septum and left lung agenesis with hypoplasia of the left and /or right lungs (Mendelsohn et al. 1994).

Recently, in a new genetic mouse model, the *Slit3* null mice, the central tendon region of the diaphragm fails to separate from liver tissue. The *Slit 3* gene belongs to the *Slit* family of guidance molecules and is expressed predominantly in the mesothelium of the diaphragm during development (Yuan et al. 2003). However, this is not the location of the classical Bochdalek hernia.

THE CLINICAL GENETICIST'S POINT OF VIEW

Both the recognition of known entities and the delineation of novel syndromes including CDH as a feature are of importance for disease prognosis, accurate genetic counseling, and search for candidate genes. Accurate genetic counseling is obviously highly dependent on our level of knowledge about the etiology and pathogenesis of the malformations concerned.
CDH for the clinical geneticist can be part of a chromosomal anomaly, a Mendelian inherited syndrome, or it can be a congenital malformation associated with other birth defects, or an isolated major congenital abnormality.
Syndromes associated with CDH can be classified as:

1. syndromes that include CDH as a mandatory feature (table 1), page 12
2. recognizable syndromes that are occasionally associated with CDH, page 12
3. rare/miscellaneous combinations of defects that are possible syndromes, page 13-15

Around 62 dysmorphological entities that include CDH have been described in Winter and Baraitser's London Dysmorphology Database 2003. Searching the OMIM (Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov) gives 23 entities where CDH is involved. Only a few syndromes are thought to be obligatorily associated with CDH, among them Fryns syndrome (OMIM 229850) (Fryns et al. 1979) and Donnai-Barrow syndrome (OMIM 222448) (Donnai and Barrow 1993). Syndromes occasionally found in combination with CDH are Beckwith-Wiedemann syndrome (OMIM 130650) (Wiedemann 1964; Enns et al. 1998), Kabuki make up syndrome (OMIM 147920) (van Haelst et al. 2000) and craniofrontal nasal syndrome (OMIM 304110) (chapter 9a)(Brooks et al. 2002).
A large number of chromosomal anomalies have been described in CDH patients (Enns et al. 1998), and reviewed by Lurie (Lurie 2003). In table 4 an overview is given of chromosomal anomalies with CDH. Trisomy 18 is by far the most frequent. Another numeric chromosomal disorder occasionally associated with CDH is the Pallister-Killian syndrome (OMIM 147920), which is tetrasomy 12p (Pallister et al. 1977; Rodriguez et al. 1994; Enns et al. 1998). So far no chromosomal interstitial deletions have been reported which led to the identification of CDH predisposing genes. However, interstitial deletions of 15q26 may hold promise for future positional cloning efforts; several authors recently hypothesized that this region might harbor genes involved in the development of the diaphragm (Schlembach et al. 2001; Lurie 2003; Biggio et al. 2004).

Genetic studies are difficult to conduct because CDH is a relatively rare birth defect. The traditional and most powerful approach for locating a disease gene in humans is linkage analysis. For these kinds of studies multigenerational families are needed, however these are not available. The familial recurrence of CDH is very low and the high mortality restricts family inheritance studies. Moreover, as CDH is considered in most cases a polygenic or multifactorial disease, with small effects of individual genes, other strategies than classical linkage approaches may be needed (Botstein and Risch 2003; Dean 2003). Therefore many questions on the genetics of CDH are still unanswered. In the future, genes may be found that play a pivotal role in the etiology of CDH. However, they will never explain all CDH cases.

THE EPIDEMIOLOGIST'S POINT OF VIEW

The prevalence of CDH is about 0.3 per 1,000 births with mortality rates that have not changed much for over 30 years, in particular in cases with associated malformations (Torfs et al., 1992; Skari et al., 2000). A recent meta-review calculated an overall mortality rate of 48%, which increases to 93% for fetuses with other anomalies (Beresford and Shaw 2000). Approximately 40 to 50% of CDH cases have other anomalies, with heart and limb defects being the most frequent (Benjamin et al. 1988; Torfs et al. 1992; Tibboel and Gaag 1996).
To perform epidemiological studies birth defects registries have been created in many countries. In California, the California Birth Defects Monitoring Program (CBDMP) monitors all live births and stillbirths. Specially trained data collection specialists examine all hospital logs, including pathology logs, from birthing hospitals and from tertiary hospital where infants may have been sent for further evaluation or for intervention. They abstract the charts of every infant or stillbirths with a structural birth defect, according to the modified list of the British Pediatric Association (BPA 6). Ascertainment continues until the infant is one year of age or has died. We used this database to study CDH in combination with other congenital anomalies. In the northern part of the Netherlands, Eurocat (European Registration of Congenital Anomalies) records data on children with congenital malformations. Additionally, since 1977, the CDH Study Group collects data on CDH cases from several countries. At this time, over 2,700 cases have been included in the database.

THE ULTRASONOGRAPHER'S AND OBSTETRICIAN'S POINT OF VIEW

Antenatal diagnosis of CDH by ultrasound has been available since the late 1970s (Hobbins et al. 1979; Touloukian and Hobbins 1980). Prenatal diagnosis of CDH has several advantages, such as the possibility of more effective resuscitation after birth, and a better survival. However, antenatal diagnosis of an isolated CDH, regardless of its timing, is of no value in predicting outcome (Wilson et al. 1994). These authors also reported that CDH

associated with another significant anomaly currently has a poor prognosis, regardless of the timing of the diagnosis. The associated anomaly was the most accurate predictor of mortality. Other predictive values for the severity of the disease, are the lung-head ratio, the timing of prenatal diagnosis, liver-herniation, and associated anomalies. CDH is mostly accompanied by lung hypoplasia which potentionally can be measured prenatally. Prenatal diagnosis sometimes leads to a termination of pregnancy. However, very often, parents will continue the pregnancy. Delivery will then occur in a tertiary hospital with acute intensive care facilities. The CDH study Group did not find a relation between prenatal diagnosis and outcome.

The Pediatric Pathologist's Point of View

The pathologist obtains CDH cases from several departments, from the children's hospital after the child's death, and from the obstetrics department when the child died immediately after birth or was a stillborn, or was the product of pregnancy termination. Detailed examination of the phenotype in CDH patients may lead to clues on the etiology, as additional malformations may be important to reach a syndrome diagnosis. Therefore, post-mortem investigation can be important. In the literature, few post-mortem studies on CDH have focused on additional malformations and chromosomal abnormalities (Benjamin et al. 1988; Bajaj et al. 1991; Bollmann et al. 1995; Enns et al. 1998). Autopsy can uncover more anomalies relevant for a clinician's diagnosis. However, with the increased possibilities of non-invasive diagnostic procedures, such as ultrasound and MRI, it might be questionable whether postnatal evaluation (autopsy) really contributes to the knowledge of the etiology of CDH. However, not all cases of CDH come to the attention of the neonatologist or pediatric surgeon. In chapter 3 we will present a study on the relevance of post-mortem examination.

The Postnatal Treatment Provider's (Pediatrician, Neonatologist and Pediatric Surgeon) Point of View

The fundamental problem in children with CDH is the increased pulmonary vascular resistance and the lung hypoplasia. Several treatment strategies have been used and very recent reviews on postnatal care have been summarized by Bohn (Bohn 2002) and Hosgor (Hosgor and Tibboel 2004) (see the table in their article). However, none of these treatment modalities, apart from nitric oxide (NO) and Extracorporal Membrane Oxygenation (ECMO), has been tested and proven tested in properly designed randomized controlled trials. Harrison's group performed a randomized trial on the survival of infants with CDH who underwent fetal endoscopic tracheal occlusion. However, this did not improve survival (Harrison et al. 2003).

Nowadays, the concept of 'gentle handling' for the prevention of iatrogenic lung damage has been adapted by most medical centers dealing with CDH patients on a regular base. For this reason, significant increased survival rates have been reported in the past couple years, up to 80-90% in cases of isolated CDH (Wilson, personal communication). Case selection is an important confounder in survival reports, as usually only isolated cases of CDH are taken into consideration.

RESEARCH OBJECTIVES / SCOPE OF THE THESIS

The objectives of this thesis are as follows:

1. To investigate the role of lung vascular morphogenesis in CDH. In chapter 2 the results from a descriptive study on the development of the pulmonary vasculature in mice are reported.
2. To establish the role of autopsy of CDH cases, in finding associated anomalies. The results of this study are described in chapter 3.
3. To establish the role of epidemiological studies in CDH. In chapter 4 and 5, epidemiological studies present data on the co-occurrence of CDH and limb defects and of CDH in combination with esophageal atresia/ tracheoesophageal fistula.
4. To determine factors that may establish a prenatal diagnosis of CDH. The prenatal value of lung-head ratio as predictor for the severity of CDH is discussed in chapter 6.
5. To evaluate genetic aspects in CDH. Apart from a case report of CDH in combination with 4p- syndrome (chapter 7), two CDH cases are described in combination with monogenic syndromes (chapter 9). A detailed analysis of the association of CDH with a deletion of chromosome 15q is presented (chapter 8).
6. To evaluate the significance of a selected number of environmental factors for the development of CDH in humans (chapter 10). A study on the possible associaton of (OH)-PCB congeners with CDH is presented, as is a case of a dolphin with a Bochdalek–type CDH (chapter 11).

This thesis is completed by a general discussion and a summary.

For the studies described in this thesis we used a study protocol 'Environmental and Genetic factors in Congenital Diaphragmatic Hernia and Esophageal Atresia', approved by the Institutional Review Board, in collaboration with the parent support groups, 'Stichting Hernia Diafragmatica' and 'Vereniging Ouders Kinderen Slokdarmafsluiting'. During admission of the patient in our hospital and via meetings of the parent support groups, patients and their parents were included. After informed consent by the parents we took blood samples from the parents for DNA-isolation and storage, and also blood from the mother for PCB-analysis. From the patients we took blood samples, if possible combined with a regular blood sample, for karyotyping and for a cell-line and DNA-isolation, or cheekswaps for DNA-isolation.

Syndrome	Inheritance	Features	References
		syn/ectrodactyly	and Gaag 1996)
Holt-Oram syndrome	AD	Upper limb defect, ASD/VSD	(Howe et al. 1996)
Hydrolethalus syndrome	AR	Hydrocephalus, microphthalmia, micrognathia, "key-hole" foramen magnum, cleft lip/palate, CHD, polydactyly	(Bird et al. 1994b)
Kabuki syndrome	AD / ?microdeletion	Everted lower eyelids, ear anomalies, CHD, renal anomalies	(Silengo et al. 1996; Donadio et al. 2000; van Haelst et al. 2000)
Lethal multiple pterygium syndrome	AR	Multiple pterygia, hypertelorism, cardiac hypoplasia, skeletal anomalies	(Froster et al. 1997)
Limb-body wall defect	?AD / AR	Multiple midline defects, occipital defect, cleft palate, horse shoe kidney, omphalocele	(Bird et al. 1994b)
Male pseudohermaphroditism congenital heart defect-horseshoe kidney-diaphragmatic hernia	Unknown	Bicornuate uterus, septate/double vagina, rhabdomyomatous dysplasia	(Maaswinkel-Mooij and Stokvis-Brantsma 1992; Toriello and Higgins 1992)
Maxillonasal dysplasia-diaphragmatic hernia-genital anomalies	Unknown	Binder anomaly, mitral valve prolaps, cervical/rib anomalies, genital anomalies	(Toriello et al. 1988)
Micrognathia-cleft palate-short neck-vertebral anomalies-mental retardation	AD	Facial asymmetry, low-set ears, cleft palate, laryngomalacia	(Mathieu et al. 1993)
Microphthalmia with linear skin defects (MLS)	XR	Oncocytic cardiomyopathy, arrhytmias, ocular defects	(Bird et al. 1994a)
MIDAS syndrome	XR	Microphthalmia, dermal aplasia, sclerocorneae, agenesis of corpus callosum	(Happle et al. 1993)
Multiple herniae	?AR	Cervical lung herniation, hiatus hernia, inguinal hernia, bladder diverticula	(Zaglul and Odita 1995)
Nasopharyngeal teratoma-Dandy-Walker malformation-diaphragmatic hernia	Unknown	Dysplastic ears, cleft palate, micrognathia, CHD	(Aughton et al. 1990)
PAGOD syndrome	AR	Pulmonary artery hypoplasia, agonadism, omphalocele, dextrocardia, sex reversal	(Kennerknecht et al. 1993)
Polycystic kidneys-microcephaly- dysmorphic facies- brachymelia-congenital heart defects	AR	Hypertelorism, large ears, cleft palate, situs inversus, genital hypoplasia	(Gillessen-Kaesbach et al. 1993)

Syndrome	Inheritance	Features	References
Pulmonary agenesis, microphtalmia, and diaphragmatic defect)PMD'			(Berkenstadt et al. 1999)
Radial ray defects-omphalocele-diaphragmatic hernia-hepatic cyst	Unknown		(Gershoni-Baruch et al. 1990)
Split-hand-obstructive urinary anomalies-spina bifida-diaphragmatic defects	AD		(Czeizel and Losonci 1987)
Thoracoabdominal syndrome	XD	Ventral midline defects, CHD	(Bird et al. 1994b; Parvari et al. 1994)

AR = autosomal recessive, AD = autosomal dominant, XR = x-linked recessive, XD = x-linked dominat, CHD = congenital heart disease, ASD = atrial septal defect, VSD = ventricular septal defect.

Table 4 Chromosome anomalies associated with CDH. (After a search in the database, kindly provided by Prof. Dr. A. Schinzel (Schinzel 2004) and partly adapted from Lurie (Lurie 2003) and G.M. Enns (Enns et al. 1998)

Syndrome	References
Monosomy / trisomy / aneuploidy	
45,X	(David and Illingworth 1976; Benjamin et al. 1988; Cunniff et al.
Turner syndrome	1990; Bollmann et al. 1995; Tibboel and Gaag 1996)
trisomy 2p	(Lurie et al. 1995)
partial trisomy 5	(Bollmann et al. 1995; Tibboel and Gaag 1996)
trisomy 11p15	(Turleau et al. 1984)
(Beckwith-Wiedemann-syndrome)	
trisomy 13	(Benjamin et al. 1988; Thorpe-Beeston et al. 1989; Fauza and Wilson 1994)
trisomy 16	
trisomy 18	(David and Illingworth 1976; Benjamin et al. 1988; Cunniff et al. 1990; Fauza and Wilson 1994; Howe et al. 1996; Tibboel and Gaag 1996)
trisomy 20p	(David and Illingworth 1976; Bird et al. 1994b; Tibboel and Gaag 1996)
trisomy 21	(David and Illingworth 1976; Bird et al. 1994b; Tibboel and Gaag 1996)
trisomy 22	(Kim et al. 1992; Torfs et al. 1992; Ladonne et al. 1996)
47,XX, +mar	(Torfs et al. 1992)
47,XY,+mar16	(Howe et al. 1996)
47,XY,+18,inv(2)(p11.2q13)	(Tibboel and Gaag 1996)
mosaic trisomy	(Fauza and Wilson 1994)
46,XY/47,XY,+14	(Howe et al. 1996)
triploidy 69,XXX	(Thorpe-Beeston et al. 1989)
tetrasomy 12p	(Adzick et al. 1989; Fauza and Wilson 1994; Tibboel and Gaag 1996)
tetraploidy 21	(Adzick et al. 1989; Fauza and Wilson 1994; Tibboel and Gaag 1996)
Deletions-translocations	
Chromosome 1	
46,XY,del(1)(q32.3q42.3)	(Youssoufian et al. 1988)
del (1)(q32q42)	(Bird et al. 1994b; Tibboel and Gaag 1996)
dup (1)(q2431.2)	(Bird et al. 1994b; Tibboel and Gaag 1996)
dup(1)q25q32)	(Schinzel 2004)
46,XY/46,XY,dup(1)(q24q31.2)	(Clark and Fenner-Gonzales 1989; Schinzel 2004)
46,XY,t(1;15)(q41;q21.2)	(Smith et al. 1994)
46,XY,t(1;21)(q32;q22)	(Howe et al. 1996)
t(1;21)	(Philip et al. 1991)
der(1)	(Benjamin et al. 1988)
Chromosome 2	
del(2)(q37.1q37.3),dup(14)(q31.2q37.3)	(Schinzel 2004)
dup(2)(p25p21)	(Heathcote et al. 1991; Lurie et al. 1995; Schinzel 2004)
dup(2)(p25p13)	(Fineman et al. 1983; Schinzel 2004)
Chromosome 3	
46,XY,del(3)(q21q23)	(Wolstenholme et al. 1994)

Syndrome	References
del(3)	(Tibboel and Gaag 1996)
46,XY,der,t(3;8)(p23;p23.1)	(Tibboel and Gaag 1996)
Chromosome 4	
4p-	(Bird et al. 1994b; Howe et al. 1996; van Dooren et al. 2004)
del(4)(p16)	(Tachdjian et al. 1992; Schinzel 2004)
del(4)(p15)	(Schinzel 2004)
del(4)(q31)	(Schinzel 2004)
dup(4)(q31q35)	(Schinzel 2004)
Chromosome 5	
dup(5)(p15p13),del(9)(p22)	(Schinzel 2004)
dup(5)(p)	(Schinzel 2004)
Chromosome 6	
del (6)(q23)	(Shen-Schwarz et al. 1989; Bird et al. 1994b; Howe et al. 1996; Schinzel 2004)
46,XY,t(6;8)(q24;q23)	(Howe et al. 1996)
Chromosome 7	
del(7)(q11q22)	(Schinzel 2004)
del(7)(q32)	(Schinzel 2004)
46,XY,-7,+der(7)t(2;7)(p25.3;q34)mat	(Enns et al. 1998)
46,XY,del(7)(q32)	(Torfs et al. 1992)
7q-	(Fauza and Wilson 1994)
cht(7)(q31.3)	(Bonneau et al. 1991)
del(7)(p21)	(Schinzel 2004)
Chromosome 8	
del(8)(p23)	(Schinzel 2004)
dup(8)(p21)	(Schinzel 2004)
dup(8)(q23q24)	(Schinzel 2004)
trisomy 8 mosaicism	(Schinzel 2004)
46,XY,del(8)(p23.1)	(Howe et al. 1996)
del(8)	(Thorpe-Beeston et al. 1989)
t(8;14)(q24;q21)	(Philip et al. 1991)
46,XX,t(8;13)(q22.3;q22)	(Temple et al. 1994)
46,XX,t(8;15)(q22.3;q15)	(Temple et al. 1994)
r(4),7q+,del(8),+mar	(Tibboel and Gaag 1996)
Chromosome 9	
del(9)(p22)	(Schinzel 2004)
trp(9)(q22)	(Schinzel 2004)
trisomy 9	(Schinzel 2004)
trisomy 9 mosaicism	(Schinzel 2004)
46,XY,-9,+t(5q;9p)	(Torfs et al. 1992; Tibboel and Gaag 1996)
46,XY,-9,+der(9)t(9;11)(p24;p12)pat	(Donnenfeld et al. 1993)
46,XY,-9+,der(9)t(9;11)(p24;p13)	(Tibboel and Gaag 1996)
Chromosome 10	
dup(10)(p15p12)	(Schinzel 2004)
balanced t(10;X)	(Cunniff et al. 1990)
dup(10)(p)	(Schinzel 2004)
Chromosome 11	
del(11)(p13)	(Schinzel 2004)

Syndrome	References
dup(11)(p)pat	(Schinzel 2004)
Chromosome 12	
trp(12p)	(Schinzel 2004)
trp(12p) mosaicism	(Schinzel 2004)
46,XY,del(12)	(Howe et al. 1996)
t(12;15)	(Fauza and Wilson 1994)
Chromosome 13	
r(13)	(Schinzel 2004)
dup(13)(p13q14)	(Schinzel 2004)
dup(13)(p13q14),dup(11)(q21q25)	(Schinzel 2004)
dup(13)(pterq21),dup(5)(p15)	(Schinzel 2004)
13q-	(Benjamin et al. 1988)
Chromosome 14	
dup(14)(q24q32)	(Schinzel 2004)
dup(14)(q32)	(Schinzel 2004)
abnormal 14 centromere	(Fauza and Wilson 1994)
Chromosome 15	
46,XY,del(15)(q24)	(Kristoffersson et al. 1987)
del(15)(q26.1)	(Rosenberg et al. 1992)
46,XX,-15,+der(15)t(15;17)(q24.3:q23.3)	(Howe et al. 1996)
47,XY,t(15;21)(p12;p12)	(Tibboel and Gaag 1996)
r(15)	(de Jong et al. 1989)
dup(15)(q15q26)	(Schinzel 2004)
dup(15)(q15q26),del(X)(p22)	(Schinzel 2004)
del(15)(q11q14)	(Schinzel 2004)
del(15)(q21q25)	(Schinzel 2004)
invdup(15)	(Schinzel 2004)
Chromosome 22	
dup(22)(p13q11),dup(11)(q23q25)	(Schinzel 2004)
Chromosome X	
del(X)(p22.2)(MIDAS syndrome)	(Schinzel 2004)
46,X,del (X) (p22.1)	(Schinzel 2004)

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CHAPTER 2 THE EMBRYOLOGY OF CDH

Introductory remarks

The major clinical problem in CDH is the abnormal pulmonary vascular resistance and as a consequence the clinical picture of Persistent Pulmonary Hypertension of the Neonate (PPHN). In contrast to the well documented abnormal morphology, the etiology of the vascular problems of the lung is sparcely understood. Children with pulmonary hypertension exhibit structural problems of the pulmonary vessels, including thickening of the media and adventitia and extension of smooth muscle into normally non-muscularized vessels. Different theories have been put forward in the literature with regards to the development of the lung vasculature (Reid 1979; Reid 1984; deMello and Reid 2000). Therefore in this chapter an observational study will be described on the development of the lung vasculature in mice.

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CHAPTER 2

DISTAL ANGIOGENESIS: A NEW CONCEPT FOR LUNG VASCULAR MORPHOGENESIS

Distal Angiogenesis: A New Concept for Lung Vascular Morphogenesis
Marta Canis Parera, Marieke van Dooren, Marjon van Kempen, Ronald de Krijger, Frank Grosveld, Dick Tibboel and Robbert Rottier

Submitted

SUMMARY

Currently, two opposing models describe the development of the lung vasculature. One suggests that both vasculogenesis and angiogenesis are involved in the formation of the vessels, whereas the second, more recent model, describes vasculogenesis as the primary mechanism. This contradiction led us to examine the development of the murine pulmonary vasculature in relation to the airways at early stages of mouse lung development through an ontogenic morphological analysis. Therefore, we analysed fetal lungs of Tie2-LacZ transgenic mice as well as serial sections of wild type lungs stained with specific antibodies. Embryos were processed in such a way that the blood circulation was kept intact, thereby maintaining the integrity of the vasculature and preventing the collapse of vessels and thus the creation of artefacts. In contrast to other studies, we show that already at the earliest morphological signs of lung development the vasculature is a network connected to the embryonic circulation. Since our observations are not consistent with the current models, we propose distal angiogenesis as a new concept for early pulmonary vascular morphogenesis. In this model, capillary networks surround the terminal buds and expand by formation of new capillaries from pre-existing vessels as the lung bud grows.

INTRODUCTION

The adult lung has a dual vascular system, the bronchial and the pulmonary system. The bronchial system oxygenates all structures of the lung except the respiratory units, whereas the pulmonary system transports deoxygenated blood to the alveoli for gas exchange. The pulmonary arteries arise from the pulmonary trunk of the heart, and closely follow the bronchial tree, giving rise to the alveolar capillary plexus. These capillaries drain into the pulmonary veins, which run through the connective tissue septa back to the left atrium of the heart. For optimal gas exchange it is required that the air-blood barrier is correctly formed by type I alveolar cells and endothelial cells. Abnormalities in this delicate architecture may lead to inadequate function, as presented in congenital anomalies of the lung, such as congenital diaphragmatic hernia (Smith et al., 2002) and alveolar capillary dysplasia (Al-Hathlol et al., 2000). The intimate anatomical structure suggests that the close interaction between the pulmonary vessels and the airways starts to be established already early in development. However, the process by which the pulmonary vascular tree develops and the factors that control pulmonary vascular development are not completely understood.

Lung development is divided into distinctive stages with the earliest stages consisting of the embryonic stage, E9-10 in mice and 4-6 weeks in humans, and the pseudoglandular stage, E10-16 in mice and 6-16 weeks in humans. Models of pulmonary vascular

morphogenesis at these early stages are derived from morphological data. Based on vascular casts and electron microscopy of murine lungs, deMello *et al.* suggested that two processes are involved in the formation of the pulmonary vessels. In their model, the central vessels are formed by angiogenesis, defined as branching of new vessels from pre-existing ones, and peripheral vessels by vasculogenesis, defined as development of blood lakes in the mesenchyme (deMello et al., 1997). A connection between the central and peripheral vascular lumen would be established through a lytic process around E13/14 and circulation would start. A comparative analysis of serial sections of human embryos suggested that the same processes would also occur in humans (deMello and Reid, 2000). In addition, they concluded that pulmonary arteries and veins were dissociated in their timing and pattern of branching, since: "distal veins are present throughout the mesenchyme and establish a central luminal connection with the main pulmonary vein before an airway or artery is present at the same level" (deMello and Reid, 2000).

Although it is generally accepted that the distal vasculature arises by vasculogenesis, recent morphological studies have questioned the basic mechanism of formation of the proximal pulmonary vessels. Using heterozygous mice with a targeted insertion of the bacterial *lacZ* gene into the flk-1 locus, Schachtner *et al.* showed that the proximal and distal vascular structures were already connected at gestational age 10.5. However, only the proximal portion of the pulmonary artery contained a lumen (Schachtner et al., 2000). They also demonstrated that lung vessel development occurred at all stages and directly corresponded to overall lung growth. In another study, Hall *et al.* used lungs from human embryos to stain serial sections with endothelial specific antibodies, and showed continuity of circulation between the heart and the distal lung vascular plexus from 38d of gestation onwards (Hall et al., 2000). They concluded that the intrapulmonary arteries originate from a continuous expansion and coalescence of the primary capillary plexus that would form by vasculogenesis during the pseudoglandular stage. In addition, they showed that the same mechanism takes place to form the pulmonary veins (Hall et al., 2002). In contrast to the definition of deMello and co-workers, they defined vasculogenesis as differentiation of angioblasts from mesoderm to form primitive blood vessels, without the formation of hematopoietic lakes.

In spite of the lack of consensus of how the lung vasculature develops, many molecular players involved in blood vessel formation are already identified. Epithelial cells from the lung bud are suggested to induce the expansion of the capillary plexus through vascular growth factors (Healy et al., 2000). Three different growth factor systems have been described to act via endothelial cell specific tyrosine kinases: VEGFs, Angiopoietins and Ephrins (Yancopoulos et al., 2000). VEGF is required for vasculogenesis and angiogenesis and VEGF isoforms are expressed in lung epithelial (Healy et al., 2000; Maeda et al., 2002) and mesenchymal cells (Gebb and Shannon, 2000; Greenberg et

al., 2002). Furthermore, lung endothelial cells express Flk-1, the receptor for VEGF-A (Gebb and Shannon, 2000; Schachtner et al., 2000) and *in vitro* experiments showed that VEGF has a potential role in lung vascular morphogenesis (Healy et al., 2000; Zeng et al., 1998). Tie-2 and its ligand Ang-1 play a role in the regulation of angiogenesis (Yancopoulos et al., 2000). Gene disruption of either *Tie-2* or *Ang-1* in mice leads to abnormal vascular network formation and immature vessels, which lack proper organization (Sato et al., 1995; Suri et al., 1996). It is likely that these factors are involved in the stabilization of the network rather than its initial formation. Colen *et al.* demonstrated expression of *Ang-1* and *Ang-2* in the mouse lung from E9.5 onwards (Colen et al., 1999).

Since two opposing models on lung vessel development exist, we have performed an ontogenic morphological study of the lung vascular development in relation to the airways in mice ranging from E9.5, when the lung starts to become morphologically discernible, until the mid phase of the pseudoglandular stage at day E13.5. We performed analysis of fetal lungs from transgenic mice expressing the bacterial *lacZ* gene under the control of the Tie-2 promoter (Tie2-LacZ), as well as serial section analysis of normal lungs using antibodies against PECAM and Fli-1 to specifically identify endothelial cells. The mouse embryos were processed in such a way that the blood circulation was kept intact, thereby maintaining the vascular tone and the integrity of the vasculature. Hence, individual vessels are part of the embryonic circulation if circulating blood cells, which at the gestational age we investigated are primitive erythrocytes formed by the blood islands in the yolk sac, are present in their lumen. We report that even the earliest vessels formed in the lung are already connected with the heart vascular structures and thus are part of the embryonic circulation. We propose distal angiogenesis as a new model for lung vascular morphogenesis, since our findings are not consistent with the current models.

MATERIALS AND METHODS

Murine embryos from wild type FVB and Tie2.LacZ mice (Schlaeger et al., 1997) were isolated between gestational age E9.5 and E13.5, considering the morning of the vaginal plug as E0.5. In order to avoid vessels from collapsing and maintain circulating blood cells, the embryos within the intact yolk sac and placenta were placed in ice cold phosphate-buffered saline (PBS) for one hour to stop the heart from beating while dissecting the embryo for 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining or for paraffin embedding. For X-gal staining, the partially dissected embryonic thoraces (E9.5, E10.5, E11.5, E12.5 and E13.5) were rinsed in PBS, placed in fixative for 30 minutes (1% PFA, 2 mM $MgCl_2$, 5 mM EGTA in PBS) at room temperature, and rinsed twice in washing solution (2 mM $MgCl_2$, 1 mM EGTA in PBS). The thoraces were stained overnight with 1mg/ml X-gal in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, and 2

mM $MgCl_2$ at 4°C. After rinsing in PBS, tissues were postfixed for 2h hours in 4% PFA, followed by complete dissection of the lungs. After imaging of the whole-mount lungs, the X-gal stained transgenic lungs were embedded and sectioned to support the determination of the structures in the lung.

For paraffin tissue sections, the yolk sac and placenta were removed and the embryos were fixed in 4% paraformaldehyde (PFA) for 30 minutes (E9.5), 45 minutes (E10.5), 2h (E11.5) at room temperature; or overnight (E12.5 and E13.5) at 4°C. After two PBS washes, the tissue was processed for paraffin embedding. Embryos were placed in coronal or transversal orientation, completely sectioned and the four-micron-thick sections were used for hematoxylin and eosin staining (H&E) and immunohistochemistry with antibodies raised against PECAM-1 (Rat monoclonal, MEC13.3, 1:50, BDPharMingen) and Fli-1 (Rabbit polyclonal, C-19, 1:1000, Santa Cruz Biotechnology) as endothelial cell markers. We also used α-SM-actin (Mouse monoclonal, 1A4, 1:400, NeoMarkers) to assess the muscularization of the vascular and airway walls. Before incubation with the primary antibody, the sections were dewaxed and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase in methanol for 20 minutes. Antigen unmasking was performed with trypsin treatment (1.25mg/ml for 5 minutes at RT) for PECAM-1 and α-SM-actin and with microwave treatment in 10 mM citric acid buffer (pH adjusted to 6.0; 9 minutes at 450W) for Fli-1. Sections were blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes and incubated with primary antibody diluted in 5% BSA in PBS overnight at 4°C. As secondary antibody we used rabbit anti-rat IgG-peroxidase (Dako) for PECAM-1 , goat anti-rabbit IgG-peroxidase (Dako) for Fli-1 and goat anti-mouse IgG-peroxidase (Dako) for α-SM-actin, all diluted 1:100 in 5% BSA in PBS 2h at RT. Antibody binding was detected by DAB and slides were counterstained with hematoxylin.

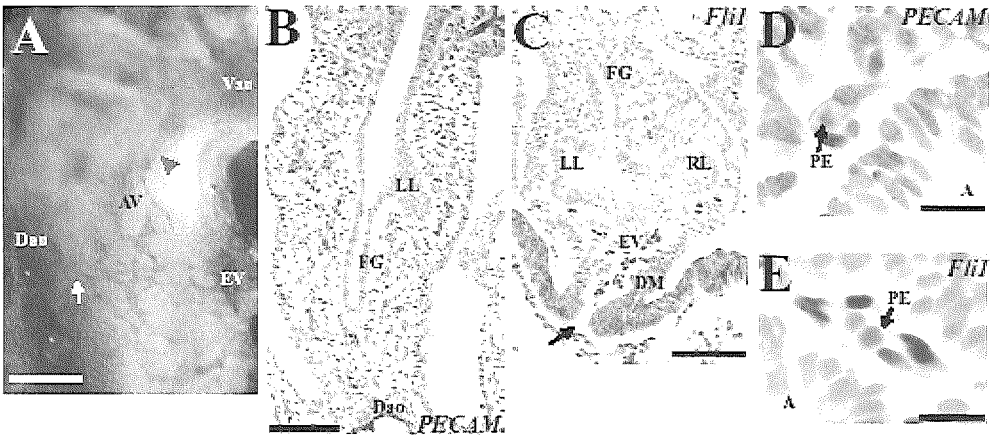
RESULTS

In order to be able to define the structures and identify the different arteries and veins, we prepared serial sections of the lungs, which were stained with antibodies against antigens specific for endothelial cells (PECAM and Fli-1) or smooth muscle cells (SM-actin). PECAM-1 (platelet endothelial cell adhesion molecule) is a transmembrane glycoprotein expressed on the surface of endothelial cells (Drake and Fleming, 2000), and Fli-1 (Friend leukemia integration-site1) is an ETS-domain containing transcription factor, expressed in hemangioblasts, angioblasts and (early) endothelial cells (Sato, 2001a). Aside from wild type murine embryonic lungs, we also used the Tie2-LacZ transgenic strain. The Tie2-LacZ transgene phenocopies the endogenous *Tie-2* expression, so X-gal positive cells can be assumed to be Tie-2 positive (Schlaeger et al., 1997). Fli-1 is an important regulator of *Tie-2* expression in the mouse embryo, since the *Tie-2* promoter contains a cluster of ETS binding sites (Sato, 2001b) and disruption of

the *Fli-1* gene in mice leads to a specific down regulation of the *Tie-2* gene (Hart et al., 2000). Complete Tie2-LacZ transgenic lungs, stained with X-gal, were analyzed and subsequently sectioned to validate our conclusions based on the whole mount analysis. So, we could identify particular structures or vessels throughout the lung with adequate precision by combining the serial sections stained with specific antibodies and the X-gal stained transgenic lungs. We divided the pulmonary vasculature in three components, the afferent, the effective and the efferent: the afferent component comprises the vessels that are proximally continuous with the vascular outflow of the heart, and distally with the capillary network, which we define as the effective component. This effective component is in close contact with the epithelium of the terminal bud of the airway. The pulmonary veins that transport the blood from the periphery of the lung back to the heart form the efferent component. The proximal part of the primitive pulmonary veins is continuous with the atrial structures and develops into the dorsal mesocardium. We have analyzed a minimum of 5 murine embryos for each gestational age and representative pictures are displayed in the figures.

The primitive gut originates after the completion of gastrulation when a crescent layer of endodermal cells starts folding to form a tube. The foregut, which is the anterior part of this gut-like structure, gives rise to a number of organs, like the thyroid glands and lungs. Immediately caudal to the fourth pharyngeal arch, the first morphological sign of the lung starts to become visible at E9.5 when a cluster of cells bud from the ventral site of the foregut and invade the surrounding splanchnic mesenchyme. Subsequently, this lung bud grows and splits into the prospective left and right lobes, running alongside the future esophagus. The dorsal mesocardium, or heart stalk, connects the atrial myocardial wall with the splanchnic mesenchyme ventral to the foregut (Webb et al., 1998). At E9.5-10, the lung vasculature is part of the splanchnic plexus surrounding the developing esophagus and airways (Figure 1). The maintenance of the blood circulation while fixing the embryos made the vessels easy to define and traceable throughout the serial sections, because they remained as an open structure. The afferent and efferent components form a Tie-2, PECAM-1 and Fli-1 positive plexus of capillaries that surrounds the proximal foregut (Figure 1A, 1B and 1C), and are continuous with the developing aortic arches (Figure 1A, arrowhead) and dorsal aorta (Figure 1A, arrow). A capillary network of Tie-2, PECAM and Fli-1 positive endothelial cells surrounds the two airway buds (Figure 1A asterisk) and is in close contact with the epithelial cells of the airway (1D and 1E). Primitive erythrocytes, produced by the blood islands of the yolk sac, are frequently observed within the main vessels, as in the dorsal aorta and heart structures (Figure 1C), and in the capillaries, indicating that even the smallest vessels are connected to the embryonic circulation (Figure 1D and 1E). The venous confluence of this network runs through the dorsal mesocardium (Figure 1C, DM), and forms an invagination at the entrance of the atrium, described as the pulmonary pit (Webb et al., 1998).

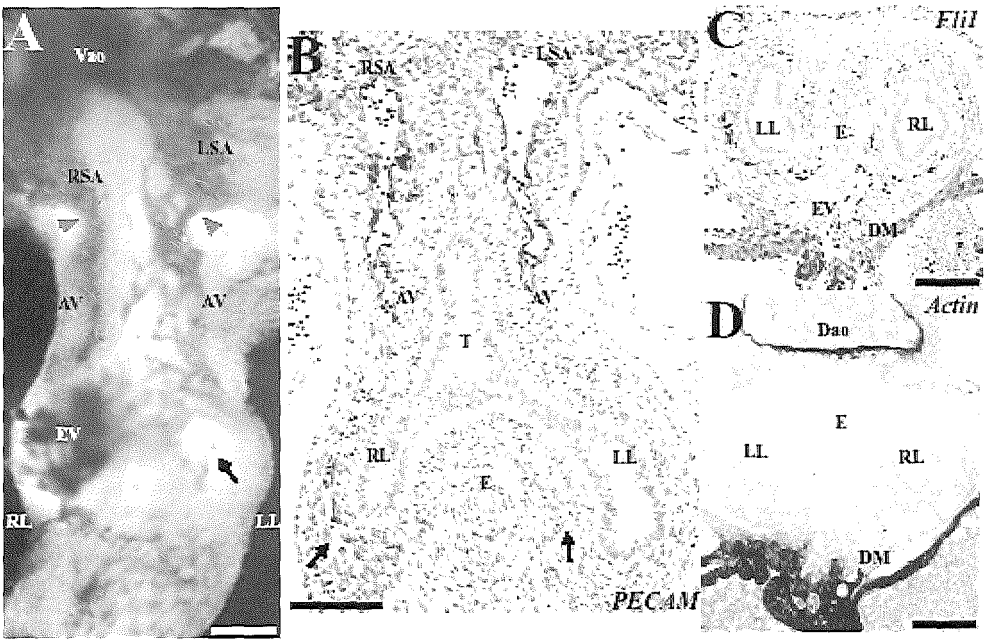
Figure 1 E9.5 lung vasculature



(A) Tie-2 driven LacZ expression in whole mount transgenic lung shows continuity of the primitive lung vasculature with the aortic arch (arrowhead) and the dorsal aorta (arrow). The network of small capillaries surrounding the growing lung bud is indicated with an asterisk (B) PECAM staining of a coronal section showing the lining of the vasculature. (C) Fli-1 staining of a transversal section. The efferent vessels (EV) run through the dorsal mesocardium (DM) into the heart structure. The arrow indicates the pulmonary pit. (D and E) Details of PECAM staining (D) and Fli-1 staining (E). Note the presence of primitive erythrocytes (PE) within the vessels of the splanchnic plexus adjacent to the airway (A). (Vao = ventral aorta, Dao = dorsal aorta, AV = afferent vessel, EV = efferent vessel, LL = left lung, FG = foregut, RL = right lung, DM = dorsal mesocardium, A = airway, PE = primitive erythrocyte). Bar in A, B, C: 100 μm. Bar in D, E: 20 μm.

At E10.5, the first airway branches are formed and the trachea is separated from the esophagus, while both are surrounded by a contiguous mesenchyme. The left and right lungs are clearly formed and positioned slightly curved dorsally at each side of the esophagus. The afferent vessels are not a defined vascular tube yet, but resemble two plexiform networks that coalesce alongside the trachea (AV in Figure 2A and 2B) and are continuous with the sixth aortic arch (Figure 2A, arrowheads, RSA and LSA). A capillary vascular network surrounds both primitive bronchi (Figure 2A, 2B, arrows) and is continuous with the larger vessels, as has been concluded from the examination of serial sections. The efferent vessels form a plexus of capillaries that drain through the dorsal mesocardium into the common atrium of the heart (Figure 2A and 2C, EV). The heart in figure 2A is removed to better illustrate the point that a vascular connection exists between the efferent vessels and the dorsal mesocardium of the heart, as shown in figure 2C. All vascular structures have a clear and open lumen filled with primitive erythrocytes, indicating their connection with the total embryonic circulation (Figure 2B). Although the endothelial cells of these vessels are positive for Tie-2, PECAM and Fli-1, they are not yet muscularized. In addition, the airways and esophagus also lack muscularization, whereas the dorsal aorta and dorsal mesocardium clearly are (Figure 2D).

Figure 2 E10.5 lung vasculature



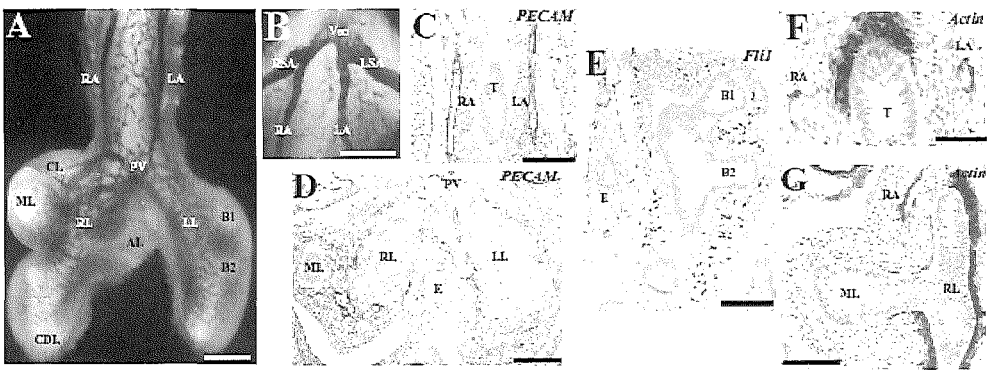
(A) Tie-2 driven LacZ expression in whole mount transgenic lung shows continuity of the primitive lung vasculature with the sixth aortic arches (arrowheads) and the network of capillaries surrounding the lung bud (arrows). (B) PECAM staining of a coronal section showing vascular structures alongside the airways filled with primitive erythrocytes (arrows). The afferent vessels form two plexiform networks that coalescence alongside the trachea (A and B). (C) Fli-1 staining of a transversal section showing that a plexus of capillaries drains through the dorsal mesocardium into the heart. (D) Alpha-SM-actin staining of a transversal section. The vessels of the lung, the airway and the esophagus are not yet muscularized, whereas the dorsal aorta and the dorsal mesocardium are clearly muscularized. (Vao = ventral aorta, RSA = right sixth aortic arch, LSA = left sixth aortic arch, RL = right lung, LL = left lung, AV = afferent vessels, EV = efferent vessels, T = trachea, E = esophagus, Dao = dorsal aorta, DM = dorsal mesocardium, A = airway). Bar: 100 μm.

At E11.5, lung asymmetry has become obvious: the left lung has one branch (Figure 3A, LL), whereas the right lung has four main branches, the primordia of the cranial, the middle, the caudal and the accessory lobe (Figure 3A, CL, ML, AL and CDL). The proximal afferent vessels can now clearly be identified as two vascular tubes that run alongside the trachea, the right and left pulmonary arteries (Figure 3A and 3C, RA and LA). Proximally, the right pulmonary artery is still connected to the right sixth aortic arch, but has been lateralized towards the left (Figure 3B). The left sixth aortic arch, which will form the ductus arteriosus, is more obvious than the right sixth aortic arch, which eventually degenerates (Figure 3B, LSA and RSA). The vessels that surround the lung buds are positive for Tie-2, PECAM and Fli-1 (Figure 3A, 3D and 3E) and contain primitive erythrocytes. The efferent vessels form a vascular tube, the common pulmonary vein (Figure 3A and 3D, PV). Smooth muscle cells start to enfold the proximal parts of the arteries, airways (Figure 3F and G) and, to a lesser extent, veins

(data not shown). However, the growing part of the distal airways and surrounding vessels are not muscularized.

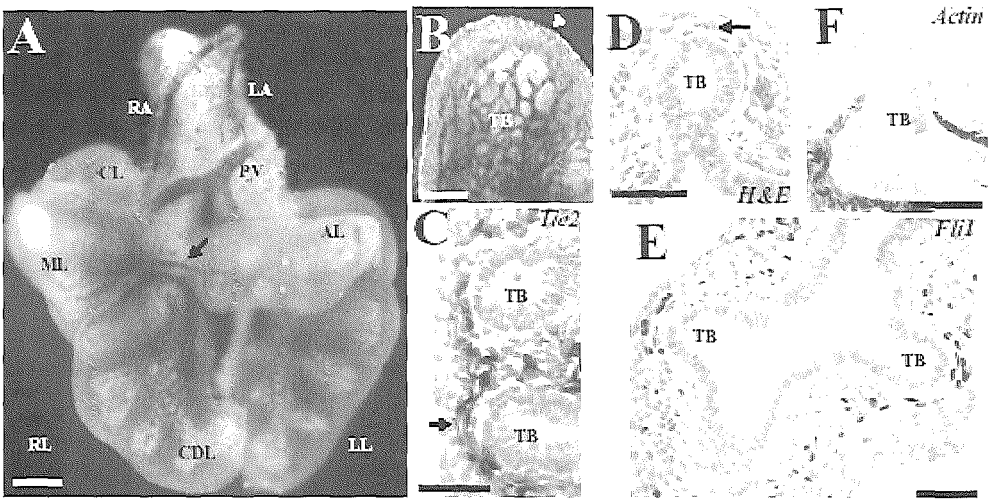
At E12.5, the esophagus and trachea are only attached by mesothelial linings (Figure 4B, arrowhead). The lung has a single left lobe and four clearly distinguishable right lobes (Figure 4A, LL, CL, ML, AL and CDL). Each lobe of the lung has undergone further branching, which occurs in an axial fashion, increasing length and number of generations from the periphery to the center. The airway terminal buds are completely surrounded by a polygonal, irregular capillary plexus (figure 4A, 4B), which contains a lumen (Figure 4C, arrow) filled with primitive erythrocytes (Figure 4D, arrows), indicating a direct and closed connection with the embryonic circulation. The endothelial cells of these vessels are close to the epithelial cells of the terminal bud (Figure 4C and 4E), but these vessels are not yet muscularized (Figure 4F).

Figure 3 E11.5 lung vasculature



(A and B) Tie-2 driven LacZ expression in whole mount transgenic lung. The right and left pulmonary arteries can now be identified as two vascular tubes that run alongside the trachea. (C-G) Coronal sections stained with PECAM (C and D), Fli-1 (E) and α-SM-actin (F and G). C is a section at the level of the imaginary line between RA and LA in figure 3A, D between ML and B1, G between ML and AL, E through LL and F between RA and LA. The common pulmonary vein is formed by several efferent vessels from the right and left lung. Alpha-SM-actin staining of a coronal section shows that the proximal arteries and airways are being muscularized. (RSA = right sixth aortic arch, LSA = left sixth aortic arch, RA = right pulmonary artery, LA = left pulmonary artery, RL = right lung, LL = left lung, PV = pulmonary vein, CL = cranial lobe, ML = middle lobe, AL = accessory lobe, CDL = caudal lobe, T = trachea, E = esophagus, B1 and B2 = branch one and two of the left lung, Vao = ventral aorta). Bar in A, B, C, D and G: 100 μm. Bar in F: 50 μm.

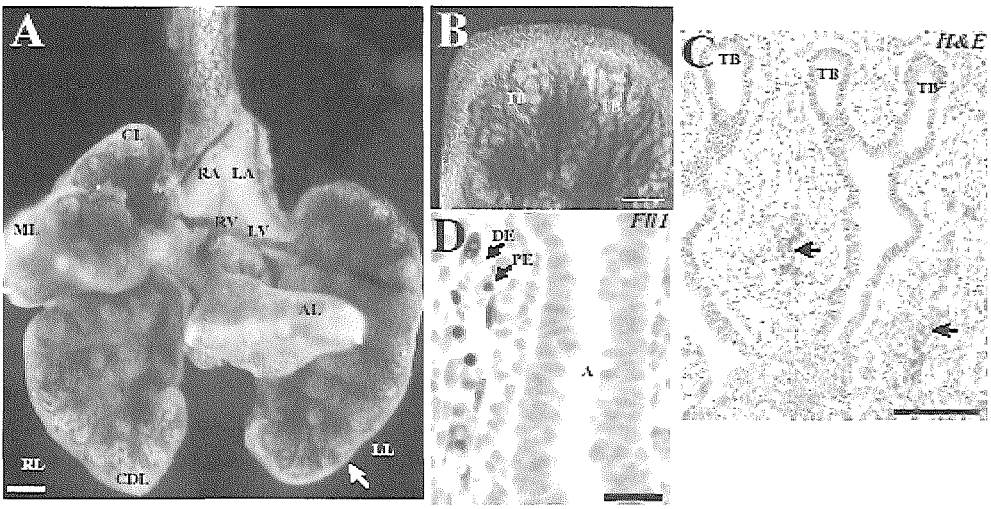
Figure 4 E12.5 lung vasculature



(A and B) Tie-2 driven LacZ expression in whole mount transgenic lung. The Tie-2/lacZ positive capillary network is the effective component that wraps the terminal buds. The arrow in A indicates the pulmonary artery of the right accesory lobe, and the arrowhead in B indicates the mesenchymal layer. (C) H&E staining of a X-gal stained Tie2-LacZ transgenic lung section demonstrating that the capillary network is close to the airway epithelium. The arrow indicates the vascular lumen. (D) H&E staining showing a primitive erythrocyte in the lumen of a capillary (arrows). (E) Fli-1 staining indicating the vasculature surrounding the growing lung buds. (F) Alpha-SM-actin staining illustrates that the terminal buds are not muscularized. (RA = right pulmonary artery, LA = left pulmonary artery, RL = right lung, LL = left lung, PV = pulmonary vein, CL = cranial lobe, ML = middle lobe, AL = accessory lobe, CDL = caudal lobe, TB = terminal bud). Bar in A: 200 μm. Bar in B: 100 μm. Bar in C, D, E and F: 50 μm.

At E13.5, further branching of the airways results in a more complex bronchial tree (Figure 5A) and the polygonal network of capillaries clearly encloses the growing lung bud (Figure 5B). The proximal pulmonary arteries are no longer associated with the capillary plexus of the trachea, and are connected with the developing pulmonary trunk and ductus arteriosus. Two proximal pulmonary veins exit the right and the left lung (Figure 5A, RV and LV), and form a common vein. The intrapulmonary arteries run in close proximity to the airway and form straight tube-like structures (Figure 5D), which are muscularized more peripherally than the veins. The intrapulmonary veins run at some cell-thickness away from the airway (Figure 5C, arrows). The venous drainage is more irregular and has a wider lumen (Figure 5C). Since the site of hematopoiesis is shifted towards the liver, a mixture of primitive erythrocytes (nucleated, blood island origin) and definitive erythrocytes (enucleated, liver origin) is present in the lumen of the vessels (Figure 5D). This clearly shows the existence of a vascular connection between the lung and other embryonic parts.

Figure 5 E13.5 lung vasculature



(A and B) Tie-2 driven LacZ expression in whole mount transgenic lung displays a capillary network wrapping the terminal buds. The Tie-2 positive vessels clearly surround the terminal lung buds (B). (C) H&E staining shows that the pulmonary veins are irregularly shaped and are a few cells-thickness away from the airway. The lumen of these veins is filled with erythrocytes (arrows). (D) Fli-1 staining displaying that the pulmonary arteries form a straight vascular tube closely associated with the airway epithelium. Note the presence of primitive (PE) and definitive (DE) erythrocytes in the vascular lumen. (RA = right pulmonary artery, LA = left pulmonary artery, RV = right pulmonary vein, LV = left pulmonary vein, RL = right lung, LL = left lung, CL = cranial lobe, ML = middle lobe, AL = accessory lobe, CDL = caudal lobe, TB = terminal bud, A = airway, DE = definitive erythrocyte, PE = primitive erythrocyte). Bar in A: 200 μm. Bar in C: 100 μm. Bar in B, D: 50 μm.

Discussion

We describe the development of the pulmonary vasculature in the mouse from the first morphological sign of lung development until early pseudoglandular stage by analysis fetal lungs of whole-mount X-gal stained Tie2-LacZ transgenic mice and immunohistochemistry of serial sections of wild type mice. The embryos have been isolated and processed without disrupting the circulation in order to leave the vascular tone and integrity intact. This procedure prevents the collapse of vessels and the putative creation of artefacts in the sections. We were able to follow individual structures throughout the serial sections, which allowed us to identify vessels and airways with accuracy. More importantly, by fixing the tissue with the blood cells still in the vascular system we could recognize primitive, and later definitive erythrocytes, in the lumen of the lung vessels. Since primitive erythrocytes are produced by the blood islands of the yolk sac and definitive erythrocytes by the fetal liver, this observation unequivocally proves the existence of a connection with the heart, yolk sac and liver, and thus the presence of a closed circulation.

Our main conclusion is that already at the onset of lung development the vasculature consists of Tie-2, PECAM and Fli-1 positive endothelial cells that are part of a capillary network in the splachnic mesoderm (figures 1 and 2). The proximal and distal structures from this plexus are connected to each other and are continuous with the heart vascular structures. The presence of erythrocytes within the vascular lumen proofs that there is already blood circulation in the lung and connection with the embryonic circulation from the earliest point of lung development (figures 1D, 1E and 2B). Remodelling of this plexus forms the main trunks of the proximal vessels, which is especially notable in the case of the pulmonary artery that changes from a plexus alongside the trachea at E9.5 into two muscularized vascular tubes at E11.5 (figures 1A, 2A and 3A). We expanded our analysis of the pulmonary vasculature using immunohistochemistry for two distinct endothelial specific cell markers, PECAM and Fli-1. Serial sections demonstrated that both the proximal and distal vessels of the afferent and efferent pulmonary vasculature were positive for both antigens from E9.5 onwards. The endothelial cells of the network of capillaries that surrounds the growing epithelial buds, the effective component, were also positive for PECAM and Fli-1 during early lung development. We hypothesize that the vasculature grows primarily by expansion of existing vessels, but we cannot exclude at this moment that a minor population of putative angioblast-like cells also contributes to the growth of the capillary network.

Vasculogenesis and hematopoiesis seem to be intimately associated extra-embryonically, and this lead to the description of the existence of a common precursor cell, the hemangioblast (Risau and Flamme, 1995). Coffin and Poole used QH-1 antibodies as a label for angioblasts to study the major vessel primordia in chick-quail chimeras (Poole and Coffin, 1989). They defined vasculogenesis as the *in situ* formation of vessels from the aggregation of angioblasts into a cord, that later acquires a lumen, and angiogenesis as the formation of new vessels by sprouting of capillaries from existing ones. Later studies concluded that vasculogenesis gives rise to the heart and the first primitive vascular plexus, whereas angiogenesis is responsible for the remodelling and expansion of this primitive plexus (Patan, 2000). A vasculogenic study in early mouse embryos identified the angioblast as a mesodermal Tal-1$^+$/Flk-1$^+$/PECAM$^-$ cell (Drake and Fleming, 2000). The presence of angioblasts in the lung has been described morphologically (deMello and Reid, 2000; deMello et al., 1997), and as isolated PECAM positive (Hall et al., 2002; Hall et al., 2000) or Flk-1 positive (Schachtner et al., 2000) endothelial cells. Flk-1 is expressed by undifferentiated endothelial cells and angioblasts, but Schachtner *et al.* showed that *LacZ* expression driven by the Flk-1 promoter in the lung overlaps with PECAM expression. deMello *et al.* suggested that the lung itself produces blood cells in hematopoietic lakes as part of a vasculogenic process (deMello and Reid, 2000; deMello et al., 1997). However, during our analysis of serial sections of the different embryonic stages of lung development, we have never encountered other blood cells than primitive and definitive erythrocytes. If

the lung produced blood cells, it would be conceivable that intermediate hematopoietic precursor cells are observed in the sections, even if their frequency is very low. Furthermore, Medvinsky *et al.* previously performed colony-forming unit spleen (CFU-S) assays on several embryonic tissues and demonstrated that the embryonic lung does not contain hematopoietic progenitors (Medvinsky et al., 1996). Hematopoiesis has been described extra-embryonically in the hematopoietic islands of the yolk sac as well as intra-embryonically in the trunk intermediate cell mass (ICM) and later in the AGM (aorta-gonad-mesonephros), liver, spleen and bone marrow (Dzierzak et al., 1998). The lung is not recognized as an organ with the capacity to produce hematopoietic cells (Medvinsky et al., 1996).

We have used an established transgenic Tie2-LacZ mouse strain to perform whole-mount analysis of the lung vascular network at earlier stages than reported before (E9.5 and E10.5). This strain has been shown to express the bacterial *LacZ* gene according to the endogenous expression of the *Tie-2* gene (Schlaeger et al., 1997). Therefore, cells that convert the X-gal substrate can be considered to be positive for the angiopoietin receptor Tie-2. Through the preparation of vascular casts, DeMello *et al.* investigated the development of the pulmonary vasculature in mouse and concluded that there was no connection between proximal and distal structures of the mouse lung before E13.5 (deMello et al., 1997). This technique, though very valuable and informative, has considerable limitations when studying vascular networks that consist mainly of capillaries and very small vessels. Schachtner *et al.* used heterozygous Flk1-LacZ knock-in mice to study the lung vasculature, starting their whole-mount analysis at E11.5, 2 days after the initiation of lung development (Schachtner et al., 2000). They concluded that only the proximal part of the growing pulmonary artery contained a lumen through analysis of E10.5 mouse embryo sections (Schachtner et al., 2000). Our data are partly in line with the work of Hall *et al.* who showed continuity from the proximal and distal structures of the lung using 3D reconstruction of immunostained 34d human lung (Hall et al., 2002). Hall *et al.* identified PECAM positive cells in vessel walls, some of which had a narrow lumen (Hall et al., 2002; Hall et al., 2000). In the work presented here, we were able to clearly show for the first time that the lung vasculature is already part of the embryonic circulation from the start of lung development using a tissue processing procedure that keeps the blood circulation intact and prevents the collapsing of the vessels. It is also clear that the lumen of the proximal arteries appears narrower than the lumen of the proximal veins, due to differences in pressure and muscularization. This may explain why deMello *et al.* observed that early veins were diffusely present throughout the mesenchyme, establishing a central luminal connection to the main pulmonary vein before airways or arteries were present at the same level, thus leading to the conclusion that veins and arteries are dissociated in their timing and pattern of branching (deMello and Reid, 2000). Schachtner *et al.* named these venous drainages, which are very clear at E13.5, "lacunaes" (Schachtner et al., 2000). However,

our analysis of serial sections revealed that these structures are in fact the pulmonary veins. Based on hematoxylin and eosin stained sections, DeMello *et al.* described the presence of "hematopoietic lakes" in E10 mouse lung (deMello et al., 1997) and 33d human lung (deMello and Reid, 2000), but analysis of serial sections through embryonic lungs fixed with intact circulation did not reveal structures that could resemble these hematopoietic lakes. We conclude that the description of hematopoietic lakes in the lung is based on morphology and these lakes most likely are collapsed vessels containing trapped primitive erythrocytes.

Our observations of the gradual muscularization of the proximal vessels as part of their maturation process confirms the work described by Hall *et al.* in human (Hall et al., 2002; Hall et al., 2000). They showed that veins acquired α-SM-actin at 56d, whereas arteries did at 38d. In mice, we first detected α-SM-actin positive cells in the vascular wall of arteries and veins at gestational age E11.5. Two muscularized pulmonary arteries run alongside the trachea at E11.5, as also shown by a confocal microscopic study in mouse lungs (Tollet et al., 2001). Two recent papers (Han et al., 2003; Maeda et al., 2002) used double immuno-labeling for PECAM and α-SM-actin in human fetal lungs and concluded that there are two populations of endothelial cells in the lung vascular system. One population of vascular plexi independent of α-SM-actin positive adjacent cells, whereas another population of vascular plexi was juxtaposed to α-SM-actin positive cells. The authors concluded that both plexi communicate at the mid-pseudoglandular stage. Our analysis of serial sections revealed that there was just one vascular network and that proximal vessels undergo muscularization as part of the wall maturation as they accommodate the increasing blood flow and pressure.

Distal angiogenesis as a new concept for lung vascular morphogenesis

Pulmonary vascular morphogenesis has been described to occur either by a combination of central angiogenesis and vasculogenesis with the formation of hematopoietic lakes (Figure 6, Model 1) (deMello and Reid, 2000; deMello et al., 1997; Stenmark and Gebb, 2003) or just by vasculogenesis with formation of new vessels from endothelial precursor cells (Figure 6, Model 2) (Hall et al., 2002; Hall et al., 2000; Hislop, 2002). However, our data do not support the existence of central angiogenesis or distal vasculogenesis in lung development. Therefore, we propose "distal angiogenesis" as a model for pulmonary vascular morphogenesis (Figure 6, Model 3 and Figure 7). Distal angiogenesis is the formation of new capillaries from pre-existing ones at the periphery of the lung. On the basis of extensive and detailed morphological observations we define the concept of the "tip zone" as the distal part of the branching airway that lacks the layer of smooth muscle cells. It is wrapped by a polygonal meshwork of capillaries -the effective component- that expands by distal angiogenesis as the lung bud grows, finally leading to the alveolar capillary plexus. We hypothesize that epithelial-endothelial interactions are decisive to induce angiogenesis at the "tip zone", which ensures the

coordinate expansion of the vascular network as the branching proceeds (figure 7). Newly formed vessels remodel dynamically, as they form part of the afferent or efferent component. This vascular remodelling implies that some vessels will grow and fuse with neighbouring vessels, while others will remain small or degenerate (figure 7).

Two basic mechanisms of embryonic angiogenesis by which the capillary network can expand have been proposed: sprouting and non-sprouting angiogenesis (Risau, 1997). Sprouting angiogenesis involves the expansion of the capillary network by the formation of vascular sprouts from opposite preexisting capillaries, sprouts meet each other by filopodia and form a solid strand, that later acquires a lumen and splits the intervascular space (Risau and Flamme, 1995). In the non-sprouting angiogenesis or intussusception, a solid mesenchymal pillar grows into a capillary, subsequently enlarges and forms a new intervascular space (Risau and Flamme, 1995). Intussusceptive angiogenesis has been described to be responsible for the postnatal growth of the lung capillary bed (Burri and Tarek, 1990). At present, the precise mechanism of angiogenic expansion of the vascular network in our model is under investigation.

In summary, we performed a detailed ontogenic morphological analysis of the pulmonary vasculature from the earliest embryonic stage onwards. The present study describes the development of the different vascular components (arteries, capillaries and veins) of the lung in relation with the developing airways. We conclude that the vasculature is part of the embryonic circulation from the moment the lung starts to develop. This implies that the presence of blood vessels could be more important for the development of the lung than previously anticipated. The endothelial cells of the splanchnic mesoderm may be involved in the pre-patterning of the presumptive lung region, like it has been shown for liver and pancreas development where endothelial cells are involved in the induction of these organs (Lammert et al., 2003). Our observations led us to propose distal angiogenesis as a new concept for lung vascular development. We defined the concept of the "tip zone", where the epithelial-endothelial interactions are crucial to determine the expansion of the lung vascular network. Based on our observations, we propose that angiogenesis already starts at the embryonic phase of lung development and is the major blood vessel forming process.

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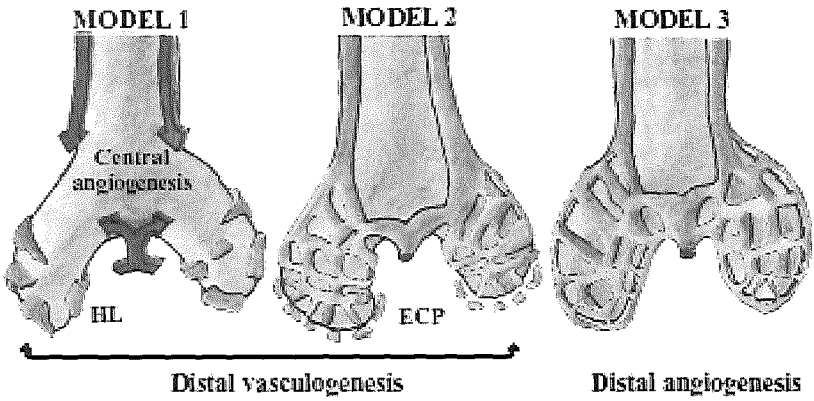
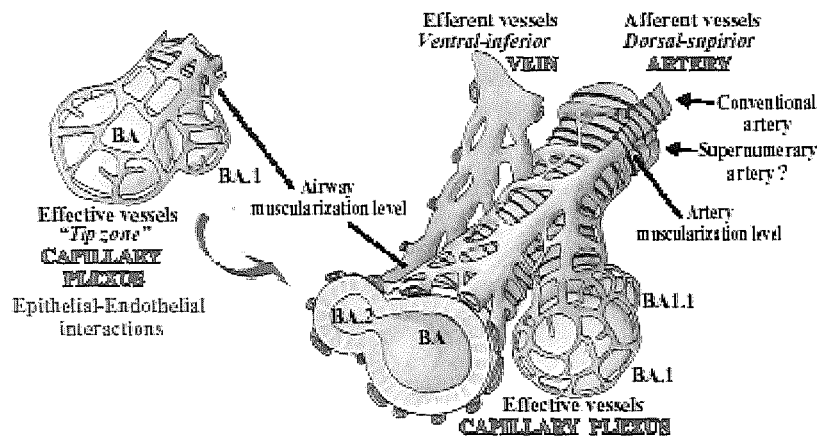
Figure 6 Cartoon of lung vascular morphogenesis models



Model 1, proposed by deMello et al., describes two mechanisms of lung vascular morphogenesis, central angiogenesis (sprouting of arteries and veins from central vascular trunks) and distal vasculogenesis (development of hematopoietic lakes in the mesenchyme) (deMello et al., 1997). Connection between the two vascular beds would occur at E13/14. Model 2, proposed by Hall et al., proposes distal vasculogenesis (development of new vessels from endothelial cell precursors) as the mechanism of lung vascularization (Hall et al., 2000). Model 3, explains our newly proposed mechanism of distal angiogenesis as the process to develop lung vasculature (formation of new capillaries from preexisting ones). (HL = hematopoietic lakes, ECP = endothelial cell precursor).

Figure 7 Distal angiogenesis



Based on the analysis of serial sections of murine embryonic lungs, we propose that the formation of new capillaries from preexisting vessels takes place at the "tip zone", where airway and capillary network expand in a coordinated way through epithelial-endothelial interactions. Newly formed vessels remodel dynamically as they form part of the afferent or efferent component. See text for detailed explanation (BA = airway branch A, BA.1, BA1.1, BA.2 = daughter branches from branch A).

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CHAPTER 3

POST-MORTEM FINDINGS AND CLINICO-PATHOLOGICAL CORRELATION IN CONGENITAL DIAPHRAGMATIC HERNIA

Post-mortem Findings and Clinico-pathological Correlation in Congenital Diaphragmatic Hernia

Marieke F. van Dooren, Natascha N.T Goemaere, Annelies de Klein, Dick Tibboel, Ronald R. de Krijger

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ABSTRACT

Congenital Diaphragmatic Hernia (CDH) is a severe life-threatening disease with an incidence of 3 per 10,000 births, which can occur as an isolated defect or in combination with other congenital anomalies. We reviewed the clinical and autopsy reports of 39 CDH patients that were autopsied in the period 1988-2001, in order to see if autopsy had an additional value in the detection of malformations in CDH patients. We compared the clinical data on congenital anomalies with the autopsy results. Before autopsy 6 structural cardiac defects, 3 anomalies of the urogenital system and 3 anomalies of the digestive tract were observed in 10 patients. However, with post-mortem examination, only 4 structural cardiac defects were confirmed, 2 cases showed another cardiac anomaly, and 7 new cardiac defects were found. In the urogenital system, 1 anomaly was confirmed, 1 was not confirmed, and 1 child showed another malformation. In addition, in 7 patients new urogenital malformations were found after autopsy. In the digestive tract all 3 malformations were confirmed, but we found 3 new malformations after post-mortem examination. All clinically established dysmorphic features, anomalies of the skeletal system and central nervous system (CNS) were confirmed by autopsy and no additional malformations were found. We concluded that post-mortem examination has a significant additional role in the detection of structural cardiac defects, malformations of the urogenital system and digestive tract in children with CDH.

INTRODUCTION

Congenital Diaphragmatic Hernia (CDH) is a severe life-threatening disease, mainly presenting in the neonatal period. The incidence is 3 per 10,000 births and mortality is high (50%) due to lung hypoplasia, pulmonary hypertension, and associated anomalies (Torfs et al. 1992; Skari et al. 2000). It can either present as an isolated birth defect, in combination with other congenital abnormalities, or as part of a defined syndrome or chromosomal disorder (Benjamin et al. 1988; Tibboel and Gaag 1996). Despite advanced treatment modalities such as High Frequency Oscillation, Nitric Oxide (NO)–treatment, and Extra Corporal Membrane Oxygenation (ECMO), mortality remains high. The commonest form of diaphragmatic defect is the classic posterolateral defect (Bochdalek hernia) in 96% of cases (Torfs et al. 1992). Although the etiology is still unknown and multifactorial inheritance has been suggested, chromosomal and syndromal CDH cases have been described. Mendelian disorders associated with CDH are Fryns syndrome, de Lange syndrome and Beckwith-Wiedemann syndrome. Chromosomal anomalies include Pallister- Killian syndrome (tetrasomy 12p) and other well-known numerical anomalies such as trisomy 13 and 18 (Enns et al. 1998). Detailed examination of the phenotype in CDH patients may lead to clues on the etiology, as additional malformations may be important to reach a syndrome diagnosis. Therefore,

post-mortem investigation can be important. In the literature, few post-mortem studies on CDH have focused on additional malformations and chromosomal abnormalities. (Benjamin et al. 1988; Bajaj et al. 1991; Bollmann et al. 1995; Enns et al. 1998) However, systematic analysis on the relevance of post-mortem examination in CDH cases has not been described. The aim of this study was to compare clinical information with the results of post-mortem examination in order to investigate the additional role of autopsy in deceased CDH patients.

Patients and Methods

In our hospital, a single institution level III perinatal center and pediatric surgical center, all 184 patients with CDH were registered in a database during the period 1988-2001. Apart from all standard clinical information, this database contains information on associated birth defects, genetic information, obstetrical history, surgical procedures, and autopsy information. Eighty-three of these 184 children with CDH were live born, but died at varying time intervals after birth. Post-mortem examination was performed on 39 children (47%). Retrospectively, we reviewed the autopsy reports and patient case record details of the 39 deceased children with CDH in the period 1988-2001. In this group, there were 23 males and 16 females. Twenty-five infants had a left-sided defect, 11 had a right-sided defect, and 3 had a bilateral defect. In 17/27 cases (63%), in which prenatal echography was performed, the diagnosis of CDH was established, and in 27/39 cases (69%) karyotyping was performed. The average gestational age was 266 days (38 weeks; range 196-294 days). The average birth weight was 2840 gram (range 735-3965 gram). The average survival time in the 32 patients in whom this was known, was 250 days (range 0-1825 days). However, 14/32 patients died within 1 day of birth. In our center, routine and standardized care of high-risk newborns with CDH involves consultation of a clinical geneticist, cardiac, renal, and cranial ultrasound. Autopsy was performed according to a standardized protocol. The nature and number of associated anomalies were identified. Associated anomalies were defined as one or more defects not directly related to the hernia. As a consequence, intestinal malrotation and persistent ductus arteriosus were not considered as separate, unassociated anomalies.

Results

The results of the comparison between clinical data and autopsy data with respect to congenital malformations are summarized in Table 1, whereas Table 2 shows the structural congenital anomalies and karyotyping results in the 23/39 (59%) patients with multiple congenital anomalies (MCA).

Table 1 Congenital anomalies before and after autopsy

	Before autopsy	After autopsy Confirmed	After autopsy Not confirmed	After autopsy Other findings	After autopsy New findings
Structural cardiac defect	6	4	0	2 - ASD type 2 - Bicuspid pulmonary valve	7 - Complete transposition of great vessels, interrupted aortic arch, hypoplastic left atrium/ ventricle, ASD - Hypoplasia left heart, preductal aortic coarctation - VSD, right aortic arch, open foramen ovale - Hypoplasia isthmus aortic arch, open foramen ovale - Persistent left superior caval vein - Double outlet left ventricle, atrioventricular septal defect (AVSD), muscular VSD, complete absence atrial septum, abnormal pulmonary venous connection, small bicuspid pulmonary valve, overriding aorta, hypoplasia truncus pulmonalis, absent ductus arteriosus - Hypoplasia heart
Urogenital system	3	1	1	1 Septated vagina, bicornuate uterus	7 - Horseshoe kidney (2x) - Cryptorchidism (2x) - Horseshoe kidney, bicornuate uterus - Cysts of the renal cortex (2x)
Digestive tract	3	3	0	0	3 - Gallbladder atresia, duodenal atresia, colon agenesis - Duplication of the stomach - Meckel's diverticle
CNS	3	3	0	0	0
Dysmorphic features	8	8	0	0	0
Skeletal system	8	8	0	0	0

In the group with cardiac malformations 4 of the 6 clinically known defects were confirmed by autopsy, whereas in 2 patients a different cardiac anomaly was found. This concerned 1 patient with a clinically detected ventricular septal defect (VSD) that proved to be an atrial septal defect (ASD) type 2 at autopsy. In the other patient an unspecificied structural cardiac defect was clinically suspected, whereas at autopsy only a bicuspid pulmonary valve was found. In 7 other patients structural cardiac defects were only observed after post-mortem investigation (see Table 1).

The most common cardiovascular anomalies in our study group were VSD (n = 4), ASD (n = 3) and hypoplastic left heart (n = 3). Anomalies of the aorta and aortic arch were found in 5 cases (hypoplasia of the aortic arch in 3 cases, interrupted aortic arch and right aortic arch in 1 case each). Atrioventricular septal defect (AVSD) and bicuspid pulmonary valve were found in 2 patients each. Other findings were transposition of the great vessels, persistent left superior caval vein, abnormal pulmonary venous connection, absent ductus arteriosus, hypoplasia of the truncus pulmonalis, and generalized cardiac hypoplasia. Taken together, 13 cardiovascular anomalies were found in 39 patients.

Urogenital malformations were clinically suspected in 3 patients, which were confirmed in 1 patient. A different urogenital malformation was found in another patient (a suspected left renal agenesis could not be confirmed, however, a septated vagina with bicornuate uterus was found). In the third patient, cryptorchidism could not be confirmed at autopsy. In 7 patients additional anomalies of the urogenital tract were found at post-mortem investigation (see Table 1).
In the entire study group, the most common urogenital malformation were cryptorchidism (n = 3), horseshoe kidney (n = 3) and a bicornuate uterus (n = 2). Other findings were septated vagina, hypospadia, atrophic testis, and cysts in the renal cortex (n = 2).
Three patients were known to have malformations of the digestive tract and these were all confirmed at post-mortem examination (see Table 2). However, in 3 other patients intestinal anomalies were only found at autopsy (see Table 1).

All malformations of the CNS and skeletal system, and all dysmorphic features were confirmed by autopsy. There were no additional or new malformations found. Anomalies of the spleen were only detected by autopsy, with 3 patients with polysplenia and 1 patient with asplenia.

Table 2 MCA and karyotypes in the autopsy group (confirmed malformations are indicated in bold)

	sex	structural cardiac defects	urogenital system	digestive tract	CNS	dysmorphic features	skeletal system	spleen anomalies	karyotype
1	m		horseshoe kidney			**low implant ears, small philtrum**	**rocker bottom feet**		47,XY,+18
2	m	complete transposition of great vessels, interrupted aortic arch,hypoplastic left ventricle, hypoplastic left atrium, ASD	horseshoe kidney		**hydrocephalus, pseudoholo-prosencephaly**	**dysmorphic face and helix, small mouth**	**rocker bottom feet, radiusaplasia left**		47,XY,+18
3	v	**cardiomyopathy, coarctation of aorta**				**dysmorphic face and helix, cleft palate**			46,XX
4	v		cysts of the renal cortex						
5	m	hypoplastic heart							46XY
6	m							accessory spleen	
7	m		cryptorchidism						
8	m	hypoplastic left heart, preductal aortic coarctation							
9	v	VSD, right descending aorta	horseshoe kidney, bicornuate uterus				**syndactyly first and second toe left**		46,XX
10	v	**ASD type 2, hypoplastic left ventricle, VSD, tubular hypoplasia of the aorta**	septated vagina, bicornuate uterus					polysplenia	46,XX
11	v	**AVSD**	cysts of the renal cortex			**protrusion tongue, simian creases bilateral, sandal gap right foot**			47,XX,+21
12	v			**omphalocele**			**deformation ribs and vertebral column**		46,XX
13	m			gallbladder- and duodenumatresia, agenesis colon		**flattening head left, pronated back of the head**	**contracture right hip, tallipes right, high thoracal scoliosis**		

14	v	ASD type 2							46,XX
15	m		**hypospadias, cryptorchidism, atrophic left testicle**						46,XY
16	m				**anencephaly, cervical rachischisis**				46,XY
17	m							accessory spleen	46,XY
18	v	bicuspid pulmonary valve		duplication of the stomach		**broad nose, hypertelorism, low implant ears, malformation left ear, micrognathia**	**rocker bottom feet, overlying tall fingers**		47,XX,+18
19	m	hypoplastic isthmus aortic arch	cryptorchidism	**laryngo-tracheo-oesophago cleft**			**hypoplastic nails of the toes**		46,XY
20	v	**muscular VSD**							46,XX
21	m	persistent left sup caval vein			**olivoponto-cerebellar atrophy**	**large, broad-based nose, flat supra-orbital ridges, low implant dysmorphic ears, micrognathia**	**multiple flexion-contractures, pterygium elbow, pectus excavatus nailhypoplasia**		46,XY
22	m			Meckel`s diverticle					46,XY
23	m	double outlet left ventricle, AVSD, muscular VSD, complete absence atrial septum, abnormal pulmonary venous connection, small bicuspid pulmonary valve, overriding aorta, hypoplasia truncus pulmonalis, absent ductus arteriosus		**abnormal position anus**		**hypertelorism, pre-auricular tag right, antihelix right , 2 pits under columella, high glabellum**		asplenia	46,XY

Discussion

Our hospital serves as a referral facility for level III perinatal and pediatric surgical care with a focus on CDH treatment (including ECMO) for the Southwestern part of the Netherlands with 3-4 million inhabitants and an annual birth rate of 35.000. Regarding the incidence of CDH (3 per 10.000), the annual number of expected cases is 11 patients. We conclude that our cases (184 patients in 13 years) are representative for our region. This is also reflected in the high frequency of children with CDH (3.4%) we found in our autopsy database from 1988-2001 (n = 1402 autopsies). Bajaj et al. describes a frequency of only 1.7% (Bajaj et al. 1991) .
To evaluate the additional value of autopsy we compared the number of malformations before and after post-mortem examination. We hypothesized that post-mortem examination would be useful to obtain significant additional information. We found a striking number of previously unrecognized malformations in the cardiovascular system (7/13, 54%), urogenital system (7/10, 70%), and digestive tract (3/6, 50%), despite prenatal ultrasound in 27/39 (69%) children. It must be noted that the previously unrecognized additional malformations were found in 14 patients, 3 of whom did not have a prenatal ultrasound. Of the remaining 11 patients, only 3 lived longer than 1,5 day (and were thus likely to receive further clinical investigations). The additional malformations found in this latter group were relatively minor: renal cysts found by microscopy in 2 and cryptorchidism in 1 patient.

Cardiovascular malformations were the most common anomalies in association with CDH, in 33% of our patient group. The incidence of cardiovascular anomalies in association with CDH in the literature ranges from 24% to 42% (Benjamin et al. 1988; Migliazza et al. 1999). In the group of structural heart defects we could not detect a specific heart defect in combination with CDH, although left ventricular outflow tract obstruction and VSD were the most commonly found abnormalities (Bollmann et al. 1995). Among our CDH patients, there were 4 patients with a VSD, which is a common congenital heart defect (1/400 full term live births). VSD can be isolated, but it also occurs in a variety of syndromes such as trisomy 13 and 18, CHARGE association, VACTERL association, Holt-Oram syndrome, and Zellweger syndrome. The 4 patients with the combination of VSD and CDH all had a normal karyotype, 1 patient had Fryns syndrome, in which septal defects have been reported (Ayme et al. 1989). Chromosomal anomalies reported in children with VSD are numerous, e.g. trisomy 8 and 22 (Copel et al. 1986).

Hypoplastic left heart has also been frequently described in patients with CDH, which has been attributed to cardiopulmonary compression due to the diapghragmatic defect (Suda et al. 2000). In the literature, several chromosomal anomalies have been detected in children with hypoplastic left heart: trisomy 13 and 18, 45,XO (Turner syndrome) and

structural anomalies of chromosomes 5 and 10 (Brackley et al. 2000). In accordance, in our study we found a trisomy 18 in one of three patients, in which karyotype analysis had been performed. In general, in 4 of our 13 cases with cardiovascular malformations there was a chromosomal anomaly (Table 2) or a syndrome diagnosis, in this case a Fryns syndrome.

In the urogenital system, 8 new anomalies were discovered at autopsy. One of the most frequently detected urogenital anomalies was cryptorchidism (n = 3, 8%). The expected incidence of cryptorchidism in normal term males varies from 0,7% to 3,3%, depending on the birth weight. Benjamin *et al* (Benjamin et al. 1988) describe cryptorchidism in 30% of autopsy patients with CDH. Horseshoe kidneys can be associated with CDH, but 2 of our 3 patients with horseshoe kidney had trisomy 18, which is also known to be associated with this anomaly. Our observed incidence of urogenital malformations (26%) is similar to that mentioned in the literature (18%-27%) (Benjamin et al. 1988) (Bollmann et al. 1995).
Malformations of the digestive tract are far less common in CDH than the previously mentioned anomalies, and include serious malformations such as agenesis of the colon or relatively innocuous ones, such as a Meckel's diverticulum. Benjamin *et al* (Benjamin et al. 1988) describe a higher incidence of Meckel's diverticulum in CDH patients (8% versus 2,3% in the general population), but we could not confirm this in our study, in which we found a frequency of 1/39 (2.6%).

All dysmorphic features, malformations of the skeleton and CNS had been detected by prenatal ultrasound, physical examination, and total body X-ray. Autopsy did not have an additional role in the detection of these malformations. This is probably related to the present policy in our hospital to consult a clinical geneticist immediately post partum to assess dysmorphic features and to establish a possible syndrome diagnosis.
Four chromosomal anomalies and 1 syndrome diagnosis were found. The overall incidence of karyotypic anomalies is 4/27 (15%) in our study, whereas in the literature the incidence ranges between 2% and 31% (Cunniff et al. 1990). All patients with an abnormal karyotype had MCA. As already described, CDH is frequently found in association with trisomy 13, 18 and 21, but it can also be associated with structural chromosomal anomalies, such as deletions, translocations, and marker chromosomes, mainly involving chromosomes 3, 8, 15, and 20 (Howe et al. 1996; Brennan et al. 2001; Schlembach et al. 2001). There is increasing evidence in the literature that the long arm of chromosome 15, especially 15q24-15q26, may play a role in the development of the diaphragm (Schlembach et al. 2001). Diaphragmatic defects are also encountered as a feature in a number of genetically determined disorders including Fryns syndrome, de Lange Syndrome, Pentalogy of Cantrell, and Pallister Killian syndrome. Fryns syndrome, characterized by CDH, digital hypoplasia, cleft palate, and autosomal recessive inheritance, was found in 1 patient in our study population, which is important to

recognize in order to counsel the parents properly.

In conclusion, post-mortem examination has an important additional role in the detection of malformations of the cardiovascular system, urogenital system and digestive tract. However, autopsy does not have such role in the diagnosis of dysmorphic features, most skeletal anomalies, and anomalies of the CNS. Close collaboration between pathologist, paediatrician, radiologist, and clinical geneticist increases the likelihood of specific syndrome diagnosis, which may have prognostic implications for future pregnancies. Since it will never be possible to obtain permission for autopsy in every child with CDH, further investigation of other examination methods, such as MRI, should be considered. However, recent articles have indicated that, despite being superior to echography, MRI is inferior to post-mortem examination, and cannot replace this as the gold standard (Alderliesten et al. 2003).

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CHAPTER 4

THE ASSOCIATION OF CONGENITAL DIAPHRAGMATIC HERNIA WITH LIMB REDUCTION DEFECTS

The Association of Congenital Diaphragmatic Hernia with Limb-Reduction Defects

Marieke F. van Dooren, Alice S. Brooks, Dick Tibboel, Claudine P. Torfs

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ABSTRACT

Background

The pathogenesis of congenital diaphragmatic hernia (CDH), a severe birth defect, is not well understood; however, both developmental genes and environmental factors have been suggested to be involved. CDH is frequently associated with malformations of other structures, such as limbs, whose embryogenesis is better understood. An examination of the co-occurrence of developmental defects may provide clues as to the origin and timing of the insult to the diaphragm and limbs. Our focus was on CDH-associated limb-reduction defects (LRDs).

Methods

For this descriptive study, we reviewed the medical records of infants with a posterolateral (Bochdalek) CDH and an associated LRD among 146 patients from the Sophia Children's Hospital, and among 810 infants and 36 stillbirths from the California Birth Defects Monitoring Program (CBDMP).

Results

In the hospital group, 14 patients (10%) had an associated limb defect, of which about one-third were LRDs (of these, most were of a nonsevere type, such as hypoplasia of fingers). In the registry group, a limb defect was found in 162 cases (18.5%), 18 of which were mostly severe LRD (usually of the upper extremities). Additional congenital anomalies were observed in all CDH-LRD cases in both groups

Conclusions

In the registry group, 77.8% of LRDs were either bilateral or ipsilateral, and were mostly preaxial, suggesting an early embryological insult affecting both precursor anlages. These results, from large numbers of cases, support the notion of a developmental association between CDH and LRD, as has been observed in several knockout mice. Future analyses of candidate genes from patients with CDH and LRD may elucidate this developmental association in humans.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe birth defect characterized by a combination of failure in the formation of the diaphragm, unilateral or bilateral lung hypoplasia, and postnatal pulmonary hypertension. The prevalence of CDH is about 0.3 per 1000 births, and mortality rates are high, particularly in patients with associated malformations (Torfs et al., 1992; Skari et al., 2000). Recently, however, survival rates have improved (Muratore and Wilson, 2000; Boloker et al., 2002). The three main

anatomical types of CDH are the posterolateral defect or Bochdalek hernia, the Morgagni hernia, and the hernia of the pars sternalis, which represent respectively about 96%, 2%, and 2% of CDH cases (Torfs et al., 1992). Because these anatomical types are thought to have different etiologies, we evaluated only the Bochdalek hernia in this study.

Approximately 40-50% of CDH patients have other malformations, and limb defects are one of the most frequent (Benjamin et al., 1988; Torfs et al., 1992). The most severe limb defects involve the absence, shortening, or hypoplasia of a limb or part of a limb, and are known as limbreduction defects (LRDs). Few epidemiological studies of the co-occurrence of CDH and LRD have been reported (Froster-Iskenius and Baird, 1989; Martinez-Frias, 1996; Migliazza et al., 1999). However, several clinical case studies of infants with both defects have been published (McCredie and Reid, 1978; Gershoni-Baruch et al., 1990; Lerone et al., 1992; Bird et al., 1994; Winter, 1996; Wallerstein et al., 1997; Herman and Siegel, 2001; Marino et al., 2002).

The etiology of CDH is poorly understood, and both genetic and environmental factors have been proposed as the underlying cause. Experimental studies of genes involved in the development of both the diaphragm and organs whose defect co-occurs with CDH have been reported. However, these genes act at different times in development and may induce defects of varying severity. Similarly, the timing of an environmental insult may determine the type of organ that is co-affected and the severity of the defect. Our descriptive study examines the type and spectrum of severity of LRDs associated with CDH in infants and stillbirths by using two databases: one from a tertiary hospital, which includes only liveborn infants with CDH, and the other from a birth defects registry, which includes both livebirths and stillbirths. The two populations were specifically chosen to represent the full spectrum of the association. Because the hospital group comes from a tertiary facility that specializes in the care of patients who are candidates for surgery, and includes a team of geneticists, dysmorphologists, and surgeons, we expected that the quality of the data would be very high, particularly with reference to minor defects. However, stillbirths and infants who are expected to die shortly after birth are usually not transferred to this hospital. Those cases are better described in the registry data, as they usually have complete reports from a pathologist and/or other medical specialists. Thus we chose high-quality data from both registries for our descriptive study of the association of CDH with limb defects. We also reviewed the findings in experimental studies, both genetic and environmental, that have induced similar combinations of defects, as a basis for further hypotheses to be tested in human populations.

MATERIALS AND METHODS

We analyzed the records of 146 infants with a posterolateral CDH who had been admitted consecutively to the Erasmus Medical Center, Sophia Children's Hospital, Rotterdam, in 1986-1998, and the records of 810 liveborn and 36 stillborn infants with CDH who were born in 1983-1995 in the area of ascertainment of the California Birth Defects Monitoring Program (CBDMP). We excluded prenatally diagnosed fetuses that had been terminated. We also excluded conjoined twins and patients with amniotic band syndrome, because these deformations have an embryologically different etiology and are not considered in epidemiological studies as being of the Bochdalek type.
The CBDMP keeps a record of all infants and stillbirths with structural birth defects in all birth and tertiary hospitals in a large area of California. Data collection specialists abstract the medical records of infants up to 1 year of age from obstetrical, surgical, pediatric, and pathological logs. They also collect data from genetic labs that serve these hospitals. All data on the same child are collated, coded, and quality controlled. For each diagnosis, the method of evaluation and the specialty of the physician are recorded. The final diagnosis is that of the most specialized physician who has attended the child, which is usually the surgeon or the pathologist. Geneticists are consulted to define syndromes, whenever possible. Chromosomal analysis is routinely performed when a chromosomal defect is suspected.

The Sophia Children's Hospital is a level III perinatal and pediatric surgical center serving as a referral facility for the southwestern part of The Netherlands, which has 3-4 million inhabitants and an annual birth rate of 35,000. A hospital-based database was created to register patients with congenital defects (particularly diaphragmatic hernia), with a focus on associated birth defects, genetic information, obstetrical history, and surgical procedures. For almost every CDH patient, with or without associated anomalies, a clinical geneticist with experience in dysmorphology was consulted throughout the whole study period. However, not all of the infants who died shortly after arriving at the hospital, or who were edematous from being on extracorporeal membrane oxygenation (ECMO), had blood drawn to determine their karyotype. Moreover, minor defects that sometimes identify a syndrome are not as apparent in an infant with edema and may be missed, although these cases are very rare. However, stillbirths and severe cases that had been diagnosed prenatally and terminated were not included in this group, as many did not reach the hospital. Therefore, we used the California population data to analyze severe cases.

We reviewed all CDH cases with a limb defect in both groups, and recorded the sex, birth status, and survival of the infant, the side of the diaphragmatic defect, and the location (ipsilateral, contralateral, or bilateral) and severity of the limb defects.

Chromosomal defects, malformation syndromes, and the presence of additional congenital anomalies were also recorded. First, a database search was performed in the registry group for all Bochdalek hernia cases with a limb defect corresponding to British Pediatric Association (BPA) codes 755.2-755.9. In the hospital group, we selected all infants who had a CDH and an LRD.

RESULTS

Table 1 presents the distribution of infants with CDH in the hospital and registry groups, according to birth status, type of associations, and gender of the infant. All cases in the hospital group were liveborn, but 36 cases (4%) in the registry group were stillborn. In both populations, males predominated slightly (55% and 60% in the hospital and registry groups, respectively). Chromosomal abnormalities and specific malformation syndromes were diagnosed in 9% of the hospital group and in 8% of the population group. In each population the majority of cases had an isolated CDH (i.e., a Bochdalek hernia without any additional major malformation), but the proportion was higher among the hospital group (71% vs. 52%, respectively). All of the subjects had lung hypoplasia, with different degrees of severity, as is expected in CDH.

In the hospital group, 14 CDH patients (11%) had a limb defect; five of these patients (3% of the total group) had an LRD (mostly nonsevere), and none had a defect with a BPA code of 755.2-755.4. In the registry group, 162 CDH patients (19%) had a limb defect, and 18 of these patients (2%) had a severe LRD (cases involving only minor limb defects are not described in this study).

Table 1 Distribution of infants with CDH in the hospital group and in the registry group, according to birth status, type of defect, and sex

	Hospital Group (n = 146) (1986-1998)		Registry Group (n = 846) (1983-1995)	
Case characteristic	Number	%	Number	%
Birth Status				
Live born	146	100	810	96
Stillborn	0	0	36	4
Type of defect				
Chromosome abnormalities/recognized syndrome	13	9	66	8
Multiple congenital cnomalies[a]	29	20	337	40
Isolated CDH	104	71	443	52
Sex				
Male	81	55	504	60
Female	65	45	342	40

[a] *One or more additional major malformations*

Table 2 Distribution of cases of CDH (Bochdalek) with limb reduction defects among 146 hospital and 846 registry cases, according to birth status, type of defect, and sex

Case characteristic	Hospital group n = 5		Registry Group n = 18	
	n	%	n	%
Birth Status				
Live born	5	100	16	89
Mortality	3	60	16	100
Stillborn	0	0	2	11
Type				
Recognized Syndrome	1	20	2	11
Chromosome abnormality	2	40	3	17
MCA[a]	5	100	18	100
Sex				
Male	3	60	11	61
Female	2	40	7	39

[a]*Multiple Congenital Anomalies: all cases studied had more than one major malformation in addition to CDH and LRD.*

Table 2 presents cases of both CDH and LRD. In both study groups the mortality rate was high: 100% in the registry group, and 60% in the hospital group. Additional major anomalies were present in all cases in both groups. The sex ratio was similar in both the hospital and registry groups (60 and 61%, respectively).

Registry cases involving both CDH and LRD are described in detail in Table 3. This table lists the type and laterality of the diaphragmatic defect and LRD, birth status, karyotype and/or syndrome, and associated anomalies, such as renal and cardiac malformations. The LRDs include the absence of entire limbs or parts of limbs, such as amelia and phocomelia (the absence of the proximal portion of a limb or limbs, the hands or feet being attached to the trunk by a small, irregular-shaped bone), and absent radius, fibula, hand, thumbs, or other digits. All infants had additional anomalies, some of which were severe (particularly heart defects and kidney defects), and all of the liveborn infants died. Two infants had trisomy 18, and one infant had an abnormality of chromosome 9 that was not clearly specified in the infant's abstract. Chromosome analysis was not performed on five of the infants. Two of the infants had a right-sided hernia, two had a bilateral defect, and the others all had left-sided hernias.

Table 4 lists cases in the hospital group involving both CDH and LRD, all of which were nonsevere. Three of five infants had a left-sided hernia. Two infants had a trisomy 18, and the other patients had a normal karyotype. Three of five infants had a renal defect, and three had a heart defect. The majority of patients with left-sided CDH had an ipsilateral or bilateral LRD of the upper limbs. However, this association was more obvious in the registry group.

Discussion

Our purpose in this descriptive study was to evaluate the full spectrum of CDH-associated LRDs–the severe and the nonsevere–using two different populations with the highest quality of ascertainment of defects. Our purpose was not to estimate the population frequency of those associations. A corollary is that the cases differed in many ways between these two populations.

In the hospital group we found more isolated cases than in the registry group (70% and 51%, respectively) (Table 1). This is probably the result of the selection of infants, as those infants with severe additional defects often died before reaching the hospital, and prenatally diagnosed fetuses were often electively terminated.

In both the hospital and registry groups, a proportion of infants with CDH of the Bochdalek type had a structural limb defect, of which a sizeable proportion had an LRD (Table 2). The two different populations were used to evaluate the association of CDH with LRD because each population had a different advantage. To obtain the full spectrum of major and minor LRDs, we evaluated all cases with an LRD from the hospital group, and all cases with an LRD included in BPA codes 755.2-755.9 from the registry group. The mortality rate of the respective cases reflects these selection criteria, being much higher in the registry group than in the hospital group (Table 2).

Chromosomal analysis was less likely to have been performed for the registry group of cases than for the hospital group, because karyotypes are seldom ordered for stillbirths or for infants that died shortly after birth (Tables 1 and 2). In both groups, the proportion of males was higher than that of females (Table 1), as previously reported for Bochdalek defects (Torfs et al., 1992; Tibboel and Gaag, 1996). Furthermore, the proportion of males with severe LRD was also substantially higher than that of females (Table 2). Limb defects occurred more often on the ipsilateral side of the hernia and in the upper limb (Tables 2 and 3), particularly among the more severe cases (77.7%) in the registry group. However, bilateral defects and a few contralateral cases were also noted. This reflects the fact that lung defects, which are usually ipsilateral to the hernia, may also be contralateral.

The association between CDH (Bochdalek) and limb defects has been reported in previous epidemiological studies of diaphragmatic hernia (Torfs et al., 1992), LRDs (Froster- Iskenius and Baird, 1989), and amelia (Froster-Iskenius and Baird, 1990). It has also been described in several case reports (McCredie and Reid, 1978; Gershoni-Baruch et al., 1990; Lerone et al., 1992; Bird et al., 1994; Martinez-Frias, 1996; Winter, 1996; Herman and Siegel, 2001). The presence of some very rare LRDs, such as amelia, phocomelia, and absent limb bones, in conjunction with CDH and lung hypoplasia, is of particular interest because it suggests a developmental relation between the diaphragm, the lung, and the limb. Below we present several arguments in support of this idea.

Table 3 CDH and limb reduction defects in cases from the Registry Group

No	Live born	Sex	Side CDH	Limb defect	Side limb defect	Karyotype Syndrome	Additional defects	Cardiac defects	Renal defects
1	Yes, died	M	R	Upper limb reduction defect* Spade like hand Club feet	Right Bilateral	46,XY	Bladder hypoplasia, Imperforate anus, Vertebral/rib-anomalies, Duodenal atresia, Tracheo-esophageal fistula	Right-sided aortic arch	Cystic kidneys bilateral
2	Yes, died	F	L	Phocomelia[b] , arm Absent digits, arm	Left Bilateral	Unknown	Absent gonads, Double vagina, Bicornate uterus, Macrocephaly, Congenital arthrogryposis multiplex	No	No
3	Yes, died	F	L	Absent fingers Claw-like hands Micromelia	Left Bilateral	46,XX. Cornelia de Lange	Face/ rib anomalies	No	Glomerulo-cystic kidney
4	Yes, died	F	L	Short radius Absent thumb Cleft digit 2-3, hands Nail hypoplasia Thumb, proximal	Left Left Right	46,XX	No	ASD	Ectopic kidney
5	Yes, died	F	L	Short thumbs Club feet	Bilateral	Unknown	Face anomalies, Hypoplasia cerebellum	VSD	Horseshoe-kidney
6	Yes, died	M	R	Phocomelia** arm Syndactyly, hands	Right Bilateral	46,XY	Imperforate anus, Hypoplasia right ear, Cryptorchidism right	No	No
7	Yes, died	M	L	Absent humerus 4 digits, hands	Left Left	Unknown	Cryptorchidism right	No	No
8	Yes, died	M	L	Hypoplasia lower limb Short great toe Syndactyly, feet	Bilateral Bilateral	47,XY, +18	Face/rib anomalies, Cryptorchidism	VSD, Transposition of great vessels	No
9	Yes, died	F	NOS	Hypoplastic thumb Short upper & lower extremities	Bilateral Bilateral	46,XX, –9, +9p+	Cleft palate, Face anomalies, Small labia minor, Hypoplastic ribs, Hydrocephalus	VSD, Hypoplastic right heart, Tricuspid atresia	No
10	Yes, died	M	L	Absent fingers	Bilateral	Cornelia de Lange	Face anomalies, Micropenis, Hypospadias	No	No
11	No	M	LR Agenesis	Absent thumb	Right	Unknown	Craniorachischisis, Pituitary/adrenal atresia, Cryptorchidism	VSD, Hypoplastic LA, Mitral atresia	Horseshoe kidneys

No	Live born	Sex	Side CDH	Limb defect	Side limb defect	Karyotype Syndrome	Additional defects	Cardiac defects	Renal defects
12	Yes, died	M	L	Short arm Hypoplastic fingers, nails	Left Bilateral	46,XY Rubella syndrome	Face anomalies	Transposition of great vessels	Horseshoe kidney
13	No	M	L	Short leg Club foot	Bilateral Left	Unknown	Cleft palate, Hypoplasia midface, Accessory spleen	No	Agenesis left kidney
14	Yes, died	M	L	Absent radius Slender radius Short forearms	Right Left Bilateral	46,XY	Cerebrum anomalies, Tracheo-esophageal fistula, Anal atresia, Vertebral anomalies, Congenital arthrogryposis multiplex, Rudimentary scrotum	AVSD	No
15	Yes, died	F	LR	Absent fibula Hypoplastic radius Absent radius	Right Right Left	46,XY	Abnormal spine, Hypoplastic optical nerve, Partial bicornate uterus, Hypoplastic thymus	Anomaly of great veins	Horseshoe-kidney
16	Yes, died	M	L	Absent thumb Syndactyly fingers Syndactyly foot dig2-3	Right Right Right	46,XY	Micropenis, Cryptorchidism right, Hydrocephalus, Face anomalies, Cloudy cornea left.	No	No
17	Yes, died	M	L	Amelia arm Dysplastic foot	Right Right	46,XY	Absent external genitals, Anal atresia, Spine/sacral anomaly	VSD	Absent kidneys/ureters
18	Yes, died	F	L	Foot, missing digit Contractures, hands Camptodactyly hands Foot syndactyly 1-4	Left Bilateral Bilateral Right	47,XX, +18	Bilateral cleft lip/palate, Microcephaly, Face anomalies, Congenital arthrogryposis multiplex	Single ventricle plus AV valve, Interruption of aortic arch	No

**The abstract carried the code for a limb reduction defect, but did not describe the defect.*
***Absence of the proximal portion of the limb, the hand being attached to the trunk by a small irregularly shaped bone.*

Table 4 CDH and limb (reduction) defects in the hospital group, according to birth status, sex, type and laterality of limb defect, and associated anomalies.

No	Live born	Sex	Side CDH	Limb defect	Side limb, defect	Karyotype/ Syndrome	Additional defects	Cardiac defects	Renal defects
1	Yes, died	M	R	Hypoplastic metacarpals Contractures hands Ulnar deviation	Bilateral Bilateral Bilateral	47,XY, +18	Face anomalies, Micropenis, Epispadia	VSD, Aneurysm atrial septum	Hydronephrosis left
2	Yes	M	R	Short upper arms Brachymetacarpia digit 1 Brachymesophalangy digit 5	Bilateral Bilateral Bilateral	46,XY, Cornelia de Lange	Pyloric stenosis, Face anomalies, Growth/mental retardation, Cryptorchidism	No	No
3	Yes	M	L	Radial deviation hand Brachymetacarpia digit 1 Brachymesophalangy digit 5	Left Bilateral Bilateral	46,XY	Hypertelorism, Vertebral anomalies	No	Agenesis left kidney
4	Yes, died	F	L	Brachydactyly, hands Hypoplastic metacarpals/ fingers/toes Syndactyly foot digits 2-3	Bilateral Bilateral Left	46,XX	Micrognathia, Bicornate uterus	Aberrant aorta, VSD	Horseshoe-kidney left
5	Yes, died	F	L	Camptodactyly Short digits 1-2, hands Hypoplastic toenails	Bilateral Bilateral Right	47,XX, +18	Eye anomalies	ASD/VSD	No

First, in human embryology there is a time overlap between the sensitive period of limb development (days 24-36 of gestation) and the period of diaphragm development (days 28-48 of gestation). The diaphragm and upper limbs derive from somites adjacent to the cervical neural crest. According to McCredie and Reid (1978), an insult to the neural crest anlage could induce an adverse development in the contiguous anlages that are dependent on it for their innervation. Although the timing of these developments correlates with this theory, no experimental work that we know of has tested it.

Second, from human and animal studies it is known that several genes are shared in the development of both organ systems. Knockout mice for different combinations of retinoic acid receptors (RARs) have either limb or diaphragmatic defects. RAR–2 double mutants have both a diaphragmatic hernia and lung malformation, while RAR– double mutants have only limb defects (Lohnes et al., 1994, 1995; Mendelsohn et al., 1994). Another gene–the Wilms' tumour 1 gene (WT1)–is required for diaphragm, limb, and urogenital development, as seen in the WT1-knockout mouse (Moore et al., 1998). This association was reported in a patient with the Denys-Drash syndrome who had a diaphragmatic hernia, limb and renal defects, and a mutation of the WT1 gene, but no tumor (Devriendt et al., 1995). Interestingly, about 50% of the severe CDH cases in the registry group also had a kidney defect, such as renal agenesis, horseshoe kidneys, ectopic kidney, and hydronephrosis (Table 3). This suggests that renal development may also be dependent on genes involved in diaphragm and limb development. Again, most of these renal defects were not ipsilateral to the hernia. Also, in knockout mice for the C-met receptor gene, myogenic precursor cells do not migrate into the diaphragm and the limb bud (Bladt et al., 1995). Although both organs are affected, the amuscular diaphragm forms fully (Babiuk and Greer, 2002); therefore, this gene defect may not be a good model for studying the Bochdalek hernia.

Based on suggestions in earlier studies by Iritani (1984), Keijzer et al. (2000) recently presented a "dual-hit" hypothesis, which proposes that in the nitrofen model, lung hypoplasia precedes the diaphragmatic defect and is subsequently aggravated by the herniation of organs in the chest cavity. One must then also consider genes that are important for both limb and lung development. Integrines 3a and 3b are developmental genes for both organs (De Arcangelis et al., 1999). Mice that are double-knockout for both integrine genes have limb abnormalities and bilateral lung hypoplasia; however, diaphragm defects have not been reported in these mice. Additionally, knockout mice for fibroblast growth factor 10 (FGF 10) die at birth from lack of lung development, and also have limb growth abnormalities (Sekine et al., 1999). FGF receptor 2 (Arman et al., 1999) is also required for limb development and lungbranching morphogenesis. However, Babiuk and Greer (2002) showed that the diaphragm can form normally in the absence of lung tissue, and furthermore that diaphragmatic defects can be induced in a nitrofen-treated FGF10 –/– mouse.

In summary, although many developmental genes have been shown in animals to be

important for both limb and diaphragm formation, it is not yet clear whether those genes are responsible for the combination of a Bochdalek hernia and an LRD in humans. It can be speculated that severe LRDs are more likely than minor limb defects to be caused by abnormal expression of genes acting early in development, and that a different set of genes may be associated with minor limb defects, as seen in Fryns syndrome.

Third, the co-occurrence of CDH and LRD is seen in several monogenic syndromes, including Fryns syndrome (Fryns et al., 1979) and Brachmann de Lange syndrome (Brachmann, 1916; Cunniff et al., 1993). A numeric chromosomal disorder associated with CDH is the Pallister- Killian syndrome, which is tetrasomy 12p (Pallister et al., 1977; Rodriguez et al., 1994). In our study, the majority of the CDH patients who had a severe LRD either had a normal karyotype or had not undergone chromosomal analysis. Skin fibroblast cultures can detect tetrasomy 12p; however, they are seldom performed and were not reported in our study. The results of recent experimental studies with developmental genes, and the association of certain syndromes with specific mutations warrant DNA analyses of patients with LRD and CDH.

Finally, it is also important to consider teratogens as a cause of both CDH and LRD. This has been reported in animal studies, but not in studies of human pregnancies. One teratogen, cadmium, when given to rats results in a combination of LRD, CDH, and renal defects (Barr, 1973). Another teratogen, the herbicide nitrofen (2,4-dichlorophenyl- p-nitrophenyl ether), when administered to the pregnant rat on days 9–11 of gestation, induces diaphragmatic defects of the Bochdalek type and lung hypoplasia in the offspring (Costlow and Manson, 1981; Kluth et al., 1990; Tenbrinck et al., 1990; Allan and Greer, 1997). Although other vertebral and rib abnormalities have been observed, no LRDs have been reported in conjunction with diaphragmatic hernia in this model (Migliazza et al., 1999). However, the timing and dosages of the teratogen have been limited. Vitamin A, co-administered to the nitrofen-treated rats, prevents the development of CDH to some extent (Thebaud et al., 1999). In various animals, maternal vitamin A deficiency causes abnormalities in development of the diaphragm (Andersen, 1941, 1949; Warkany et al., 1948; Wilson et al., 1953). Extreme deficiency in vitamin A also results in defects of the eye, lung, and cardiovascular and urogenital systems, as well as in forelimb reduction defects (Wilson et al., 1953; Morriss-Kay and Sokolova, 1996). Vitamin A is transformed in the placenta into retinoic acid by retinaldehyde dehydrogenase 2. Mouse embryos deficient in this enzyme have highly reduced forelimbs, but apparently normal hindlimbs (Niederreither et al., 2002). Moreover, a lower than normal retinol binding protein (RBP) level in umbilical cord blood of CDH infants has been observed, suggesting the involvement of vitamin A or its binding protein in the embryogenesis of CDH (Major et al., 1998). However, no similarly collected data are available from other research centers. To our knowledge, no recognized teratogen has been associated with CDH in humans.

Table 5 Genes and teratogens associated with CDH, lung defects, and/or LRD defects in animal studies

	Diaphragm defects/ lung defects[a]	Limb defects
Genes		
Retinoic Acid Receptors $\alpha\beta 2$[b]	yes	no
Retinoic Acid Receptors $\alpha\gamma$[b]	no	yes
Wilms' Tumour 1 gene	yes	yes
C-met-encoded receptor tyrosine kinase	yes	yes
Integrins 3 $\alpha\beta$	yes	yes
Fibroblast Growth Factor 10	yes	yes
Fibroblast Growth Factor Receptor 2	yes	yes
Retinaldehyde Dehydrogenase 2[b]	unknown	yes
Retinol Binding Protein	unknown	yes
Teratogens		
Cadmium	yes	yes
Nitrofen	yes	yes
Vitamin A deficiency	yes	yes

[a] *Unknown means that the phenotype of the diaphragm was not described in those studies.*
[b] *Double knockout mice*

In our descriptive study we have presented the range of CDH-LRD combinations, and speculated about a few ideas. The association between the limb and the diaphragm may depend on several predisposing genes or exogenous factors, as summarized in Table 5. There may, of course, be more. Severe LRDs must result from a very early developmental insult to the anlage that will give rise to limbs through a cascade of signals mediated by different enzymes and genes. At each point in the pathway, variations can occur. Sometimes the insult occurs early and also involves the diaphragm, whereas sometimes it involves only one of the anlages. The fact that the defects occur together more often than expected is an indication that, in some cases, both anlages can be affected at the same embryological time or by the same factor. Many syndromes (e.g., Cornelia de Lange) and sequences (e.g., VACTERL) have variable phenotypic expression. What induces this variability? And why is the combination of limb and CDH occasionally seen in trisomies 13 and 18, but never (to our knowledge) in the most common syndrome, namely Down syndrome? We know from experimental animal studies that there are several teratogens that can induce a CDH. Many of these teratogens can also induce either severe or mild limb defects, yet some exposed cases do not have either or both defects. Several teratogens affect the diaphragm and the kidney, but not the limb. The fact that both humans and animals exhibit similar combinations is what makes the comparison between species interesting, as it suggests similar pathogeneses. Consequently, laboratory research is being conducted to determine the various pathways that result in these different combinations of defects, at both the genetic and the environmental level. In conclusion, our epidemiological study confirms in a large sample the co-occurrence of CDH and LRD,

and suggests both genetic and environmental etiologies. Based on animal models, genetic analysis of candidate genes from patients with CDH and LRD may in the future elucidate the developmental relation between the diaphragm and limbs in humans.

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CHAPTER 5

THE CO-OCCURRENCE OF CONGENITAL DIAPHRAGMATIC HERNIA (CDH), ESOPHAGEAL ATRESIA (EA) / TRACHEOESOPHAGEAL FISTULA (TEF) AND LUNG HYPOPLASIA (LH)

The Co-Occurrence of Congenital Diaphragmatic Hernia (CDH), Esophageal Atresia (EA) / Tracheoesophageal Fistula (TEF) and Lung Hypoplasia (LH)
Marieke van Dooren and Claudine Torfs

Submitted

ABSTRACT

Two severe birth defects, esophageal atresia (EA) with or without tracheoesophageal fistula (TEF) and congenital diaphragmatic hernia (CDH) have traditionally been analyzed separately in epidemiological studies. Lung hypoplasia (LH), part of the CDH spectrum, is not usually associated with EA/TEF, yet both are foregut malformations. We conducted an epidemiological study of two combinations of the defects. In the population of 3,318,966 live births and stillbirths monitored from 1983 to 1996 by the California Birth Defects Monitoring Program, 433 cases had a Bochdalek type CDH (0.13 per 1,000 births), 893 had EA/TEF (0.27 per 1,000 births), and 646 had LH (0.19 per 1,000 births). Among them, 18 cases had CDH with EA/TEF (0.01 per 1,000 births), and 53 had EA/TEF and LH (0.02 per 1000 births); both prevalences are significantly higher than expected. Only one of 17 infants with CDH and EA/TEF, and six of 40 infants with EA/TEF and LH, survived. Of infants who died, 72% and 74% respectively had an autopsy. The male to female sex ratios were 1.25 and 1.13 respectively. Most infants had additional severe defects whose proportions were similar in both groups, except for kidney defects and recognized syndromes which were higher in the EA/TEF with LH group. To evaluate the co-occurrence of the defects, we reviewed studies, mostly from experimental animal models, of genetic and environmental factors reported to affect one or more of the defects. Future studies should include storage of patients' biological materials for DNA analysis, karyotyping, and environmental exposure evaluation.

INTRODUCTION

Two severe birth defects, esophageal atresia (EA) with or without tracheoesophageal fistula (TEF) and congenital diaphragmatic hernia (CDH) have traditionally been analyzed separately in epidemiological studies. A variable amount of lung hypoplasia (LH) is considered to be one of the key features of CDH, but has only occasionally been reported with EA/TEF. However, in the past few years, the results of experimental studies in molecular biology have suggested that some common early embryological pathways may be shared by all three defects (Litingtung et al. 1998). Both the trachea and the esophagus develop from a bud of the ventral endoderm of the foregut, as does the septum that separates them and whose malformation leads to an EA/TEF. Several researchers now think that some cases of CDH may be a consequence of the faulty development of the branchial airways that results in bilateral lung hypoplasia (Iritani 1984; Keijzer et al. 2000), which is usually more prominent on the side of the thoracic cavity where abdominal organs have penetrated. However, lung hypoplasia without CDH has been reported in one animal model, the knockout FGF9 -/- mouse (Colvin et al. 2001). In human cases of CDH, both defects co-occur with great predictability.

Experimental animal studies have also reported that mutations of several genes, such as Gli2 in combination with Gli3 (Motoyama et al. 1998), Sonic Hedgehog (Shh) (Litingtung et al. 1998), and Retinoic Acid Receptor –beta (RAR-beta) (Mendelsohn et al. 1994) can result in foregut malformations, including EA/TEF and LH. Other animal studies have shown that several environmental risk factors, such as Nitrofen (Ambrose et al. 1971a), vitamin A deficiency (Wilson et al. 1953) and cadmium (Barr 1973) can disrupt fetal development and cause CDH and LH, although their effect on the development of EA/TEF has not been reported. In contrast, adriamycin, an antibiotic and chemotherapeutical drug, can cause EA/TEF without LH in animals (Diez-Pardo et al. 1996).

These observations suggest that one might find a larger than expected number of infants with a combination of these defects among the birth population. However, there are no epidemiological studies of these combinations, although there are a few case-reports of CDH with TEF that have been reviewed by Thakral (Thakral and Sajwani 1998).
As these defects must originate early in embryogenesis and involve major organs, one would expect additional defects in a majority of cases, and a high neonatal mortality or stillbirth rate. In a large birth population we reviewed all cases with two combinations of these defects, either EA/TEF with LH, or CDH with EA/TEF and LH.

Materials and Methods

Description of the population and methods of ascertainment

Cases were ascertained from the population of 3,318,966 live births and stillbirths monitored by the California Birth Defects Monitoring Program from 1983 to 1996. For this registry, specially trained data collection specialists examine all hospital logs, including pathology logs, from birthing hospitals and from tertiary hospitals where infants may have been sent for further evaluation or for intervention. They also examine the records of genetic labs that service those hospitals. The specialists then abstract the charts or pathology reports of every infant or stillbirths with a structural birth defect, and record the specialty of the physician who made the diagnosis, and the procedures used to make the diagnosis. Only confirmed diagnoses are retained and classified, according to the modified list of the British Pediatric Association (BPA 6). Ascertainment continues until the infant is one year of age or has died. All abstracts for the same child are collated. For each defect, the diagnosis of the most specialized physician was chosen as the most accurate for this study.

Definition of cases

CDH diagnoses include the Bochdalek type hernia, unilateral or bilateral. They do not include eventration of the diaphragm or the Morgagni type of CDH because these two forms are not thought to have the same pathogenesis. EA/TEF diagnoses include all cases of esophageal atresia with or without fistula, esophageal stenosis and esophageal web. All cases with a diagnosis of LH are included, but LH is sometimes not reported separately in cases of a Bochdalek type CDH as it is assumed to be present. We shall make the same assumption in our analyses.

RESULTS

Among the 3,318,966 live births and stillbirths in the surveillance area of the CBDMP, there were 433 cases with a Bochdalek type CDH, for a prevalence rate of 0.13 per 10,000 births, 893 cases with a EA/TEF, for a prevalence rate of 0.27 per 1,000 births, and 646 cases with lung hypoplasia, for a prevalence rate of 0.19 per 1,000 births (Table 1).The prevalence rate of the combination of CDH with EA/TEF is 0.01 per 1,000 births and that of EA/TEF with LH is 0.02 per 1,000 births. The prevalence of either combination is significantly higher than expected. ($p<0.4\ 10^{-7}$ and $p<0.5\ 10^{-7}$, respectively). The co-occurrence of EA/TEF and LH is three times as prevalent at birth as that of CDH and EA/TEF.

Table 2 shows the infants' birth characteristics. Of 17 cases with CDH and EA/TEF, 16 were live births and one was a stillbirth; every infant but one died shortly after birth. The male to female sex ratio was 1.48. Of the 53 cases with EA/TEF and LH, 40 were live births and 34 of them died. The sex ratio was 1.13. A relatively high proportion of infants who died (72% and 74% respectively) had an autopsy. There were no particular maternal race or age differences, but numbers in each category were small.

Table 1 Birth rates of infants with a Bochdalek CDH, EA/TEF, LH and of the combination of these defects, in a California population of 3,318,966 births, from 1983 to 1996

Defects	n	Rate/1,000 births	95% CI
Congenital diaphragmatic hernia	431	0.13	0.10-0.18
Esophageal atresia or stenosis with or without fistula	893	0.27	0.25-0.29
Lung hypoplasia	646	0.19	0.19-0.21
Congenital diaphragmatic hernia and tracheoesophageal atresia	17	0.005	0.00-0.01
Lung hypoplasia and tracheoesophageal atresia	53	0.02	0.01-0.02

Table 2 Birth characteristics per combination of defects: EA/TEF + DH +LH and EA/TEF + LH

	EA/TEF + DH + LH (n = 17)		EA/TEF + LH (n = 53)	
Characteristics	**n**	**%**	**n**	**%**
Sex, male	10	59	27	51
female	7	41	24	45
Ambiguous/unknown	0	0	1/1?	4
Live birth	16	94	40	75
Live birth, died	15	88	34	64
Stillbirth	1	6	13	25
Autopsy	11	64	39	74

One set of conjoined twin with multiple severe malformations was not included

Table 3 shows the distribution of associated anomalies, for each combination of defects. Most infants and stillbirths had additional severe defects whose proportions were very similar in the two groups. An equal proportion (11%) of cases with each combination of defects had a trisomy, all of which were trisomy 18, except for one trisomy 21 among the EA/TEF with LH cases.

Table 3 Associated anomalies per combination of defects: EA/TEF + DH +LH and EA/TEF + LH

	EA/TEF + DH + LH (n = 17)		EA/TEF + LH (n = 53)	
Anomalies	**n**	**%**	**n**	**%**
Trisomy	2	11	6	11
Syndrome/sequence	2	12	18	34
Heart defect	13	76	39	76
Limb defect	5	29	18	34
Kidney	6	35	28	53
Lung hypoplasia	16	100*	53	100
Anal defect	6	35	27	51
Genital defect	8	47	35	66
Orofacial clefts	2	12	8	15
Vertebral or ribs	13	76	30	57
CNS	6	35	22	41
Urinary tract	3	18	21	40
Digestive tract	9	53	22	41

** In the abstract of one infant, a Bochdalek type congenital diaphragmatic hernia was reported without a mention of the lung hypoplasia, which has been assumed*

However, the proportions of recognized or diagnosed syndromes were much higher in the EA/TEF with LH group than in the CDH with EA/TEF and LH group. In the latter group, several cases presented with the multiple anomalies that are included in the VACTERL association. Congenital heart defects were the most common defects in both groups, 76% in each group.

Of interest to our study is the relatively high proportion of infants (35% and 53% respectively) with kidney defects, which included unilateral or bilateral kidney agenesis, horseshoe kidney, ectopic and cystic kidneys.

Discussion

Embryological studies and experimental animal studies have reported gene mutations and environmental factors that affect the development of organs derived from the endoderm of the foregut. Affected organs include the branchial airways and the esophagus, and the defects include EA/TEF and LH. A few of those factors also affect the diaphragm and result in CDH. If these defects share some common pathogenic factors, their co-occurrence should be higher than expected from their individual occurrence.
In a large population of births we evaluated the co-occurrence of two combinations of these defects, namely, EA/TEF with LH, and CDH with EA/TEF and LH. Our epidemiological data (Table 1) clearly show that the combinations of the defects occur significantly more often than expected from their individual prevalence in the population. To our knowledge, this is the first epidemiological study of the combination of these defects, although one epidemiological study of EA/TEF reported 2.7% of cases with a CDH (Torfs et al. 1995), and a study of CDH reported 2.2% of cases with EA/TEF (Torfs et al. 1992).

Because these defects of early embryological development result in a high mortality rate, both in utero and after birth (Table 2), it is possible that these combinations of defects are missed in many clinical situations unless the infants or fetuses have had an autopsy or intensive treatment after birth and before death.
There are only about 20 individual case reports of EA/TEF with CDH in the literature (Ahmed 1970; Gibon et al. 1978; Bowen 1983; Rawlings et al. 1984; Udassin 1987; Takehara 1993; Sapin et al. 1996; al-Salem et al. 1997; Thakral and Sajwani 1998). The need for a very large population of monitored births to ascertain such rare cases is the probable reason for the lack of epidemiological studies of the combinations of these defects.
Both CDH with EA/TEF cases and EA/TEF/LH cases in our study had additional defects of other organs (Table 3). It is known that approximately 40 % of cases of CDH (Torfs et al. 1992; Tibboel and Gaag 1996a; Kaiser and Rosenfeld 1999) and of EA/TEF (Torfs et al. 1995; McMullen et al. 1996) have additional malformations. For EA/ TEF, there is a well known association, VACTERL that involves many organ systems, including renal defects, but not LH; no genetic or environmental factors have been established at this time.

Except for one case with a trisomy 21 in the group with EA/TEF with LH, trisomy 18 is the only trisomy that was reported for both combinations of defects. The association of trisomy 18 with both EA/TEF and CDH is well known (Jones 1996), as is the association of trisomy 21 with TEF/EA (Kallen et al. 1996; Bianca et al. 2002). The absence of trisomy 21 cases among the CDH with EA/TEF cases is congruent with the lack of association of trisomy 21 with a Bockdalek type CDH (Honore et al. 1993). There is a higher proportion of cases with a syndrome in the EA/TEF/LH group compared to the CDH with EA/TEF group (34% and 17% respectively), in part attributable to the cases with a VACTERL type of sequence. The high proportion of infants with severe kidney defects in both groups in of interest because both the lung and the kidney are branching organs that share several developmental genes (Quaggin et al. 1999; Burrow 2000; Lin et al. 2001; Affolter et al. 2003).This could signify that genes responsible for the branching of both the kidney and the lung are pathogenic for the LH in the EA/TEF cases, and in a proportion of the Bochdalek CDH cases with TEF/EA. In an epidemiological study of CDH, 8% of all cases of Bochdalek type CDH had a kidney defect (Torfs et al.1992). However, these genes will not be causal for LH in those CDH cases that do not have an additional kidney defect.

All three defects are the consequence of a very early embryological insult, the timing of the budding of the endoderm of the foregut into trachea and esophagus. However, the diaphragm does not bud from the endoderm. Then why is there a significantly higher combination of CDH with EA/TEF and LH than expected? There are several hypotheses to explain this occurrence. The first is that the hernia occurs as a result of the lung hypoplasia, as has been suggested by several researchers (Iritani 1984; Keijzer et al. 2000). However, as most cases of CDH with LH do not have other foregut malformations, other possible lung pathogeneses would have to be invoked. Another hypothesis is that the same genetic mutations or environmental factors can affect the three structures independently and in various degrees, but that the LH per se does not produce the CDH. However, it is possible that both mechanisms may operate in different circumstances and that cases of CDH with many additional defects, as seen in this study, are among those resulting from LH defects, whereas isolated CDH does not.
For this reason we reviewed the literature of both experimental animal studies and human studies for possible mechanisms of shared pathogenesis between the three defects.

Animal studies of genes resulting in more than one of the three defects.
Murine knock-out models of transcription factors, signaling molecules, and their receptors indicate a number of putative candidate genes for the pathogenesis of these defects: the fibroblast growth factors fgf-7, fgf-9, and fgf-10, thyroid transcription factor 1(ttf1), bone morphogenetic protein 4 (bmp-4), forkhead 1(foxf1), hepatocyte nuclear factor-3B (hnf-3B), sonic hedgehog (shh), a combination of gli2 and gli3, and some

isoforms of the retinoic receptors rar-α and -β genes (Mendelsohn et al. 1994). These genes regulate foregut development. Shh knockout mice have TEF and hypoplastic lungs (Litingtung et al. 1998; Pepicelli et al. 1998; Warburton and Lee 1999; Warburton et al. 1999). Mice deficient in gli2 have foregut defects, including defects of the esophagus and the lung, and double mutants gli2-/-/gli3-/- do not form an esophagus, trachea, or lung (Motoyama et al. 1998). However they do not have CDH. Recently, it was shown that a knockout mouse for the combined shh and gli-3 genes also had a diaphragmatic defect (oral communication). Fgf-9 and Fgf–10 are important in lung development. However, Fgf-9 -/- mice have lung hypoplasia and an intact diaphragm (Colvin et al. 2001). Double mutants RAR α/β2 have LH, TEF, and a small proportion of them also have CDH, the phenotype depending on the strain of mice (Mendelsohn et al. 1994). In summary, recent animal models suggest several genes that may affect all three organs, but no human studies of those genes are presently available.

Human Chromosomal anomalies associated with the defects.

CDH can occur with several numerical and structural chromosomal anomalies, most commonly trisomy 18 and 13, or monosomy 9p and 4p (Cunniff et al. 1990; Howe et al. 1996; Tibboel and Gaag 1996b; Hilfiker et al. 1998; Witters et al. 2001), as recently reviewed by Lurie (Lurie 2003). However, with the exception of trisomy 13 and 18, none of the phenotypes of CDH cases with a chromosomal anomaly have included TEF or EA, although they have included many other structural defects. Familial cases are very rare and are mostly sib recurrences, notably for Fryns syndrome (Langer et al. 1994), or found in consanguineous families (Donnai and Barrow 1993; Gibbs et al. 1997; Gripp et al. 1997; Mitchell et al. 1997) Parent-to-child recurrence has only sporadically been described (Frey et al. 1991), probably because the successful repair of CDH has only occurred in the past twenty to thirty years. None of those studies mentions EA/TEF as an associated defect.

Genetic studies of infants with EA/TEF have reported autosomal imbalances, among them a locus on 2p23p24 (Celli et al. 2000) and del 17(q22q23). However, none of these studies have reported the co-occurrence with CDH.

It is noteworthy that, except for cases with trisomies 18, there are no genetic studies of infants with the combination of EA/TEF with CDH. Nor do we know of any genetic study of cases of EA/TEF with LH. The fact that those cases are very rare and are either stillborn or die shortly after birth, may explain this lack of data. However, future genetic investigations of such cases will be possible now that new technologies such as array-CGH (Comparative Genome Hybridisation) are available for analyses of various prenatal and postnatal patient materials.

Environmental factors associated with the defects

Several environmental factors are thought to affect the formation of foregut derived organs and of the diaphragm. Nitrofen, a pesticide, is the most studied animal model of CDH: The administration to the pregnant rat of nitrofen will result in a proportion of pups with a CDH and LH (Ambrose et al. 1971b); but TEF has not been reported in this model. The exact mechanism of action of nitrofen is not completely understood. Interactions between nitrofen, vitamin A (Thebaud et al. 1999) and thyroid hormone (Manson 1986) have been proposed. Concomitant administration of vitamin A, thyroxine, and nitrofen to pregnant rats decreases the incidence and severity of nitrofen-induced CDH (Manson et al. 1984; Thebaud et al. 1999; Thebaud et al. 2001). Vitamin A deficiency has been show in experimental animal studies to predispose to CDH and to TEF (Andersen 1941; Wilson et al. 1953). The group of Greer found reduction in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid (Babiuk et al. 2004) and proposed the retinoid hypothesis (Greer et al. 2003). Also, they found that nitrofen inhibits retinal dehydrogenase-2 (RALDH2), expressed in the developing diaphragm, a key enzyme necessary for the production of retinoic acid. However, there is no clear evidence that vitamin A deficiency and thyroid hormone imbalance influence CDH in humans (Bos et al. 1994; Tibboel and Gaag 1996b), although Major found lower retinol and retinol binding protein levels in children with CDH (Major et al. 1998).

Cadmium, a heavy metal, is known to be teratogenic in several animal models. It causes congenital malformations that include CDH, limb defects and defects of the kidneys (Barr 1973). However, no defects of the trachea or esophagus are reported. Cadmium has not been reported as a teratogen in human studies.

Conclusion

Our epidemiological data show that the occurrences of two combinations of defects, CDH/LH with EA/TEF, and EA/TEF with LH, are higher than expected. We evaluated a few possible explanations for this co-occurrence, both genetic and environmental. Future studies of patients with those combinations of defects should include karyotypes, storage of DNA for evaluation of gene mutations, and investigation of possible environmental risk factors.

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CHAPTER 6

CONGENITAL DIAPHRAGMATIC HERNIA: AN EVALUATION OF THE PROGNOSTIC VALUE OF THE LUNG-TO-HEAD RATIO AND OTHER PRENATAL PARAMETERS

Congenital Diaphragmatic Hernia: An Evaluation of the Prognostic Value of the Lung-to-Head Ratio and Other Prenatal Parameters
Laudy JAM, Van Gucht M, Van Dooren MF, Wladimiroff JW, Tibboel D

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ABSTRACT

Objectives

A retrospective analysis of the prognostic significance of the lung-to-head ratio (LHR) and other prenatal parameters on the outcome of fetuses with left-sided congenital diaphragmatic hernia (CDH).

Methods

26 Fetuses with isolated left CDH without chromosomal abnormalities were included. 21 LHR measurements could retrospectively be calculated from the last available ultrasonographic recordings before birth. The relationship between LHR and fetal outcome and the gestational age dependency of this relation was tested. Cut-off levels as previously published were applied to determine their predictive value in this population. The association between other prenatal predictive variables and fetal outcome was also determined. Survival was defined as discharge from the hospital.

Results

The overall survival rate was 50%. There was a statistically significant difference between the mean LHR of the survivors compared to the mean LHR of the non-survivors (1.78 vs 1.02), whereas the mean gestational age of these two groups did not differ. LHR was not gestational age dependent in the prediction of fetal outcome. The cut-off levels LHR < 1, 1-1.4, > 1.4 showed a good applicability in the prediction of fetal outcome within the present study population with a 100% survival if LHR > 1.4 and a 100% mortality if LHR < 1. An intrathoracic position of the stomach, mediastinal shift, polyhydramnios as individual variables and early diagnosis (< 25 weeks' gestation) revealed to be poor sonographic predictors for fetal outcome.

Conclusion

LHR proved to be a good predictor for fetal outcome, independent from gestational age at time of the measurement. To substantiate our observation a prospective multi-center study is warranted.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe and major anomaly with unknown etiology with an incidence of 3.3 in 10,000 births (David and Illingworth, 1976; Torfs et al., 1992). In a recent meta-analysis the median overall mortality was 58% for a fetus diagnosed in utero, 48% if born alive and 33% if the child managed to get to the operating room for surgical repair (Beresford and Shaw, 2000). The most important responsible factors for this mortality rate are pulmonary hypoplasia and therapy resistant

pulmonary hypertension (Molenaar et al., 1991). There is a wide range in severity of pulmonary hypoplasia and parents face difficult decisions following prenatal diagnosis ranging from termination of pregnancy to fetal surgery. In order to improve the clinical care and counseling reliable prenatal parameters predicting fetal outcome in CDH are highly desirable.

Over the years many sonographic parameters have been suggested as prenatal predictors of fetal outcome in CDH such as the presence of a polyhydramnios (Adzick et al.,1985), mediastinal shift (Dommerques et al., 1996), an intrathoracic stomach (Hatch et al., 1992), intrathoracic lobe of the liver (Boodstaylor et al., 1995), visceral herniation (Stringer et al., 1995), underdevelopment of the left heart (Sharland et al., 1992) and quantification of the contralateral lung area (Guibaud et al., 1996). Early diagnosis (< 25 weeks' gestation) has also been suggested as a predictor of fetal outcome (Adzick et al., 1989). The predictive value of these (sonographic) parameters is still a matter of ongoing debate and not sufficiently enough investigated for application in daily clinical practice. (Dommergues et al., 1996; Geary et al., 1998; Witters et al., 2001)

More recently, the lung-to-head ratio (LHR) has been suggested as a predictor for fetal outcome in CDH in three publications, all coming from one institution in San Francisco, California, performing fetal repair (Metkus et al., 1996; Lipshutz et al., 1997; Sbragia et al., 2000). Metkus et al. reported that no fetus with a LHR ratio less than 0.6 mm survived, those with a LHR between 0.6 mm and 1.35 mm had a survival rate of 61% and all fetuses with a LHR higher than 1.35 survived. Lipshutz et al. reported slightly different cut-off levels. In their study no fetuses survived with a LHR below 1.0 mm, those with a LHR between 1.0 and 1.4 mm had a survival rate of 38% and all fetuses with a LHR above 1.4 mm survived. In their most recent paper, in which Sbragia et al. tested the predictive value of LHR measurements in fetuses with an isolated left-sided CDH without liver herniation into the chest, only one cut-off level was used (Sbragia et al. 2000). A LHR below 1.4 mm was predictive for a poor prognosis and a LHR above 1.4 mm was predictive for a good prognosis. In a study from an other center for fetal repair (Philadelphia, Pennsylvania), the cut-off levels of LHR presented by Metkus et al. were used to select a group of fetuses with severe CDH for fetal intervention by tracheal occlusion. From the fetuses with isolated left sided CDH either who did not fit the criteria for fetal surgery or whose mothers chose conventional postnatal management, all fetuses with a LHR below 1.0 died. The survival rate was 56% and 85%, when the LHR was between 1-1.4 or above 1.4 respectively. (Flake et al., 2000)
In this study the significance of the LHR in prenatal prediction of fetal outcome in isolated left-sided CDH has been evaluated retrospectively. We also studied the prognostic value of the intrathoracic position of the stomach and liver, mediastinal shift, polyhydramnios and early diagnosis.

and Sbragia et al. (2000), were applied to determine their predictive value in our population. Finally, the association between the earlier mentioned other prenatal predictive variables and fetal outcome was determined (Chi-square/Fisher's Exact test). A P value < 0.05 was considered statistically significant. Data are expressed as mean ± 1 SD.

RESULTS

The maternal age at delivery varied from 23 to 35 years (median 30 yr), gravidy ranged from 1 to 4 (median 2) and parity from 0 to 3 (median 1). The median gestational age at birth was 38 weeks (range: 34-40 weeks) and the mean birth weight was 2839 ± 436 gram (range: 2200-3800). 54% of the newborn was female. In the present study population overall survival was 50% (13 of 26); death was caused by progressive respiratory failure in all cases, in many cases combined with therapy refractory pulmonary hypertension. Autopsy was performed in only 5 newborns, presenting a mean lung weight / body weight index of 0.005 ± 0.0034 (range 0.003-0.011) which was considered prove for severe pulmonary hypoplasia. Pulmonary hypertension was diagnosed clinically in 8 out of 13 (62%) survivors and in 7 out of 13 (54%) non-survivors according to predetermined objective criteria including repeated cardiac ultrasonography as published before by our group (Shehata et al., 1999). In 4 newborns, objective evaluation of pulmonary hypertension was impossible because they died within one hour after birth. All 26 newborns were high risk patients because they presented with respiratory failure within 6 hours requiring endotracheal intubation and artificial ventilation. ECMO was required in 14 cases (54%), containing 6 out of 13 survivors (46%) and 8 out of 13 non-survivors (62%). Surgical correction of the defect of the diaphragm was performed in all 13 survivors and one non-survivor, according to the concept of delayed repair (Hazebroek et al., 1988).

The LHR could be determined in 21 (81%) of the 26 cases of CDH. In 5 cases the LHR could not be accomplished since the lung area measurements from the video recordings failed due to unfavourable fetal position and poor quality of the recordings. Within these 5 cases there were 2 survivors and 3 non-survivors. From the remaining 21 cases, the mean gestational age at which the last measurement of LHR was performed before birth was 34.8 wk ± 2.1 weeks (range 28-37 weeks). The mean LHR was 1.42 ± 0.57 (range 0.87-2.88). The survival rate in these 21 cases was 52% (11 out of 21). There was a statistically significant difference between the mean LHR of the survivors compared to the mean LHR of the non-survivors (1.78 ± 0.58 vs 1.02 ± 0.14, t-test P = 0.001), whereas the mean gestational age of these two groups did not significantly differ.
(Table 1)

Table 1 LHR of survivors and non-survivors

Outcome (n = 21)	Percentage	Mean LHR* (mm)	SD	Range	Mean GA (wk)	SD	Range	ECMO
Survivors	11 (52%)	1.78	0.58	1.30-2.88	34.04	2.42	28.14-36.29	5 (45%)
Non-survivors	10 (48%)	1.02	0.14	0.87-1.35	35.48	1.51	32.29-37.14	7 (70%)

**, p = 0.001; SD = Standard deviation, GA = gestational age*

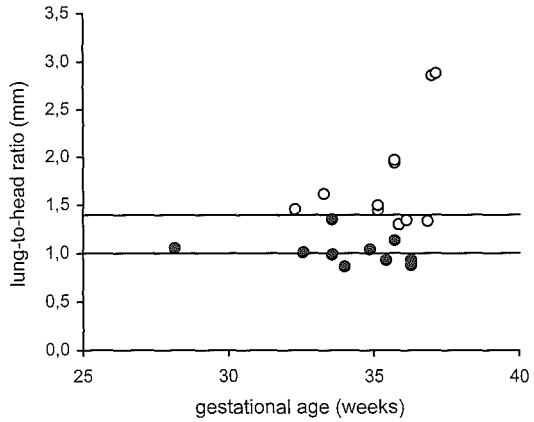
Using analysis of covariance, adjusting for gestational age at time of measurement, non-survivors have a mean LHR which is 0.70 lower (95% CI: 0.27-1.13) as compared to survivors (P = 0.003). (Figure 3) This difference is comparable to the outcome when the gestational age is not taken into account (P = 0.001). The analysis of covariance further showed no statistically significant relation between LHR and gestational age (P = 0.39). In the LHR subset, ECMO was required in 45% of the survivors and in 70 % of the non-survivors. This difference was also not statistically significantly different. (Table 1)

In the present study all fetuses with an LHR above 1.4 mm survived. A 100% mortality rate was shown in all fetuses with a LHR below 1.1. Table 2 presents the results from our study compared to the data from Metkus et al. and Lipshutz et al.. The cut-off levels as presented by Lipshutz et al. (1997) showed better applicability to our particular study population in respect to prediction of fetal outcome compared to the cut-off levels as presented by Metkus et al. (1996), with 100% survival (8/8) at a LHR above 1.4 mm, 100% mortality (5/5) at a LHR below 1 mm and a 38% survival at a LHR between 1 and 1.4 mm (Figure 3, Table 3). When the cut-off of 1.4 mm as a predictor for good or poor prognosis as proposed by Sbragia et al. (2000) was applied to our study population, a statistically significant difference in survival was found between the group with a LHR above this level and the group with a LHR below this level (100% (8/8) vs 23% (3/13) survival).

Table 2 A comparative analysis of LHR as prenatal predictor for outcome of left-sided CDH.

Source	Metkus et al. (1996)	Lipshutz et al. (1997)	This study.
Analysis:	Retrospective	Prospective	Retrospective
n =	55	15	21
Survival:	65%	47%	52%
Mean LHR survivors	1.33 ± 0.50 mm	1.46 ± 0.33 mm	1.78 ± 0.58 mm
Mean LHR non-survivors	0.87 ± 0.32 mm ($p < 0.001$)	1.05 ± 0.30 mm ($p < 0.05$)	1.02 ± 0.14 mm ($p = 0.001$)
100% survival as	LHR > 1.35 mm	LHR > 1.4 mm	LHR > 1.4 mm
100% mortality as	LHR < 0.6 mm	LHR< 1.0 mm	LHR < 1.1 mm

Figure 3 Relation between lung-to-head ratio and gestational age in respect to fetal outcome



Closed dots = non-survivors, open dots = survivors.
The 2 horizontal lines represent the cut-off levels 1.0 and 1.4. (Lipshutz et al, 1997)

Table 3 The cut-off levels as suggested by Metkus *et al.* (1996), Lipshutz *et al.* (1997) when applied to the present population.

n = 21	**Metkus et al. (1996)**			**Lipshutz et al. (1997)**		
LHR (mm)	< 0.6	0.6 – 1.35	> 1.35	< 1.0	1 – 1.4	> 1.4
Survivors	0	3 (25%)	8 (89%)	0	3 (38%)	8 (100%)
Non-survivors	0	9 (75%)	1 (11%)	5 (100%)	5 (62%)	0

The relation between other prenatal sonographic parameters and fetal outcome was also assessed in all 26 isolated left-sided CHD. Polyhydramnios was present in 12 cases (46%). In 7 of these 12 cases (58%), the newborn died. Of the 14 cases in which no polyhydramnios was diagnosed, 6 fetuses died (43%). Mediastinal shift was present in all 26 cases of which 13 died (50%). Mean gestational age at the time of diagnose of the CDH was 29.3 wk (SD 5.13) and varied between the 19 and 39 weeks. In 27% (7/26) the diagnosis CDH was made before 25 weeks of gestation of which 71% (5/7) died. Of the 19 cases, which were detected after 25 weeks' gestation, mortality rate was 42% (8/19). Intra-thoracic herniation of the stomach was present in 24 cases (92%) of which 50% died. The mortality rates associated with the presence or absence of these variables are presented in Table 4. None of these suggested predictors showed significant differences in mortality rates between groups with or without these variables. No accurate data could be obtained on the position of the fetal liver in the medical records.

Table 4 Mortality rates by other parameters (n = 26)

Mortality ratio	Variable Present n (%)	Variable not present n (%)	Significance
Polyhydramnios	7/12 (58)	6/14 (43)	N.S.
Mediastinal shift	13/26 (50)	0	
Early diagnosis*	5/7 (71)	8/18 (44)	N.S.
Intrathoracic stomach	12/24 (50)	1/2 (50)	N.S.

**) < 25 weeks' gestation; N.S. = not significant*

Discussion

The present study focuses on the retrospective evaluation of the value of LHR in the prenatal prediction of fetal outcome in cases of isolated left sided CDH. Despite early prenatal diagnosis, planned delivery at a center with ECMO-facilities and important progress in treatment of both neonatal and surgical intensive care, the associated morbidity and mortality of these patients remain high.
In our study population the overall survival rate of 50% was more or less comparable to similar studies. Metkus et al. (1996) reported a survival rate of 65% in their retrospective study. Lipshutz et al. (1997) found a survival rate of 47% in their prospective study. An explanation for the variance in survival is perhaps the wide range in severity of the anomaly in the newborns. In our study all newborns had severe respiratory problems from birth, illustrated by 4 cases dying within one hour after birth and the documented extreme pulmonary hypoplasia (low lung/bodyweight ratio) in our series. It is not possible to distract this kind of information from the articles of Metkus et al. (1996) and Lipshutz et al. (1997) to identify their patients as belonging to the same subset of patients.

The LHR has recently been proposed to provide a reliable measure to help determine postnatal survival and may serve as a counseling tool for clinicians and parents facing difficult decisions in prenatal and postnatal care (Lipshutz et al., 1997). This study showed that the survivors of isolated left CDH had a statistically significant higher mean LHR compared to the non-survivors (1.78 vs. 1.02 mm). According to Lipshutz et al. the LHR results in their study are only applicable to those fetuses in which the LHR is determined between 24-26 weeks. It is of great importance to find reliable cut-off's for the prediction of fetal outcome outside this period, because, at least in our situation, most referrals for level III ultrasound assessment do not take place between 24 and 26 weeks' gestation. Another reason is that it is only allowed to offer parents termination of pregnancy until 24 weeks' gestation, at least according to Dutch law, with exception of some anomalies. The present study showed no significant relation between LHR and gestational age. This is not remarkable since the addition of the head circumference in

this ratio corrects for gestational age dependent changes of lung growth. We speculate therefore that LHR measurements can be extrapolated to different gestations and are not restricted to the 24-26 weeks' gestation as earlier suggested.

The mean LHR of the survivors (1.78 ± 0.58 mm) in our study was higher than the mean LHR in the studies of Metkus et al. (1.33 ± 0.5 mm) and Lipshutz et al. (1.46 ± 0.33 mm). The mean LHR of the non-survivors (1.02 ± 0.14 mm) is the same as reported by Lipshutz et al., (1.05 ± 0.30 mm) but a little higher than the mean LHR for non-survivors reported by Metkus et al. (0.87 ± 0.32 mm).
In our series the cut-off levels of 100% survival and 100% mortality (above 1.4 and below 1.1 respectively) are more comparable with cut-off levels suggested by Lipshutz et al. (1997) than by Metkus et al (1996) and Sbragia et al (2000). Differences in definition of LHR cut-off levels may depend on the characteristics and number of cases in a particular study group. Further, one should realize that LHR measurements may be influenced by the angle of the transducer in the four-chamber view of the heart and by the placement of the measuring calipers, which may also induce differences in LHR cut-off levels. Applying the cut-off levels as proposed by Lipshutz et al (1997) to our data, accurate prediction of fetal outcome could be achieved. Consequently, we conclude that these specific cut-off levels can be used for prediction of fetal outcome irrespectively of gestational age at time of the measurement. However, whether they are sufficient enough for clinical practice, may need further prospective evaluation.

In our retrospective evaluation, in nearly all cases no accurate information on the position of the fetal liver was available in the medical records. As a consequence, the prognostic value of the liver position for fetal outcome could not be assessed and as such no comparison could be made with the paper of Sbragia et al. (2000). Sbragia et al. reported that fetuses without liver herniation into the chest have a favorable prognosis, even in the presence of a low LHR. None of the other prenatal sonographic parameters assessed in the present study, such as intrathoracic position of the stomach, mediastinal shift, polyhydramnios and early diagnosis, proved to be accurate in predicting the fetal outcome. However, the mortality rate was considerably higher within the group with a diagnosis of CDH before the 25th week of gestation compared to the group with a diagnosis of CDH after the 25th week (71% vs. 42%). All these parameters have been evaluated in the international literature and consequently none has been widely accepted or clinically applied (Bahlmann et al., 1999; Geary et al., 1998; Guibaud et al., 1996; Witters et al., 2001).
Other promising methods for prenatal prediction of fetal outcome associated with CHD are, next to LHR, Doppler velocimetry (Laudy and Wladimiroff, 2000) and fetal lung volume measurement by magnetic resonance (Mahieu-Caputo et al., 2001; Rypens et al., 2001).

Accurate prediction of fetal outcome associated with isolated left-sided CDH is still difficult, but for clinical practice highly desirable. We agree with Lipshutz et al that the LHR may serve as a reliable tool to help in the prediction of fetal outcome and consequently in the counseling for clinicians and parents. Nevertheless, at present, the LHR still faces difficulties with regards to continuation or termination of the pregnancy. The specific LHR cut-off levels should therefore be validated for different gestational ages preferably in a prospective international multi center study. The latter is of great importance because publications on the significance of the LHR are derived from one center up till now. Perhaps the future of the prenatal prediction of the fetal outcome in cases with CDH lies in the combination of various predictive sonographic values, LHR, Doppler velocimetry and fast spin-echo magnetic resonance lung volume measurement as recently suggested. (Rypens et al., 2001)

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Genetic aspects of Congenital Diaphragmatic Hernia

Part 2

CHAPTER 7

EARLY DIAGNOSIS OF WOLF-HIRSCHHORN SYNDROME TRIGGERED BY A LIFE-THREATENING EVENT: CONGENITAL DIAPHRAGMATIC HERNIA

Early Diagnosis of Wolf-Hirschhorn Syndrome Triggered by a Life-threatening Event: Congenital Diaphragmatic Hernia

M.F. van Dooren, A.S. Brooks, A.J.M. Hoogeboom, T.L. van den Hoonaard, J.E.M.M. de Klein, C.H. Wouters, D. Tibboel

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ABSTRACT

Wolf-Hirschhorn syndrome (WHS, OMIM 194190) is a chromosomal disorder characterized by retarded mental and physical growth, microcephaly, Greek helmet appearance of the facies, seizures/epilepsy. Closure defects of lip or palate, and cardiac septum defects occur in 30-50% of cases. Its cause is a deletion in the short arm of chromosome 4. We present a male patient, born after 37 weeks gestation, as the fourth pregnancy of non-consanguineous healthy parents, with unilateral cleft lip and palate, hypertelorism, a right-sided ear tag, and mild epispadias. At age 10 weeks he developed acute respiratory distress and acute bowel obstruction requiring emergency laparotomy. This revealed a left-sided posterolateral diaphragmatic defect, type Bochdalek, with incarceration of the small intestines necessitating major bowel resection. Clinical genetic investigation suggested a chromosome anomaly, but regular karyotyping was normal. However, FISH analysis showed a microdeletion in the short arm of chromosome 4 (4p-), consistent with WHS. A combination of this syndrome with congenital diaphragmatic hernia (CDH) has been rarely described. CDH can present either as an isolated defect at birth, or with multiple congenital abnormalities, or as part of a defined syndrome or chromosomal disorder. Therefore CDH, although not common in WHS, can lead to its diagnosis relatively early in life. We strongly recommend a clinical genetic evaluation of each CDH patient with facial anomalies taking into consideration 4p- deletion syndrome.

INTRODUCTION

Wolf-Hirschhorn syndrome (WHS, OMIM 194190) is a well-known chromosomal disorder, first described in 1961, characterized by Greek helmet appearance of, the facies, severe growth deficit, mental retardation of, variable degree, microcephaly, seizures/epilepsy (Cooper, and Hirschhorn, 1961). Closure defects of lip or palate, and cardiac septum defects occur in 30-50% of individuals, (Battaglia et al., 2001, 2002). It is caused by a, deletion of the short arm of chromosome 4. The currently considered critical region (WHSCR) for WHS is located on chromosome 4p16.3. WHS is allelic with Pitt-Roger-,Danks syndrome (PRDS) although the latter has a, milder phenotype (Battaglia and Carey, 1998; Wright, et al., 1998). Usually, diagnosis of WHS is considered, when patients are investigated to evaluate the cause of, their developmental delay and typical facial anomalies. However, early diagnosis of 4p- deletion syndrome, is also feasible when a life-threatening birth defect is present, as in our report.

Clinical Report

We present a male patient, born after 37 weeks uncomplicated pregnancy, with a birth weight of 2,330 g (-1.4 SD), a length of 48 cm (-0.3 SD), and a head circumference of 31 cm (-2.3 SD). This boy was the fourth pregnancy of non-consanguineous healthy parents. The first pregnancy ended in a mola, the second in a spontaneous abortion, and the third in a termination on parental request after 24 weeks of gestation because of multiple congenital anomalies including lung hypoplasia, megabladder, and a urethral stricture. After birth our patient was referred to a local hospital with a unilateral cleft lip and palate, a right-sided ear tag, and mild epispadias (Figure 1). Genetic analysis was not performed at that time. After 2½ months he developed acute respiratory distress due to an acute bowel obstruction, requiring admission to our level III pediatric surgical unit. X-ray of the abdomen showed dilatation of the intestines and pneumoperitoneum (Figure 2). On suspicion of a bowel perforation, an emergency laparotomy was performed. This revealed a classic left-sided posterolateral diaphragmatic hernia, type Bochdalek, with incarceration of the small intestines. The diaphragmatic defect was closed and the incarcerated intestines were resected. The latter resulted in a so-called short bowel syndrome. During his first year of life he also developed severe epilepsy–a usual feature in the clinical spectrum of WHS–, with both generalized and febril seizures, necessitating combinations of anti-epileptic drugs. It was problematic, however, to control the epilepsy. At present he is almost 4 years-old with a height of 102 cm (-0.38 SD), weight 18.7 kg (0.92 SD), and a head circumference of 45.1 cm (-3.51 SD). He has severe psychomotor retardation, being not yet able to sit unsupported, and has no speech.

Figure 1 Typical "Greek helmet" appearance of face with unilateral cleft lip and palate, hypertelorism, and in detail the right sided ear tag (indicated by an arrow)

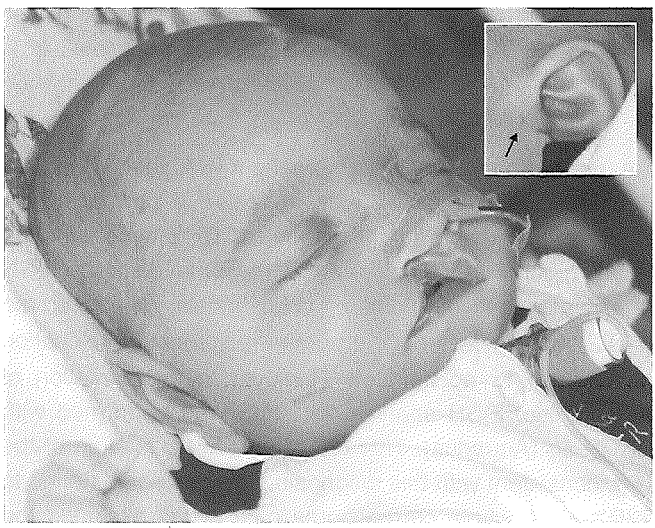
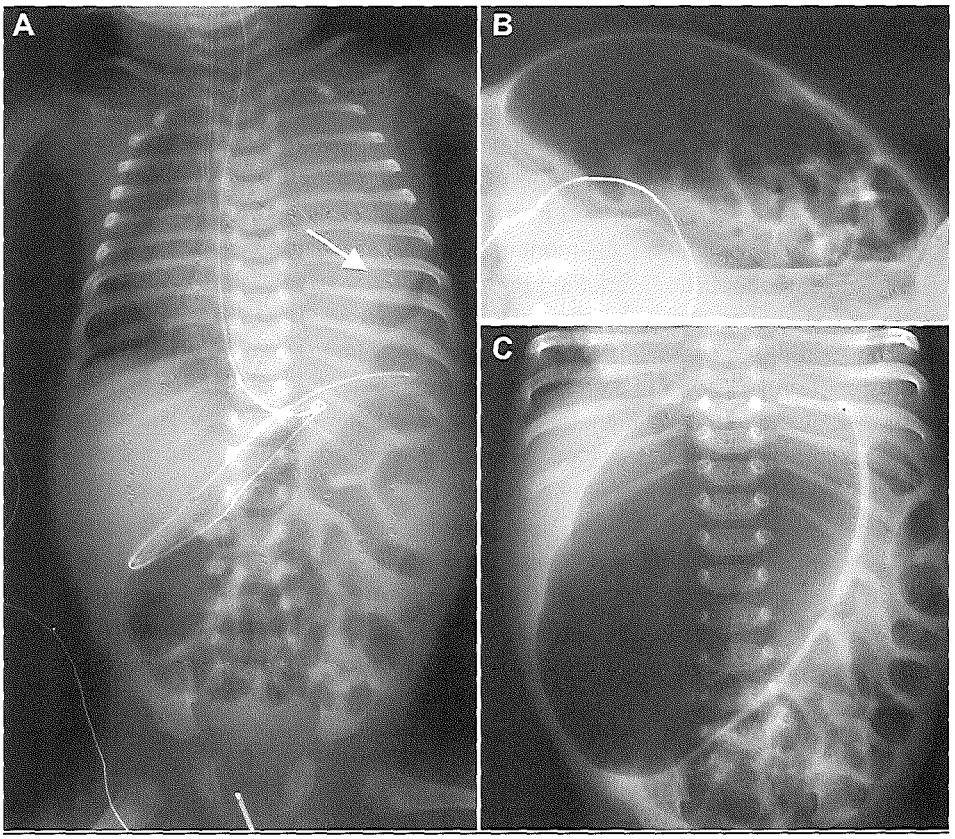


Figure 2 A. Plain X-ray shows a left-sided diaphragmatic hernia with moderately dilated intestines in the left hemithorax (arrow) with atelectasis of the left lung
B. A transverse X-ray shows a pneumoperitoneum and signs of bowel obstruction
C. Massive dilatation of the stomach before placement of a nasogastric tube



Genetic Aspects

During the postoperative course, we performed a clinical genetic investigation. Regular chromosome analysis, i.e., G banding with > 550 bands, revealed a normal 46,XY karyotype. The combination of congenital diaphragmatic hernia (CDH), hypertelorism, ear tag, and cleft lip and palate was highly suggestive for a chromosomal disorder. FISH analysis for trisomy 13, 18, and 21 was negative. In differential diagnosis, we considered an unbalanced product of a t(11;22) – the most frequent human reciprocal translocation – and microdeletions such as a 22q11 or 4p deletion. However, a microdeletion of chromosome 22q11 was excluded. Also, FISH analysis for WHS was performed with probe D4S96 (Oncor) and WHSCR-aq (Aquarius). This revealed a microdeletion of the short arm of chromosome 4 (4p-), consistent with this diagnosis. The deletion extends to 4p16.1 (RP274B16-; RP11-173B23+). A whole chromosome paint wcp4 excluded the

presence of an unbalanced translocation involving choromosome 4. Both parents had normal chromosome patterns, and FISH results with chromosome 4p telomeric probes, indicated a de novo deletion in the proband (46,XY. ish del(4)(p16.1).
To conclude, while an obstetrical history that included severe congenital malformations in a previous child of this family, suggested that one of the parents might have been a carrier of a balanced translocation between chromosome 4 and an unknown chromosome, in our patient the 4p- syndrome should be considered a de novo chromosomal disorder.

DISCUSSION

CDH can either present as an isolated defect at birth, or in co-occurrence with multiple congenital abnormalities, or as part of a defined syndrome or chromosomal disorder. The incidence is 0.33 per 1,000 births and mortality is high (50%) due to lung hypoplasia, pulmonary hypertension or associated anomalies (Torfs et al., 1992). Well-defined Mendelian disorders associated with CDH are Fryns, Brachman de Lange, and Beckwith-Wiedemann syndrome; chromosomal anomalies also include Pallister-Killian syndrome (tetrasomy 12p) and other well-known numerical anomalies such as trisomy 13 and 18 (Enns et al., 1998).
There are a few reports on the combination of CDH and WHS (Lazjuk et al., 1980; Tachdjian et al., 1992; Bird et al., 1994; Howe et al., 1996; Sergi et al., 1998). Prenatal diagnosis of WHS in combination with CDH has been made in patients karyotyped for severe intrauterine growth retardation (Tachdjian et al., 1992). It is usually a child's developmental delay together with the typical facial appearance that suggests the diagnosis of WHS. However, since this patient presented with lifethreatening CDH and had a thorough clinical genetic examination, he was subjected to specific FISH analysis at a very early age. This revealed the chromosomal disorder, which normal karyotyping failed to show.

Our patient revealed also an epispadias. A few cases of WHS have been reported with extrophy of the bladder (Nicholls and Duffy, 1998), which is thought to be in the same birth defect spectrum as epispadias. This is even more interesting since the epispadias/bladder extrophy complex is very rare in aneusomy syndromes. Therefore, the short arm of chromosome 4 might not only harbour genes for the development of the diaphragm, but also for that of the fetal abdominal wall.
The early diagnosis of WHS had important consequences for the support of the parents and follow-up of this patient. We propose that Wolf-Hirschhorn syndrome should always be considered in a patient with a diaphragmatic hernia and facial anomalies.

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CHAPTER 8 CHROMOSOMAL ANOMALIES AND CDH IN THE ERASMUS MC SOPHIA

CDH may be associated with chromosomal anomalies, for instance numerical ones such as trisomy 13 and 18, and also tetrasomy 12p, known as the Pallister Killian syndrome. Furthermore, CDH has been described in combination with abnormalities in all chromosomes, reviewed by Lurie (Lurie 2003). Schlembach and colleagues have reviewed published cases of terminal deletions of the long arm of chromosome 15 in combination with CDH (Schlembach et al. 2001). In chapter 8 the relation of CDH with 15q deletions is discussed.

A database set up in 1998 stores data from all patients with a diaphragmatic defect treated in Erasmus MC Sophia since 1988, including those with a posterolateral (Bockdalek) and medial (Morgagni) hernia but also those with an eventration of the diaphragm. This patient material (PBL or skin-biopsy) is routinely cultured, and part of it is used for DNA extraction and storage of cell suspensions.

We analysed 338 patients with CDH included so far, on conventional karyotyping results and their clinical features. However, if conventional karyotyping had not been done, in some cases we performed Comparative Genome Hybridisation (CGH) after isolation of DNA from autopsy material.

RESULTS

The results of our chromosomal analyses are summarized in Table 1. Karyotyping had been performed in 169 patients (50%). A normal karyotype was found in 151 of these (90%). In 11% (n = 18) an abnormal karyotype was found.

Chromosomal anomalies in our study included trisomy 13 and 18, 4p- syndrome, 8p+ syndrome, and also three cases of chromosome 15 anomalies, i.e. two with ring chromosome 15 and one with a translocation of chromosome 15.

Chromosome patterns of CDH-cases in our hospital from 1988-now

Number (n)	Karyotype
86	46,XY
65	46,XX
7	47,XX,+18 and 47,XY,+18
4	47,XX,+21 and 47,XY,+21
1	47,XX,+13
1	47,XY,+der(22)t(11;22)(q23.3;q11.2)
1	46,XY,der(8)t(3;8)(p23p23.1)
1	46,XY.ish del(4)(p16.1)
1	46,XY,t(1;14)(p22;q13),inv(6)(p25q22),del(15)(q26)
2	46,XY,r(15)

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CHAPTER 8

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) AND CHROMOSOME 15q26: A SPECIFIC REGION?

M.F. van Dooren, B. Eussen, R-J. Galjaard, Ch. Lee, B.A. Oostra, N. Goemaere, C. Wouters, D. Tibboel, A. de Klein

ABSTRACT

The etiology of Congenital Diaphragmatic Hernia (CDH), a severe birth defect combining faulty diaphragm formation with lung hypoplasia and postnatal pulmonary hypertension, is poorly understood. CDH carries a high mortality (50%) and its incidence is one per 3000 births. Chromosomal aberrations are observed in 30% of the cases, particularly in patients with multiple congenital anomalies (MCA). The latter mainly present in the cardiovascular and renal systems. The commonest genetic changes among CDH patients with CDH seem to be an extra chromosome 18 or 13 (trisomy 18 and 13). Other recurrent, structural chromosomal changes point to other CDH-associated loci, for example chromosome segment 15q24-26. A survey of cytogenetic data from over 150 CDH patients in our hospital revealed three cases of a small deletion of chromosome 15 (15q24-26). Furthermore, we obtained material from four other, published CDH patients with a chromosome 15q24-26 deletion. Using FISH and CGH techniques, we found a common deletion of chromosome band 15q26.1-q26.2. The fact that RLBP1 (retinol binding protein1) is located in this part of chromosome 15, corroborates the hypothesis that retinoic acid is involved in the formation of the diaphragm, as appeared from findings in teratogen-induced rodent mouse models of CDH. These preliminary results instigated us to continue our research. There is a strong indication for a critical region on chromosome 15 and this is potentially relevant for our understanding of the etiology of CDH.

INTRODUCTION

Congenital Diaphragmatic Hernia (CDH) is a life-threatening congenital anomaly often associated with varying degrees of lung hypoplasia and therapy-resistant pulmonary hypertension. CDH can present as an isolated defect, or in association with multiple congenital abnormalities, or as part of a defined syndrome or chromosomal disorder. The incidence is one in 3000 births (Torfs et al. 1992), and multifactorial inheritance has been suggested. The importance of genetic factors is brought out by the co-occurrence of numeric chromosomal disorders, such as trisomy 13 and 18, and also tetrasomy 12p, causing the Pallister-Killian syndrome. Monogenic syndromes, e.g. Fryns syndrome and Simpson-Golabi-Behmel syndrome, are often associated by a diaphragmatic defect (Enns et al. 1998). CDH has been described in combination with abnormalities in all chromosomes (Lurie 2003). Several cases of terminal deletions of the long arm of chromosome 15 in combination with CDH have been reported (Schlembach et al. 2001).

The terminal region of chromosome 15q harbors a number of genes related to retinoic acid metabolism, which is intriguing because vitamin A metabolism has been shown to play an important role in the pathogenesis of CDH (Greer et al. 2003). Maternal vitamin

A deficiency causes CDH in rats (Andersen 1941; Warkany and Roth 1948). Vitamin A interacts with Nitrofen-induced diaphragmatic hernia: concomitant administration of vitamin A and Nitrofen to pregnant rats decreases the incidence and severity of Nitrofen-induced CDH (Thebaud et al. 1999). Nitrofen inhibits the key enzyme for retinoic acid production, retinaldehyde dehydrogenase-2 (RALDH2), in vitro (Mey et al. 2003). While in humans, RALDH2 is located on 15q21.2, the 15q deletions co-occurring with CDH are usually located towards the terminal part of chromosome 15q. This 15q24-26 region harbors genes involved in retinoic acid metabolism, i.e. CRABP1 (Cellular Retinoic Acid Binding Protein-1), and RLBP1 (Retinaldehyde-binding protein 1). Located on 15q24, CRABP1 is involved in several retinoic acid-mediated developmental processes. (Vaessen et al. 1989; Vaessen et al. 1990; Flagiello et al. 1997). Located on chromosome 15q26, RLBP1 is involved in the visual system (Saari et al. 2001). Mutations in RLBP1 cause nonsyndromic autosomal recessive retinitis pigmentosa (Maw et al. 1997). In both CRABP1 and RLBP1 knockout mice, CDH has not been reported. So far, CDH has not been described in patients with mutations in RLBP1. We screened CDH patients treated in our hospital on chromosome anomalies and in detail on 15q abnormalities. In addition we analyzed material from four published cases with chromosome 15 deletions.

MATERIALS

Since 1988 data from all patients with a diaphragmatic defect treated in the Erasmus MC are stored in a database with information on clinical features, chromosomal analysis, obstetric history and autopsy files. According to standard clinical practice and after clinical genetic consultation, karyotyping was performed when a patient presented with MCA. We analysed karyotypes from 169 patients together with the clinical features of these patients. In 3 cases CDH co-occurred with a small deletion of chromosome 15 (15q24-26). In one case (case 1) we also found a balanced translocation t(1;14)(p22;q13),inv(6)(p25q22). Inviting the authors of previously published cases of CDH in combination with a chromosome 15q24-26 deletion to participate in this study, we obtained material from four other cases. Thus, our patient 4 is case 2 described by Rosenberg, and our patients 5, 6 and 7 are the cases reported by de Jong, Chen and Schlembach, respectively (de Jong et al. 1989; Rosenberg et al. 1992; Chen et al. 1998; Schlembach et al. 2001).

METHODS

We used different materials, such as cell-lines, DNA or paraffin blocks, depending on its availability. Table 1 lists the material and techniques used for every patient, such as

conventional chromosomal analysis, Fluorescence In Situ Hybridisation (FISH) and Comparative Genome Hybridisation (CGH). In the coming section the techniques are shortly described. A set of BAC-clones in the 15q23-ter region have been ordered for performing FISH.

Conventional G-banding

Metaphase chromosomes were used for chromosome banding by a standard trypsin-Giemsa method.

Fluorescence In situ Hybridisation (FISH)

Metaphase chromosomes and interphase nuclei obtained from synchronized phytohemagglutinine (PHA)-stimulated cultures of peripheral blood lymphocytes were used for FISH analysis. Conform standard procedures in the department of Clinical Genetics, we performed FISH on interphase nuclei with a set of BAC clones in the region 15q24-26.

CGH

DNA was isolated from the cultured cells or from formaline-fixed paraffin-embedded tissue (30 5μm sections, DNAeasy Tissue kit (Qiagen). Test DNA and male reference DNA was digested with EcoR1 and 5μg was labelled directly with Spectral Green and Alexa 594-UTP, respectively, using the random primed labelling kit (Invitrogen). The probe mixture was denatured and after pre-annealing with Cot-1 DNA (Invitrogen) hybridised to normal human metaphases chromosomes for 3 days. After washing 15-20 metaphases were analysed using CGH software (Metasystems, Isis 2000).

RESULTS

Table 2 lists the clinical features of the seven patients. All had other congenital anomalies, and none survived beyond the neonatal period. Pregnancy was terminated in case of patients 3 and 6, patient 5 was stillborn, and the other four died in the neonatal period.

Table 1 Material and techniques

	Karyogram	Cell-line	Paraffin	DNA	G-banding	FISH	CGH
1	46,XY,del(15)(q26)	+		+	+	+	+
2	46,XY,r(15)		+	+	+		+
3	46,XY,r(15)	+		+	+	+	+
4	46,XX,der(15)t(3;15)(q29;q26.1)	+		+	+	+	+
5	46,XY,r(15)(p11q26)		+	+	+		+
6	46,XX,der(15)t(8;15)(q24.1;26.1)		+	+	+		+
7	46,XX,del(15)(q25q26.2)		+	+	+		+

Table 2 Clinical features

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Intra-uterine growth retardation	+	+	+	+	+	+	+
Hydrocephaly						+	
Brachycephaly					+		
Microcephaly		+					+
Facial anomalies		+	+	+	+	+	+
Cardiac anomalies		+	+	+		+	
Diaphragmatic hernia	+	+	+	+	+	+	+
Renal anomalies						+	+
Hypoplastic genitals	+	+			+		
Cryptorchism		+			+		
Clinodactyly				+		+	+
Club feet/talipes equinovarus		+			+		+
Scoliosis or kyphosis						+	

All patients had a left-sided diaphragmatic defect and all showed facial anomalies, such as micrognathia, abnormal ears and other dysmorphic features. Five patients also showed limb defects (rocker bottom feet and clinodactyly), and four also cardiac defects (VSD, monoventricle with interrupted aortic arch). All patients had intra-uterine growth retardation, as shown by ultrasound studies or low birthweight (<3th percentile). Three patients had cerebral anomalies (hydrocephaly, microcephaly).

With FISH, using BAC clones from 15q24-26, we found an interstitial deletion of chromosome band 15q26.1-q26.2 (table 3). To define the deletion more precisely we selected an extra set of BAC clones from the 15q26.1-q26.2 areas and will perform these experiments in the next future.

Table 3 Schematic representation of the FISH results

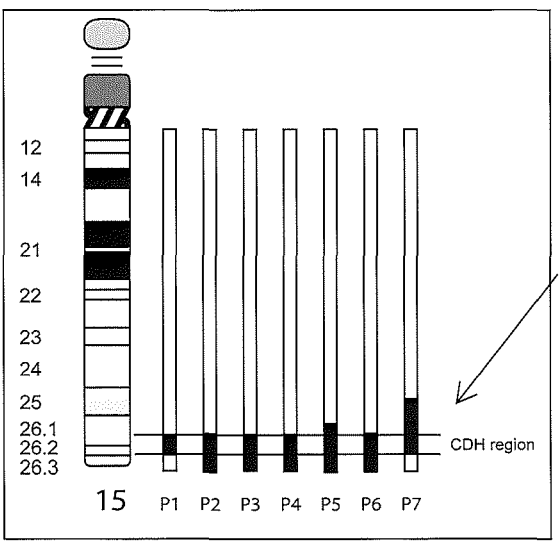


Figure 1 Chromosomal pattern of patient 3, a ring chromosome 15

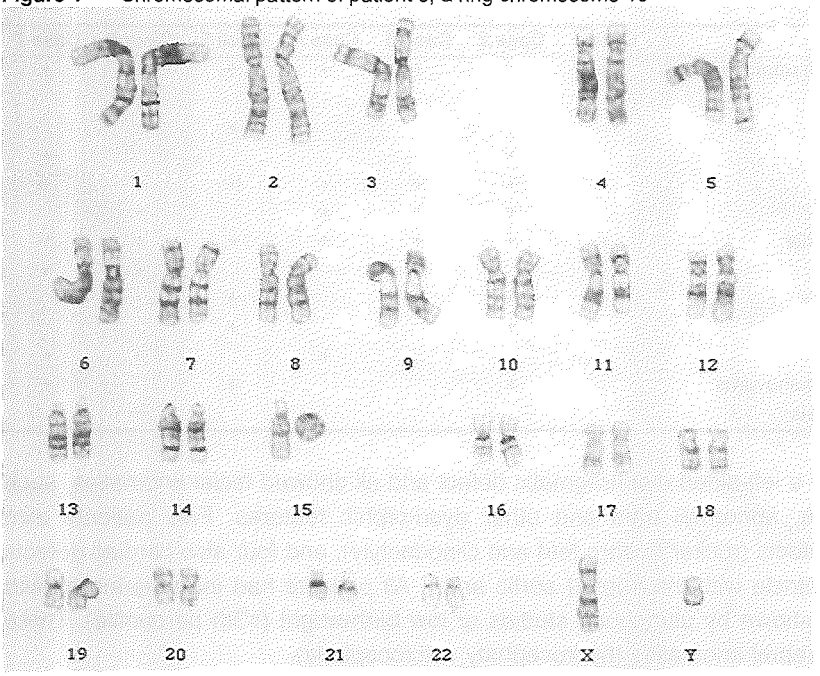
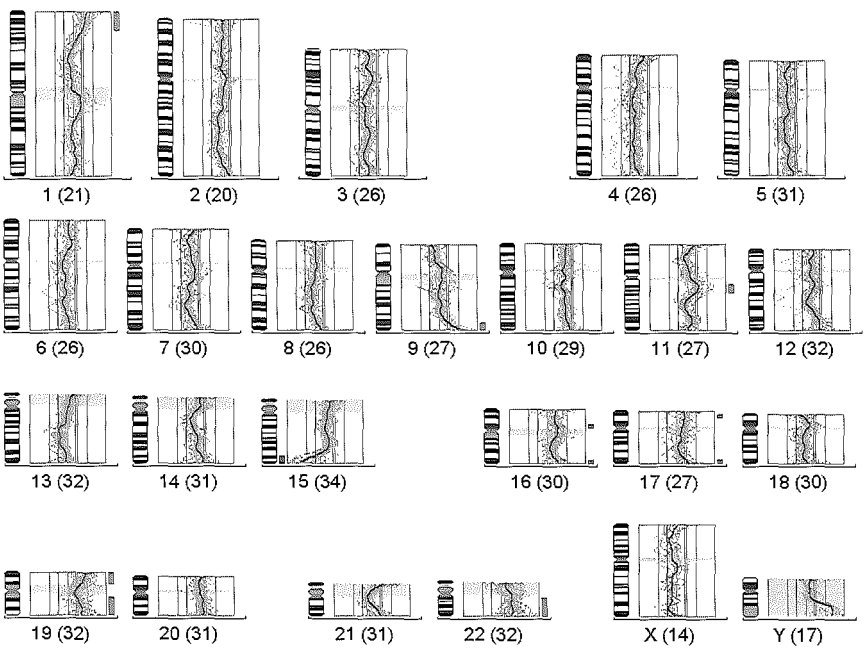


Figure 2 Results of CGH in patient 3



Discussion

Clinical evaluation revealed a left-sided CDH, intra-uterine growth retardation and other congenital anomalies in all seven patients. Only in one other case a right sided defect was reported (Biggio et al. 2004). However, we doubt whether this is relevant, because CDH predominantly occurs on the left side.
After accurate evaluation of all FISH data, we found a deletion of chromosome band 15q26.1-q26.2 both in our three patients and in the four published cases. This deletion was either interstitial in patients 1 and 7, or a deletion of the terminal part of 15q.
The shortest region of overlap was around 5 Mb. Cases of CDH co-occurring with a deletion in 15q have been reported previously. Biggio and colleagues proposed to have the smallest deletion of 15q26.1, with a right-sided CDH and coarctation of the aorta, in a patient who survived the neonatal period (Biggio et al. 2004). This deletion may overlap with our common region. Further, they suggested Myocyte-specific Enhancer Factor 2 (MEF2a) as a candidate gene for CDH, because it plays a role in muscle differentiation and development. However, this gene is located on 15q26.3 and is outside, distal of our target deletion.

Our two patients with ring chromosome 15 and the one described by De Jong *et al.*(de Jong et al. 1989), had overlapping features, such as left-sided CDH, facial anomalies and intra-uterine growth retardation . However, not all patients with terminal deletions of 15q or ring chromosome 15 have diaphragmatic defects. Patients with unbalanced translocations may have different clinical features due to a deletion of another chromosome. Reports on clinical manifestations of patients with ring 15 chromosome include severe pre- and postnatal growth retardation, triangular face and mild mental retardation (Horigome et al. 1992), but no CDH. Fukushima presented a patient with the phenotype of Silver-Russell syndrome with microcephaly, low-set ears, atrium septum defect, ventricular septum defect, rocker bottom feet and, remarkably, relaxation of the diaphragm (Fukushima et al. 2002). However, it is not known whether relaxation of the diaphragm and the classical posterolateral diaphragmatic hernia have a common underlying etiology.

Remarkably, growth retardation is observed in all cases. Roback suggested the cause of growth retardation to be loss of one functional copy of insulin-like growth factor 1 receptor (IGF1R), located on 15q26 (Roback et al. 1991). However, this deletion is unlikely to be the single candidate gene for CDH, as we expect our common deletion to be located more proximal on chromosome 15.
A gene of interest, also located in our smallest region of overlap, is RLBP1. No CDH has been observed in the RLBP1 knockout mice (Saari et al. 2001). In CDH patients no mutations in RLBP1 have been described.

In conclusion, in our cases we found a small common deletion on 15q26.1-q26.2. We will use tiling resolution arrays to further investigate the breakpoints and then perhaps be able to identify candidate genes. In addition, we will use these arrays to test other important CDH-specific regions (3q, 4p, 8p and 15q), including genes involved in Nitrofen-induced CDH mouse models and other possible candidate genes such as SHH/Gli3; SLIT3, CRABP1, RALDH2 and RLBP1.

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CHAPTER 9a

CONGENITAL DIAPHRAGMATIC HERNIA IN A FEMALE PATIENT WITH CRANIOFRONTONASAL SYNDROME

Congenital Diaphragmatic Hernia in a Female Patient with Craniofrontonasal Syndrome

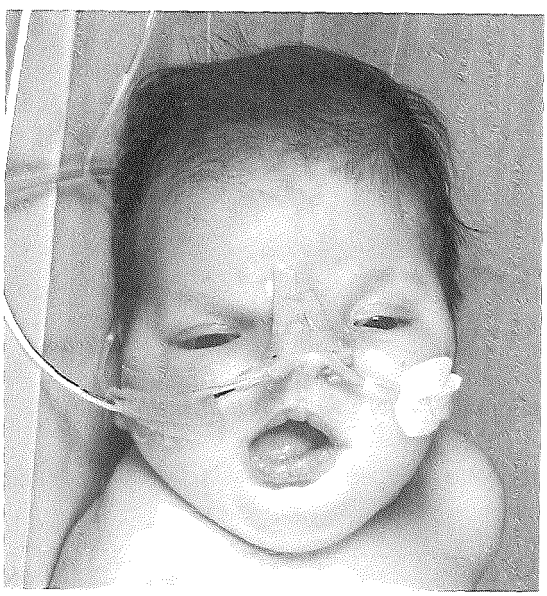
Alice Brooks, Marieke van Dooren, Jeannette Hoogeboom, Saskia Gischler, Patrick Willems, Dick Tibboel

Clinical Dysmorphology 2002,11:151-153

Craniofrontonasal syndrome or dysplasia (CFNS, OMIM 304110) is characterised by craniosynostosis of the coronal sutures, hypertelorism, longitudinal grooves of the nails and various skeletal abnormalities (e.g. syndactyly of toes) (Cohen, 1979, Morris et al., 1987). Pedigree analysis is consistent with an X-linked dominant mode of inheritance, whereby all daughters but none of the sons of male patients are affected. Remarkably, females are more severely affected than males. Affected males usually only show hypertelorism. Recently linkage analysis in 14 unrelated families has mapped the CFNS gene to Xp22 (Feldman et al., 1997, Pulleyn et al., 1999), but the gene has not yet been isolated.

We recently observed a girl with clinical features consistent with CFNS and a posterolateral defect of the diaphragm (type Bochdalek); a life-threatening congenital anomaly often associated with a variable degree of lung hypoplasia and therapy resistant pulmonary hypertension. Congenital diaphragmatic hernia (CDH) has been reported in only two CFNS patients (two out of six affected males in a four-generation family reported by Morris et al. (1987). To our knowledge no females with the combination of CFNS and CDH have been reported so far. It is well known that CDH is often associated with other major congenital abnormalities, predominantly of the heart and limbs. In a minority of cases CHD may be part of a syndrome caused by a chromosomal anomaly (e.g. Pallister Killian syndrome due to a partial tetrasomy 12p, and partial trisomy 22) or a monogenic disorder (e.g. Fryns syndrome and Simpson-Golabi-Behmel syndrome) (Enns et al., 1998). CFNS is one of a growing number of monogenic syndromes associated with CDH.

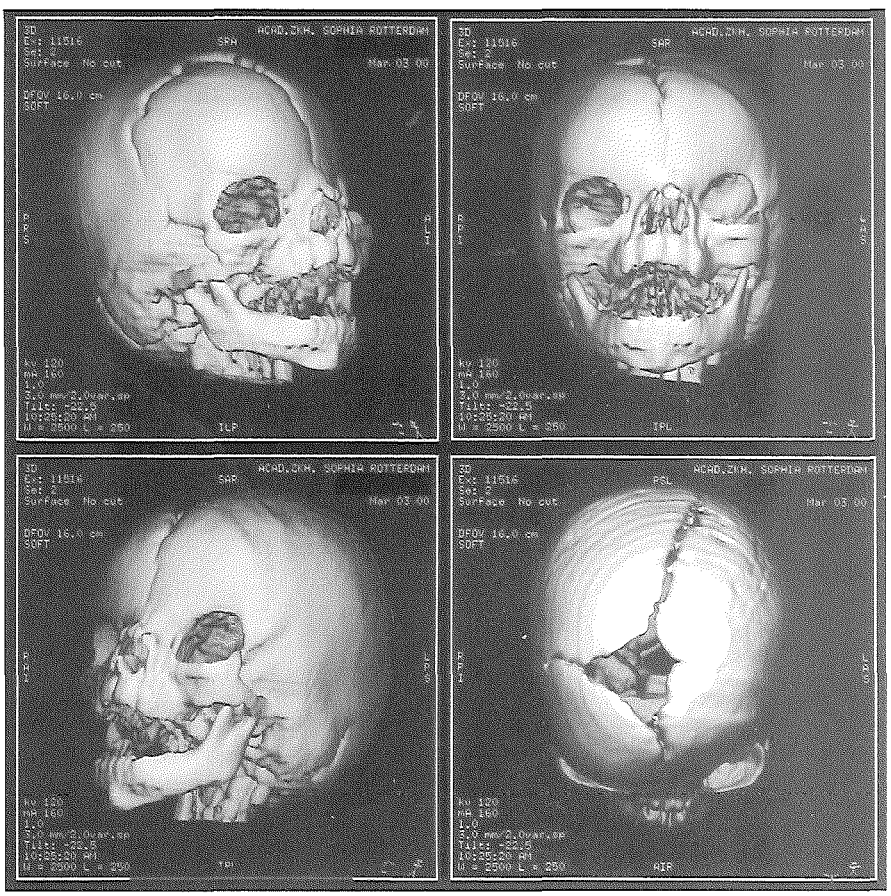
Figure 1 Frontal view of the patient showing asymmetry of the skull and hypertelorism



Our patient is at present a 21-months-old girl. She was born to healthy, non-consanguineous Caucasian parents, after an uncomplicated pregnancy. Birth weight was 4050 grams (97th centile), head circumference 33,5 cm (50th centile). Hypertelorism and a broad nasal tip were noted shortly after birth. She was admitted to our hospital at day 2 with respiratory distress. Clinical examination showed hypertelorism with evident facial asymmetry (Figure 1), a broad nose, and grooved nails (of the hallux and thumb, syndactyly of the third and fourth toes) . The nipples were widely spaced.

Radiology of the thorax showed a left-sided diaphragmatic defect with herniation of the intestines into the thorax. Skull X-rays showed craniosynostosis of the coronal suture at the left, which was confirmed with a 3-dimensional CT scan (Figure 2). MRI showed an agenesis of the corpus callosum. Cytogenetic analysis was normal.

Figure 2 3 dimensional CT scan at the age of three months showing synostosis of the left coronal suture



At operation, a posterolateral diaphragmatic defect (type Bochdalek) was surgically closed with a Goretex patch. At the age of 11 months, the craniosynostosis was operated on.
The most likely diagnosis in our patient is CFNS based on the facial features, the grooved nails and the unilateral coronal synostosis. To our knowledge CDH has only be reported twice in CFNS and never in a female patient, which is remarkable since the CFNS phenotype is more pronounced in female patients. The presence of CDH in three cases of CFNS, a rare syndrome which has only been reported in ± 100 cases, is probably not a coincidental co-occurrence as the frequency of CDH is estimated 1 in 3000-4000 live births (Torfs et al., 1992).
Therefore this case report confirms that CFNS should be added to the expanding list of syndromes that include CDH as an infrequent feature.

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CHAPTER 9b

CONGENITAL DIAPHRAGMATIC HERNIA AND SITUS INVERSUS TOTALIS

Congenital Diaphragmatic Hernia and Situs Inversus Totalis
Tesselaar CD, Postema RR, van Dooren MF, Allegaert KM, Tibboel D

Pediatrics 113; 3 (2004)

INTRODUCTION

Congenital diaphragmatic hernia is a relatively rare disorder (1:3000 newborns) that frequently presents with respiratory distress in the immediate neonatal period due to severe pulmonary hypertension and lung hypoplasia. Extracorporeal membrane oxygenation (ECMO) can be used as a last resort when artificial ventilation and/or modulation of the pulmonary vascular tone fail to improve the clinical condition.
Situs inversus totalis is a rare condition in which orientation of all asymmetrical organs in the body is a mirror image of the normal morphology (Morelli et al. 2001). Diaphragmatic hernia may be caused by predisposing genes that are involved in left-right axis determination.
A few cases of an eventration of the diaphragm combined with situs inversus totalis have been described in the literature. We report for the first time a patient with a right-sided posterolateral diaphragmatic hernia, type Bochdalek, and a situs inversus totalis for which contralateral cannulation for the institution of venoarterial ECMO (VA-ECMO) was warranted.

CASE REPORT

A girl with birth weight 3810 g, born at the postmenstrual age of 39 + 6 weeks, developed acute respiratory distress, cyanosis and lethargy within thirty minutes after birth. Physical examination revealed a scaphoid abdomen and decreased breathing sounds at the right side of the thorax. A chest-radiography revealed that heart and mediastinum were displaced to the left and that the right pleural cavity was filled with intestines (Figure 1).
Ultrasound confirmed that the right hemithorax was almost completely filled with loops of intestine, mesenterial fat and the spleen, due to a large defect in the right hemidiaphragm. An abdominal situs inversus was documented; i.e. a normally formed liver positioned left and the spleen on the right side. Echocardiography confirmed a left descending aorta and a mirror image dextrocardia; i.e. a structurally normal heart mirrored and unusually positioned in the right chest. At first, there were no signs of clinical relevant pulmonary hypertension on ultrasonography.
Karyotyping revealed a normal female (46XX). Family history showed no parental consanguinity and no congenital anomalies or minor laterality defects.
Initial management consisted of emergency endotracheal intubation. Next, different ventilatory settings were used to optimize oxygenation, ranging from conventional mechanical ventilation to high frequency oscillation with these following maximum settings: frequency 10 Hz; mean airway pressure, 17; δ P, 50; fractional inspired oxygen concentration, 100%. Supportive therapy consisted of exogenous surfactant (Alvofact 50 mg/kg, Boehringer Inc, Ingelheim, Germany), cardiovascular support with inotropic agents (maximum dosages dopamine, 20 µg/kg/min; dobutamine 20 µg/kg/min; noradrenaline

0,3 mg/kg/min), and pulmonary vasodilators such as inhaled nitric oxide (maximum dosage: 20 ppm).

Because the girl showed progressive respiratory failure complicated by a left-sided pneumothorax, a pneumoperitoneum, and therapy-resistant pulmonary hypertension, she was referred to our ECMO center on day 9 of life (Figure 2). On day 10, VA-ECMO was initiated. The abnormal position of the heart and heart chambers necessitated a left-access approach. The venous cannula for drainage was inserted in the left internal jugular vein, and the left common carotid artery was cannulated for arterial access (Figure 3). Maximal ECMO flow was 130 ml/kg/min. There were no technical complications during ECMO. Flow was weaned progressively, and the girl was decannulated after 9 days.

Sixteen days after decannulation (day 35), she underwent elective surgical correction. By right-subcostal incision, the diaphragmatic defect was exposed and repaired with interposition of a Gore-Tex patch. At surgery the malrotation of the small bowel and colon was corrected by adhesiolysis and derotation and accompanied by appendectomy. She was extubated twelve days after operation (day 47). At the age of 3 months (day 91), she was discharged. Until the age of 4 months she needed a nasogastric tube because of ongoing feeding problems.

Figure 1 Chest radiography demonstrating a displaced heart and mediastinum due to a diaphragmatic hernia

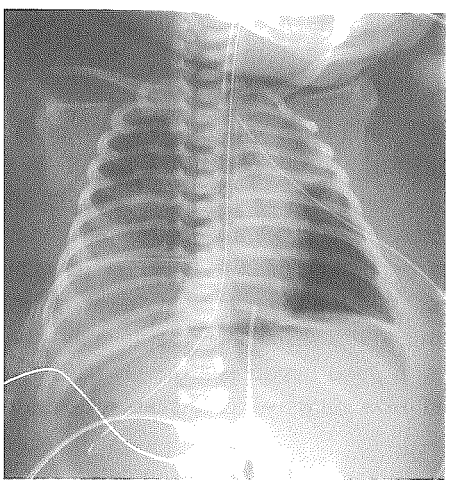


Figure 2 Chest-radiography demonstrating pneumothorax and pneumoperitoneum, revealing the situs inversus of the abdominal viscera.

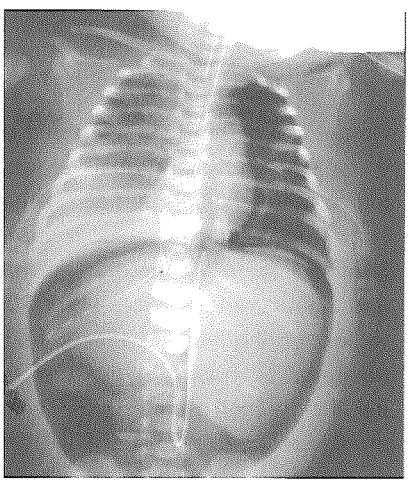
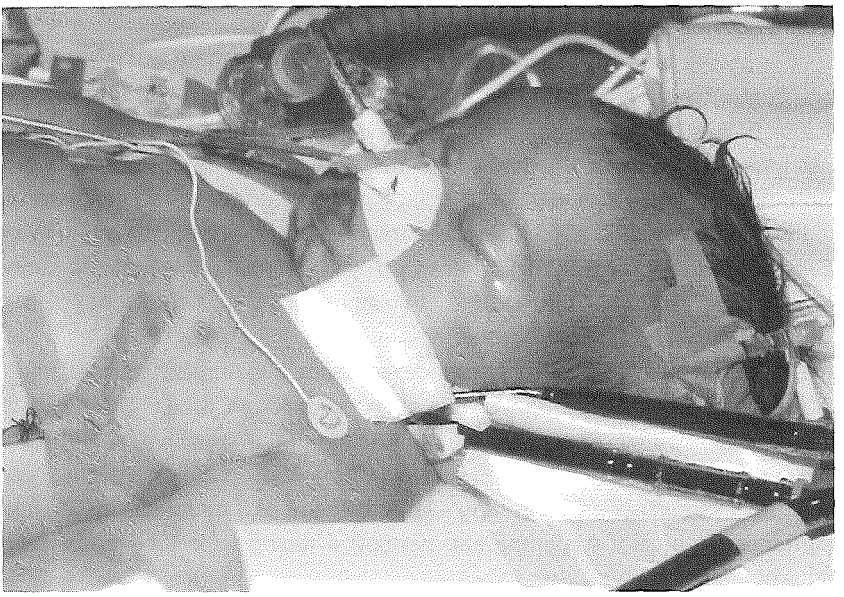


Figure 3 The individualzsed VA-ECMO approach, with cannulation of the left jugular vein and the left carotic artery.



At the age of 6 months, there were no signs of hernia-associated pathological gastroesophageal reflux. Growth and neurological and motor development corresponded to her age. She appeared to be doing well, with no apparent complications of the hernia, the ECMO procedure itself, or the operation.

Discussion

Congenital diaphragmatic hernia presents in ~ 1 in 3000 newborns. In most cases (83-94%) it is a left-sided posterolateral (Bochdalek) defect (Torfs et al. 1992). About 1% of the congenital diaphragmatic hernias is bilateral.

Notwithstanding advances in surgical and ventilatory support techniques, diaphragmatic hernia is still a life-threatening malformation with a mortality of 25% to 60% (Beresford and Shaw 2000; Boloker et al. 2002). Although competition for space by the abdominal viscera during fetal life affects the bronchial growth and the vascular elements in the lung, postnatal pulmonary hypertension and the degree of lung hypoplasia greatly determine survival(Keijzer et al. 2000). Before, during, and after surgery, ECMO be required to provide respiratory and circulatory support.

A defect in the normal development of the human left-right asymmetry during embryogenesis results in laterality defects with cardiovascular, abdominal, and pulmonary malformations (Casey 2001). Mirror-image dextrocardia is a common form of cardiac

malposition and has been estimated to occur in 1 in 8000 live births. Mirror-image dextrocardia is almost always associated with situs inversus of the abdominal organs (Gutgesell 1997). The prevalence of situs inversus totalis appears to range between 1 in 8000 and 1 in 25000 (Splitt et al. 1996). It is a condition in which the morphologic right atrium is on the left and the morphologic left atrium is on the right. The normal pulmonary anatomy is reversed so that the left lung has 3 lobes and the right lung has 2. In addition, the liver and gallbladder are located on the left, the spleen and stomach on the right. The remaining internal structures also mirror the normal situation. One fourth to one fifth of patients with situs inversus totalis have the Kartagener syndrome, which is characterized by ciliairy dyskinesia, bronchiectasia, sinusitis, and infertility.

Etiological factors in situs inversus are unknown; familial occurrence suggests multiple inheritance patterns. Genes involved in human situs anomalies include ZIC3 (zinc finger transcription factor), LEFTB (transforming growth factor B-related factor), ACVR2B (human activin receptor type IIB) and Cryptic (Bamford et al. 2000). Mutations in these genes have been found in patients with laterality defects (Kosaki et al. 1999). Strikingly, the inversin knockout mouse showed, in addition to a situs defect, an eventration of the diaphragm in one of the offspring, suggestive of a common developmental pathway (McQuinn et al. 2001). Several individual chromosome anomalies have been reported in co-occurrence with situs inversus (Aylsworth 2001). The karyotype of our patient was normal, and the family history mentioned no other cases of laterality defects. Retinoic acid must be assumed as a potential environmental risk factor for the 2 defects, because it is involved both in development of the diaphragm and in ACVR2B metabolism (Mendelsohn et al. 1994).

Only 2 case reports described laterality abnormalities in combination with a right-sided diaphragmatic defect. However, these concerned eventrations in combination with other midline defects such as a total laryngotracheoesophageal cleft or a heart defect such as an atrial septum defect (Itoh et al. 1987; Heitmann et al. 1988). The patient we present is, to our knowledge the first reported neonate with a Bochdalek diaphragmatic hernia and situs inversus totalis. In this case, an abdominal situs inversus was associated with a right-sided diaphragmatic hernia, that had significant consequences for the placement of the ECMO cannulas. The individualized approach for ECMO cannulation resulted in an uneventful clinical course.

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Environmental Aspects of Congenital Diaphragmatic Hernia

Part 3

CHAPTER 10

ARE HYDROXYLATED POLYCHLORINATED BIPHENYLS (PCBS) RELEVANT FOR THE ETIOLOGY OF CDH: A PILOT STUDY

Are hydroxylated Polychlorinated Biphenyls (PCBs) relevant for the etiology of CDH: a pilot study

Marieke van Dooren, Martin van Velzen, Janine Felix, Claudine Torfs, Paul Mulder, Bram Brouwer, Dick Tibboel

Submitted

ABSTRACT

Introduction

Congenital diaphragmatic hernia (CDH) is a severe birth defect whose etiology is unknown. An animal model for CDH consists of the administration of the herbicide 2, 4-dichlorophenyl-p-nitrophenyl ether (nitrofen) to pregnant rats whose offspring then develop a diaphragmatic hernia. Polychlorinated biphenyls (PCBs) and their hydroxylated metabolites (OH-PCBs) resemble nitrofen in structure and, at least partly in, mechanism of action, are persistent lipid-soluble contaminants present in food, and easily cross the placenta. Therefore we hypothesized that OH-PCBs levels would be higher in mothers of infants with CDH than in mothers of controls.

Methods

Cases were CDH patients born from 1999 to 2003 in the area served by the Sophia Children's Hospital in Rotterdam. Controls were infants with EA or without a birth defect. For this pilot study, we randomly selected 31 infants with CHD out of a total of 58 cases, 24 with EA and 30 normal infants. Maternal blood samples were used for the analyses of levels of p,p-DDE (1-dichloro-2, 2 bis (p-chlorophenyl) ethylene), PCB153 (2,2',4,4'.5,5'-hexachlorobiphenyl), 4OH-PCB107 (4OH-2,3,3',4',5-pentachloro-biphenyl), 4OH-PCB146 (4OH-2,2',3,4',5,5'-hexachloro-biphenyl), 4OH-PCB187 (4OH-2,2',3,4',5,5',6-heptachlorobiphenyl) and pentachlorophenol by gas chromatography using a ECD detector.

Results

Neither unadjusted analyses nor multiple regressions with adjustment for covariates showed any risk of CDH for elevated levels of any of the OH-PCBs congeners in maternal bloods.

Conclusion

The results of this pilot study do not suggest that high levels of PCBs in maternal blood are a risk factor for CDH.

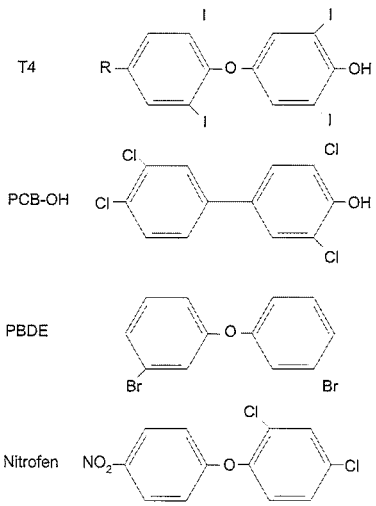
INTRODUCTION

Congenital Diaphragmatic Hernia (CDH) is a severe birth defect, characterized by failure of closure of the diaphragm, unilateral or bilateral lung hypoplasia and postnatal pulmonary hypertension. The prevalence of CDH is about 0.3 per 10,000 births with relatively high mortality rates, in particular in cases with associated malformations (Torfs et al. 1992; Skari et al. 2000). The etiology of CDH is poorly understood and genetic and environmental etiologies have been hypothesized. There are several animal models of

teratogens for CDH. In one of these models, the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether), when administered to the pregnant rat between the 9th and 11th day of gestation, induces in the offspring a diaphragmatic hernia of the Bochdalek (posterolateral) type with lung hypoplasia (Costlow and Manson 1981; Kluth et al. 1990; Tenbrinck et al. 1990). Nitrofen belongs to the group of polyhalogenated aromatic compounds (PHAHs). Its structure and mechanism of action resemble both those of the thyroid hormone and those of hydroxylated polychlorinated biphenyls (OH-PCBs) (figure 1). Thus nitrofen interferes with both the thyroid pathway and vitamin A metabolism (Manson 1986; Brouwer et al. 1998; Thebaud et al. 1999). Co-administration of vitamin A and nitrofen to pregnant rats results in a lower incidence or CDH in the pups (Thebaud et al. 1999).

PCBs were commercially produced as complex mixtures for a variety of applications such as dielectric fluids for capacitors and transformers, heat transfer fluids, and as additives in pesticides, paints, carbonless copy paper, flame retardants and plastics. Their commercial use was based largely on their chemical and physical stability, including low flammability. Most countries stopped their production in the 1980s. However, PCBs can also be formed as unintentional byproducts in a variety of chemical processes that contain chlorine and hydrocarbon sources (de Voogt and Brinkman 1989). They consist of a biphenyl ring and, depending on the number and position of chlorine atoms on the two rings there are 209 theoretically possible discrete PCB compounds, called congeners. Prior to cessation of their production in 1977, billions of tons of PCBs were manufactured all over the world.

Figure 1 General structures of thyroid hormone (T4), hydroxylated polychlorinated biphenyls (OH-PCB), Polybrominated Biphenyl Ethers (PBDE) and Nitrofen



PCBs are known as endocrine disruptors because they interfere with several receptors of the endocrine signal transduction pathways such as the aryl-hydrocarbon receptor (AhR) and receptors of the thyroid and estrogen pathways. Thyroid hormone plasma levels decrease in rats and humans after exposure to OH-PCBs due to competitive binding to the plasma thyroid hormone carrier protein transthyretin (Koopman-Esseboom et al. 1994b; Brouwer et al. 1995; Brouwer et al. 1998). Hydroxylated PCBs also inhibit estrogen sulfotransferase (Kester et al. 2002).
In 90% of cases, human exposure to PCBs occurs through food, mostly of animal origin. PCBs are lipid-soluble, accumulate in fat tissue and degrade very slowly. Half-life time in humans ranges from 1.8 years to 9.9 years (Steele et al. 1986; Taylor and Lawrence 1992). During pregnancy, PCBs are transferred from mother to child through breastfeeding and via the placenta (Masuda et al. 1978; WHO 2000). Hydroxylated PCBs, the primary metabolites of PCBs, easily cross the placenta and are able to cross the blood-brain barrier (Brouwer et al. 1998).

Synthetic organic hydrocarbons such as PCBs can be toxic for mammals (Guo et al. 1995). Their persistent presence in our environment and in our food chain was, and still is, a concern in the medical community. The toxicity of PCBs for humans has been clearly shown in Japan and Taiwan ('Yusho' in 1968 and 'Yu Cheng' in 1979 respectively) when the accidental contamination of cooking oil by PCBs resulted in congenital yusho in infants of mothers who had ingested the oil. Congenital yusho is characterized by low birthweight, intra-uterine growth retardation and a deep pigmentation of the skin and nails, and also by neurodevelopmental deficits (Rogan 1982; Rogan et al. 1988).

However, the possible effect of OH-PCBs in maternal blood on the occurrence of structural congenital malformations has not been studied extensively. In chicken embryos, PCB's altered growth and development; the observed malformations were hydrops, torticollis, brevicollis, and short legs (Hatano and Hatano 1994). Also, a potent thromboxane A2-receptor blocker AH23848, whose metabolites resemble OH-PCBs, induces diaphragmatic defects in the offspring of exposed female rats (Sutherland et al. 1989). However, other than the Japanese study of congenital yusho, no studies on the possible effect of OH-PCBs on human fetuses have been reported. Consequently, we decided to investigate in a pilot study the possible association between (OH)-PCB concentration in the mother's blood and the presence of a congenital diaphragmatic hernia in her offspring. We hypothesized that mothers of CDH patients would have higher OH-PCB blood levels than mothers of healthy children or mothers of children with another birth defect, esophageal atresia, that is unrelated to CDH and occurs at the same embryonic developmental stage. We conducted a case-control study to test this hypothesis.

METHODS

Subjects

All subjects in our study were ascertained from the Sophia Children's Hospital records. The hospital is the level III perinatal and pediatric surgical center serving as a referral facility for the Southwestern part of the Netherlands, a highly industrialized and densely populated area with 3 to 4 million inhabitants and an annual birth rate of 35,000. The study population consisted in a case group of children with CDH and two control groups, one of infants with EA and one of age-matched infants without birth defects who visited the day care facility of our hospital for minor surgery. We asked all parents of children in the three groups, either at the time of birth of the infant or later, by recruitment through the parents' support groups, to participate in the study and to donate a blood sample from both themselves and the child. Those samples were stored for future DNA analyses and, in the case of mothers only, were also used to evaluate the blood levels of OH-PCBs. We randomly selected 31 children with CDH, 24 with EA, and 30 without a birth defect for our pilot study from a group of 65 children in the CDH group, 58 in the EA group and .49 in the control group. For the CDH and EA group, we included cases with or without additional structural defects. For the statistical analysis we also compared maternal levels of OH-PCBs in two groups of children, those with isolated defects to those with MCA. The medical ethics committee of the Erasmus MC Sophia approved the study design. All parents signed consent forms.

Assessment of exposure variables

Glass tubes free of PCBs were used for the collection of the blood for PCB-measurements. The exposure variables studied in this analysis included p,p-DDE (1, 1-dichloro-2, 2 bis (p-chlorophenyl) ethylene), PCB153 (2,2',4,4'.5,5'-hexachlorobiphenyl), 4OH-PCB107 (4OH-2,3,3',4',5-pentachloro-biphenyl), 4OH-PCB146 (4OH-2,2',3,4',5,5'-hexachlorobiphenyl), 4OH-PCB187 (4OH-2,2',3,4',5,5',6-heptachlorobiphenyl) and pentachlorophenol. Samples were stored in -20 ^{0}C prior to measurement.

Chemicals

Standard solutions containing the OH-PCB's and the phenols were a gift from professor Åke Bergman of the Department of Environmental Chemistry, University of Stockholm. PCB 153, 198 and p,p-DDE were purchased from Dr. Ehrenstorfer. Other chemicals used in the sample analyses were Hexane (Ultra-resi, Baker); methyl *tert*-butyl ether (MTBE, Ultra-resi, Baker); 2-propanol (Chromasolv, Riedel-deHaën); dichloromethane (Suprasolv, Merck); Diethylether (p.a. Merck); Silicagel 60 (0.063-0.200mm, Merck); Sulphuric acid (p.a. Fluka); Hydrochloric acid (instra analyzed, Baker); potassium hydroxide (p.a., Riedel-deHaën); potassium chloride (baker-grade, Baker). Ethereal diazomethane, used for the derivation of phenolic compounds, was generated from N-

methyl-N-nitroso-ureum and 50% w/v potassium hydroxide in diethylether according to Vogel (Vogel 1967).

The serum samples (± 5 g) were extracted based on a previously described method by Hovander et al (Hovander et al. 2000).
Prior to extraction PCB198 (2,2',3,3',4,5,5',6-octachlorobiphenyl), 2,4,5-trichlorophenol and 4OH-PCB193 (4OH-2,3,3',4',5,5',6-heptachlorobiphenyl) were added as internal standards. The neutral compounds were subsequently separated from the phenolic compounds by a liquid/liquid partitioning with potassium hydroxide (0.5 M in 50% Ethanol). After adding 2M HCl, the phenolic compounds were extracted from the potassium hydroxide with a mixture of MTBE:hexane (1:9 v/v). The phenolic fraction was methylated overnight with ethereal diazomethane at 4 °C. Both neutral and phenolic fractions were shaken with concentrated sulphuric acid to remove lipids. A second clean-up step was performed by using a sulphuric acid-silica gel column (1 g of concentrated sulphuric acid: silicagel (1:2 w/w)). The neutral fraction was eluted with hexane and the phenolic fraction with hexane:dichloromethane (8:2 v/v). After elution the extracts were quantitatively transferred into an autosampler vial for measuring on the GC-ECD. With every 10 samples one blank sample (water, HPLC-grade, Baker) and one reference sample (serum, Academic Hospital, Vrije Universiteit Amsterdam) was included. The levels of the compounds were corrected for the blank values. Recovery of the internal standards 2,4,5-trichlorophenol, PCB 198 and 4OH-PCB 193 was 74% (SD 23%, n = 83), 100% (SD 5%, n = 85) and 103% (SD 11%, n = 82) respectively.

Assessment of covariables
Variables that could confound an association or interact with PCBs included the time interval from the infant's birth to the blood sampling, maternal age, maternal smoking during pregnancy (yes/no), history of breast feeding (yes/no), and parity (one/more).
The timing of blood sampling after birth is relatively unimportant because of the long half-life of PCBs. We controlled for nursing because nursing a child for more than three months decreases the maternal body burden of PCBs by 30%. We controlled for parity because mothers who had nursed more that one child would have further decreased their body burden of PCBs (Koopman-Esseboom et al. 1994a; Koopman-Esseboom et al. 1994b). We controlled for parity because mothers who had nursed more that one child would have further decreased their body burden of PCBs.

Statistical analysis
To test each variable separately we used an ANOVA test on the logarithmic scales because the standard deviations of the groups were not very different and the groups were more or less of the same size. If not, we used a Kruskal-Wallis test to compare single variables between the three groups. Results were considered significant if $p \leq 0.05$.

ANCOVA, analysis of covariance, was used to test the variables in a larger model. Variables that were likely to affect PCB-levels were included in the statistical model as a fixed set of explanatory variables. These variables were: maternal smoking, time interval from birth to blood sampling, maternal age, breast feeding and parity.

RESULTS

We analyzed 31 samples in the CDH group, 24 in the EA group and 30 in the control group. The characteristics of mothers and children in the three study groups are described in table 1. The median maternal age was 30.9 years in the CDH group and 32.5 years in both the control and EA group. The average time intervals of mothers' blood sampling were significantly different between the three groups: blood sampling in the EA and normal control groups took place about 6 and 8 months later, respectively, than in the CDH group. About 50% of mothers in the CDH and EA group were primipara, whereas only 37% were primipara in the normal group. Maternal smoking was reported in 15% of the CDH-group, in 24% of the EA-group, and in 38% of the normal control group (not significant). In the CDH group, six (19%) children had multiple anomalies whereas in the EA group 14 (58%) children had MCA. In both the CDH- and EA-group the male:female ratio showed a male predominance, 1.6 and 2.0 respectively. The ratio was 0.9 for the control. The median blood concentrations of the PCBs and their range are described in table 2. There were no statistically significant differences between study groups. Table 3 shows the results of the multiple regression analyses that included all the covariables. There were no statistically significant differences between groups for all PCB congeners evaluated.

Table 1 Characteristics of mothers and children in the study population, according to the child's birth status, either congenital diaphragmatic hernia (CDH), esophageal atresia (EA) or no birth defect

Characteristics	CDH (n = 31)	EA Control (n = 24)	Normal Control (n = 30)
A. Mothers			
Mean Age at birth of child (y ears)	30,9 (21,0-39,32)	32,5 (18,6 40,63)	32,5 (22,7-41,14)
Timing of blood sampling, in months after birth of child (minimum-maximum)	2,4 (0,3-46,2)	10,2 (0,1-49,4)	8,0 (0,7-23,2)
Smoking during pregnancy*, n (%)	4 (15)	5 (24)	11 (38)
Parity one, n, (%)	15 (48%)	12 (50%)	11 (37%)
Breast feeding, yes n, (%)	15 (50%)	15 (71%)	20 (67%)
B. Children			
Survival, n (%)	20 (65%)	24 (100%)	30 (100%)
MCA**, n (%)	6 (19)	14 (58)	N A
Sex, ♂ : ♀, ratio	19:12 (1.58)	16:8 (2.0)	14:16 (0.88)

** If smoking stopped ½ year or more before conception, mother was classified as non-smoker*
*** Multiple Congenital Anomalies*

Table 2 Levels of exposure to OH-PCBs for mothers of children with either CDH, EA, or no birth defect. (normal controls)

Exposure variables*	CDH Median (range)	EA Controls Median (range)	Normal Controls Median (range)	*P* value
P,p-DDE	600 (90-5279)	894 (248-5092)	560 (105-3364)	ns
PCB153	339 (52-841)	457 (141-765)	344 (86-958)	ns
OH-PCB107	91 (19-282)	100 (42-246)	102 (19-456)	ns
OH-PCB146	127 (39-343)	164 (55-388)	139 (46-610)	ns
OH-PCB187	105 (37-201)	118 (79-234)	112 (59-260)	ns
Pentachlorophenol	746 (234-3864)	883 (222-4928)	707 (84-5317)	ns

**Values are in pg/g, median and range*
*** ANOVA or Kruskal Wallis test*

Table 3 Results of multiple regression analyses of the different PCB compounds, comparing mothers with a child with either CDH or EA to mothers with an unaffected control child *

Chemical type	CDH/ control β (95% CI)	EA/CDH β (95% CI)	EA/ control β (95% CI)	Overall P-value
p,p-DDE	1.18 (0.79- 1.77)	0.80 (0.50-1.28)	0.95 (0.61-1.48)	0.58
PCB153	1.12 (0.86-1.45)	1.00 (0.76-1.40)	1.15 (0.86-1.54)	0.56
OH-PCB107	0.91 (0.62-1.32)	0.80 (0.52-1.24)	0.73 (0.48-1.11)	0.32
OH-PCB146	0.98 (0.72-1.33)	0.99 (0.69-1.41)	0.97 (0.69-1.36)	0.98
OH-PCB187	1.02 (0.84-1.22)	0.94 (0.76-1.16)	0.95 (0.77-1.17)	0.83
Pentachloro-phenol	1.41 (0.87-2.28)	0.85 (0.48-1.48)	1.19 (0.70-2.03)	0.37

** Covariant analysis with ANCOVA with maternal smoking, parity, time of blood sampling after infant's birth, age, and breast feeding as covariables*

Discussion

In this study, we could not detect significant differences in levels of the PCB congener 153, OH-PCB 107, -146, and -187 between mothers of CDH patients and mothers of children in both control groups, However, because of the great variability of levels of PCB congeners within each group, and the large confidence intervals around the median values (table 2), the possibility for a type II error, the failure to detect a true risk, remains. This study should be considered as a first attempt to obtain a better insight in potential environmental pollutants relevant for CDH.

Of the few studies evaluating the effect of PHAHs in humans, none have reported the occurrence of CDH or of other structural birth defects, except for neurodevelopment defects in children with a prenatal exposure (Koopman-Esseboom et al. 1997; Patandin et al. 1999; Vreugdenhil et al. 2004) reviewed by Schantz (Schantz 1996). Although nitrofen causes CDH in some animal species, the administration of OH-PCBs to the same animal species does not cause a diaphragmatic defect in the offspring. Therefore it is possible that our hypothesis is not correct. Because this is a pilot study with small

numbers of cases and controls, it is possible that a small effect, if present, would not have been detected.

As far as we know, no similar study has been done before and it is thus not possible to compare our data with that of other's. If we would consider a larger international survey, the problem is that comparison of our study exposure levels to those in different countries is difficult. For instance, the level of PCB exposure in the Faeroe Islands was higher than in studies from Holland, North Carolina, and Germany (Koopman-Esseboom et al. 1994b; Osius et al. 1999; Longnecker et al. 2000; Steuerwald et al. 2000). On the other hand, different levels of exposure to similar compounds in different populations may provide options to evaluate dose-dependency if an effect would be observed.

Our study is a pilot study, the numbers are small and therefore the power of the study is low. Furthermore, even within a geographic area where PCB exposure is thought to be uniform, the variance in PCB levels between individuals is very high (table 2 and 3). Although the exposure to PCBs in the Netherlands differs by geographic area, reported levels of OH-PCBs in a population group in the Northern part of the Netherlands (n = 100) were similar to those of our controls, confirming our results (article in preparation). Large epidemiological studies to test our hypothesis will be difficult given the low incidence of CDH in the general population, 1 per 3,000. This contrasts with approximately 10 cases per year in our hospital. Controls will have to be chosen from the same area as cases. Another point is that fairly large differences in level of exposure exist between individuals. PCBs do not vary a lot per individual because they are very stable compounds, however, there is a steady increase over time.
The time interval to blood sampling was not completely standardized in our study, but this variable was controlled for in the multiple regression. Furthermore, OH-PCBs are persistent metabolites and degradation occurs slowly over the years. A prospective study that uses a narrow window of time for the collection of maternal bloods would add necessary precision.

Another unanswered question relates to the extent that maternal OH-PCB levels correlate with levels in the CDH newborn, as we did not measure OH-PCB levels in the patients. However, as OH-PCBs pass the placenta easily we would expect comparable concentrations in mothers and their children (Brouwer et al. 1998).

Although OH-PCBs do not seem to be teratogenic for CDH in humans, it cannot be concluded that environmental factors do not cause some cases of CDH. Vitamin A, for example, is thought to play a role in the etiology of CDH, as shown by the group of Greer in animal studies. RALDH2 (retinaldehyde dehydrogenase 2), the key enzyme to produce retinoic acid, is inhibited by nitrofen (Greer et al. 2003; Babiuk et al. 2004) .Major and collegues found lower retinol and retinolbinding protein levels in newborns

with CDH in comparison with normal controls (Major et al. 1998). However, no further publications have confirmed these findings. Also the classical teratological studies of Warkany (Warkany and Roth 1948) and Andersen (Andersen 1941) on the effect of vitamin A deficiency suggest that further human studies into this retinoic acid pathway are warranted.

In conclusion, in this pilot study, we could not show higher OH-PCB concentrations in the bloods of mothers of patients with a CDH than in mothers of controls. The hypothesis of a strong association between hydroxylated PCBs and diaphragmatic defects is not confirmed by this study and therefore a similar study in larger populations is not warranted at this time.

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CHAPTER 11

CONGENITAL POSTEROLATERAL DIAPHRAGMATIC HERNIA IN A JUVENILE STRIPED DOLPHIN (STENELLA COERULEOALBA)

Congenital Posterolateral Diaphragmatic Hernia in a Juvenile Striped Dolphin (Stenella Coeruleoalba)

R.A. Kastelein, M.F. van Dooren, D. Tibboel, and J. Dubbeldam

CASE

A male striped dolphin (Stenella coeruleoalba) stranded alive on the Dutch coast. The animal had a standard length of 159 cm, a girth at the eyes of 72 cm, a girth in front of the flippers of 85 cm, a girth at axilla of 90 cm, a girth in front of the dorsal fin of 88 cm, a girth at the anus of 47 cm, and weighed 50.2 kg. Based on body weight and length and growth layer groups of the teeth, the animal was estimated to be between two and three years old (Di-Méglio, Romero-Alvarez et al. 1996) (Willy Dabin, personal communication).

The dolphin was moved to the Dolfinarium Harderwijk for veterinary treatment and rehabilitation. During transport, he remained very stressed (high skin temperature and high respiration rate) despite the Valium (Diazepam) which was administered intramuscularly. Blood analysis showed no signs of dehydration (Hematocrit: 0.54 l/l, Hemoglobin 11.5 mmol/l, Erythrocytes 5.1 x 10^{12}/l) or infection (Leucocytes 7.3 x 10^{9}/l). After arrival in the treatment pool, the animal appeared to have many muscular cramps followed by strong (labored) respirations.

The animal died 6 hours after arrival at the rehabilitation center. The carcass was frozen immediately after death. Some time later the carcass was thawed and autopsy was performed. After opening of the thorax, both the stomachs and the intestines were found to be in the thoracic cavity (Figures 1 and 2). The intestines were 22 meters long and varied in color over their length. The fore stomach and fundic stomach appeared to be normal. The dislocation of the abdominal organs into the thoracic cavity organs was judged to be congenital, based on the fact that the left lung was less than half the volume of the right lung (700 ml (left) versus 1600 ml (right); measured by water displacement in a measuring beaker; Figure 3). Also, there was a dorsolateral opening in the left side of the diaphragm (Figure 4). The left side of the diaphragm was about 1/3 the surface area of the right side, and had a smooth (rounded) thick edge dorsally (Figure 5). These observations indicated a congenital diaphragmatic hernia (protrusion of an abdominal organ through the diaphragm into the thoracic cavity). The animal had also hypoplasia of the penis (less then 10 % of a normal sized penis of an animal of this age and size). The heart (435 g) appeared to be normal, as did the kidneys (250 and 240 g each). The blubber thickness mid-dorsal was 13 mm, mid-lateral 12 mm and mid-ventral 11 mm. This is in the range of healthy striped dolphins.

Based on the normal blood parameters, the muscular cramps and labored respirations observed during the last hours of the animal's life, the varied color of the intestines, and the limited space for the stomach and intestines, the most likely cause of death was determined to be a congenital diaphragmatic hernia, with lung-hypoplasia and congestion of the intestines.

Figure 1 The right side of the striped dolphin (right rib cage removed) showing the intestines in the thoracic cavity.

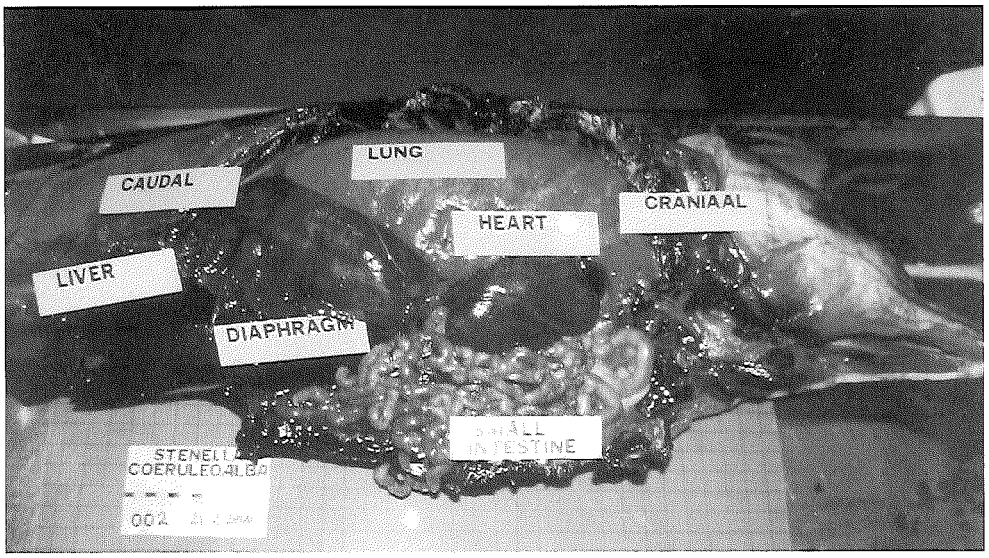


Figure 2 The right side of the striped dolphin, showing the fore stomach in the thoracic cavity after the intestines were removed.

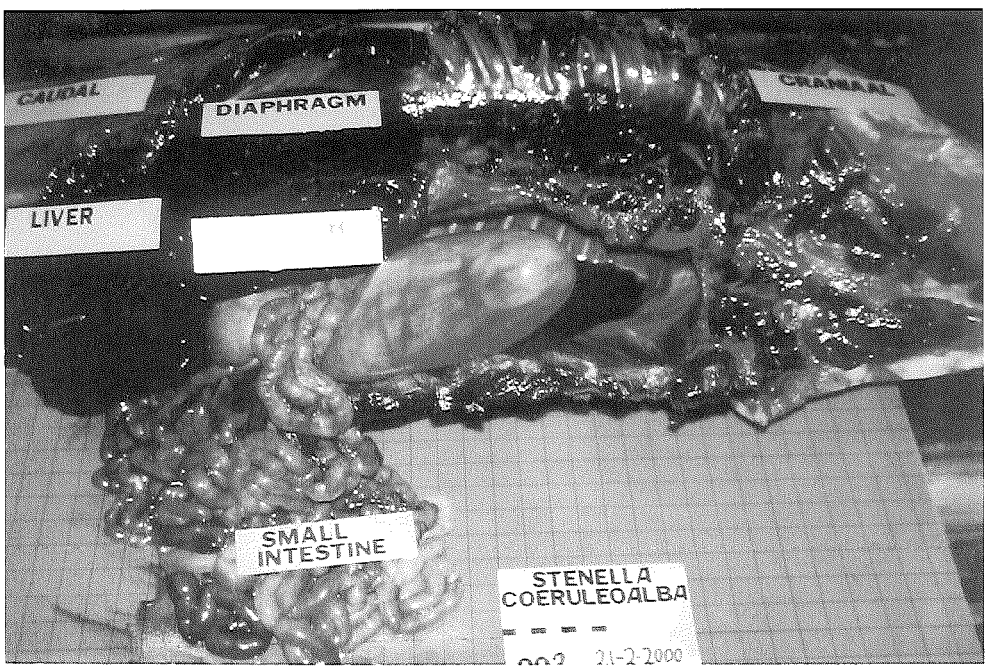


Figure 3 The left and right lungs of the striped dolphin. Note the clear asymmetry.

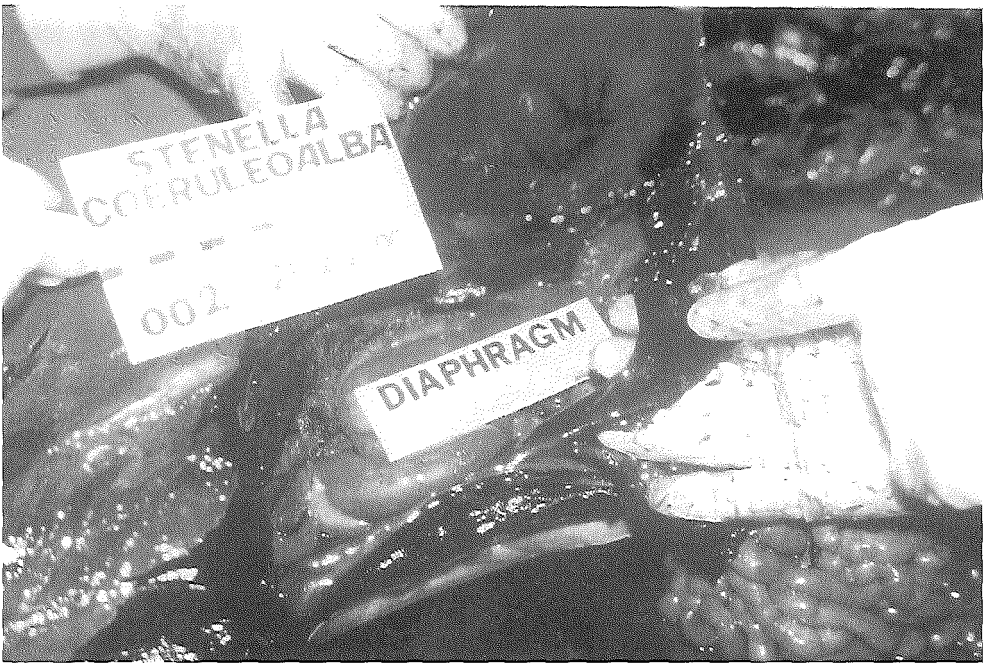


Figure 4 The hole in the left side of the diaphragm (indicated by the fore and middle fingers of the researcher) through which the stomachs and intestines were protruded into the thoracic cavity.

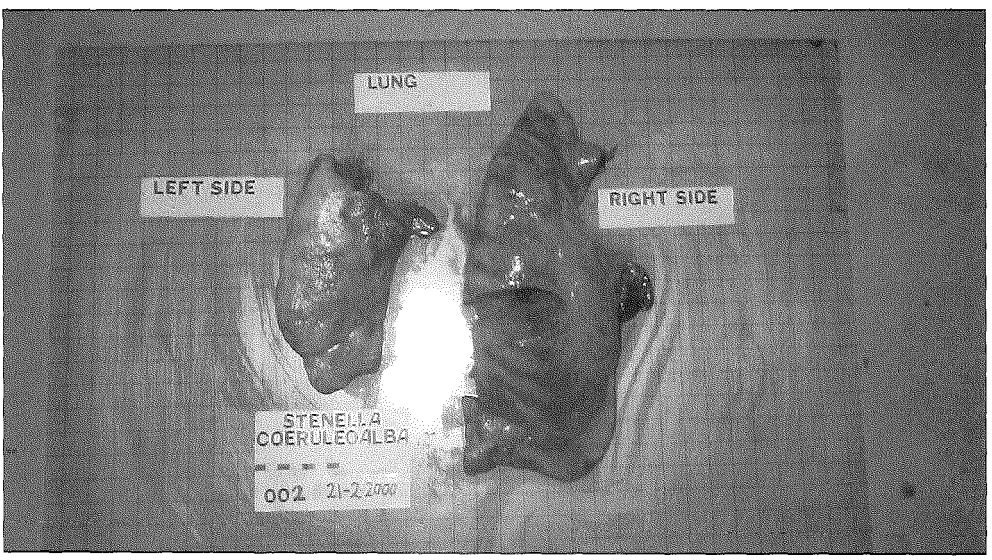
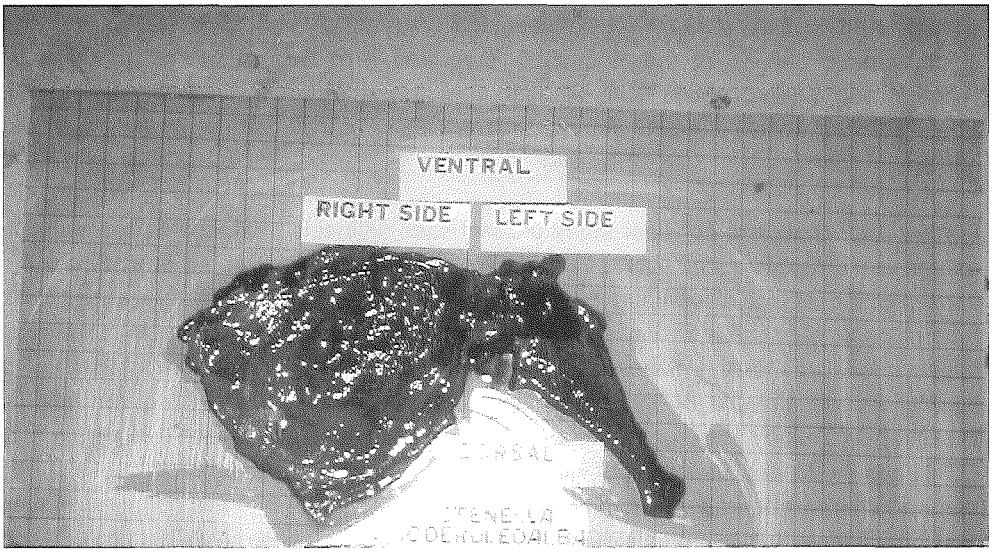


Figure 5 The left and right sides of the diaphragm of the striped dolphin. Note the clear asymmetry.



Discussion

It is remarkable that the animal was in such good condition when it stranded. It must have been feeding recently, despite the fact that its left lung was half the normal size, and that after eating a meal the fore stomach must have reduced the volume of the left lung further. Also it is curious that congestion did not occur sooner. Maybe the congestion which led to the animal's death was due to growth of the internal organs.

Congenital Diaphragmatic Hernia (CDH) is a severe birth defect in humans, characterized by a combination of failure in the formation of the diaphragm, unilateral or bilateral lung hypoplasia and postnatal pulmonary hypertension. The prevalence of CDH is about 0.3 per 1000 births with high mortality rates, in particular in cases with associated malformations (Torfs, Curry et al. 1992). Although significant efforts have been undertaken to unravel the pathophysiology of CDH, our current understanding of the etiology remains spare. The most common anatomical type of CDH is the posterolateral defect or Bochdalek hernia, which represents about 96 % of the cases. The defect of the diaphragm is predominantly unilateral and on the left side. In animals, spontaneous occurrence of CDH has been reported, mostly in case reports. Diaphragmatic defects are found in albino rats (Andersen 1941), rabbits (Pasquet 1974), dogs and cats (Wilson, Newton et al. 1971) and also in golden lion tamarins. In the latter there is a familial occurrence of ventromedial to lateral diaphragmatic hernia's (Bush, Montali et al. 1980). However, there is also a teratogen which induces diaphragmatic

hernia in rats. The herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether), when administered to the pregnant rat between the 9th and 11th day of gestation, induces diaphragmatic defects of the Bochdalek type and lung hypoplasia in the offspring (Costlow and Manson 1981; Kluth, Kangah et al. 1990; Tenbrinck, Tibboel et al. 1990). Thus, both environmental and genetic factors can be considered as important in the etiology of CDH.

Further investigation to consider in the present case animal is teratogenic analysis such as analysis of toxicological agents in serum or plasma or in fat tissue. For instance, the concentration of polychlorinated biphenyls (PCBs) will be measured because of its resemblance of nitrofen in its mechanism of action and general formula. One may expect a similar effect on diaphragm development. Since we also have material of a healthy striped dolphin of the same age, we can compare these concentrations. However, in a study in humans, we could not find increased PCB-levels in mothers of CDH patients (M. van Dooren et al, other research project, chapter 9 of this thesis).
Also, another consideration is to study the chromosomal pattern in this dolphin because the combination of CDH and micropenis suggests a genetic syndrome. This combination has been observed in humans as well, sometimes in combination with a deletion of the terminal part of the long arm of chromosome 15. Although a certain degree of homology exists between human and dolphin chromosomes (Bielec, Gallagher et al. 1998), it will be difficult to find small deletions.

In conclusion, the finding of this dolphin with CDH and a micropenis is unique. Since tissue and blood from this dolphin is available, further toxicological and genetic studies can be considered.

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- General discussion
- Summary/Samenvatting
- CV
- Dankwoord
- Stellingen

Part 4

CHAPTER 12

GENERAL DISCUSSION

CDH remains a birth defect with major clinical problems. Its cause, is poorly understood, although a variety of significant efforts have been undertaken to unravel its etiology.
The overall aim of the studies presented in this thesis was to obtain more knowledge on the etiology of CDH. Therefore a number of studies were performed with the following aims:

1. To investigate developmental processes, such as lung vascular morphogenesis, that may have a role in the etiology of CDH.
2. To evaluate the benefit of postmortem examination, in CDH-To evaluate the role of epidemiological studies in our understanding of the etiology in CDH, by studying the co-occurrence of CDH with limb defects and with esophageal atresia/ tracheoesophageal fistula.
3. To determine predictive factors of severity and survival in the prenatal diagnosis of CDH. Can parameters such as lung-head ratio predict the clinical outcome of CDH-patients.
4. To evaluate some of the genetic components of CDH; in particular the chromosomal and monogenic syndromes with CDH.
5. To test the role of environmental factors in the development of CDH. Are levels of hydroxylated PCB relevant for the etiology of CDH?

Animal studies

Developmental biological studies in animals can be of help to investigate the molecular background of processes such as lung and diaphragm morphogenesis in humans. From chapter 1 it can be concluded that the re-evaluation of 'common' textbook knowledge, such as the development of the early vascularisation of the lung, can give us new insights into developmental processes of early foregut formation. .Additionally, animal studies of gene loss of function have recently reported interesting findings concerning CDH. For example, *Slit3* $^{-/-}$ mice develop hernias through the septum transversum. *Slit3* encodes a member of the Slit family of guidance molecules and is expressed predominantly in the mesothelium of the diaphragm during embryonic development (Yuan, Rao et al. 2003). Remarkably, these *Slit3* $^{-/-}$ mice have other malformations including several grades of kidney agenesis in 20% of the cases (Liu, Zhang et al. 2003) and an enlarged right ventricle in the heart. *SLIT* is an interesting candidate gene because CDH in humans is often seen in co-occurrence with kidney defects, as observed in our study of CDH in combination with EA/TEF (chapter 4). The prevalence of kidney abnormalities was 35% in that study.

Another gene which might be involved in the pathogenesis of CDH is Friend of Gata 2 (*fog2*). *Fog2* $^{-/-}$ mice die of the circulatory consequences of tricuspid atresia (Svensson, Huggins et al. 2000). Additionally they have an eventration-like deformity of the diaphragm (Ackerman et al., personal communication). This is interesting because, in humans, CDH is often associated with heart defects.

Genes involved in vitamin A metabolism can be promising for further studies of CDH. For instance, retinal dehydrogenase-2 (RALDH2), a key enzyme necessary for the production of retinoic acid, expressed in the developing diaphragm, is inhibited by nitrofen and by other compounds that induce CDH (Mey, Babiuk et al. 2003). While in humans, RALDH2 is located on 15q21.2, the 15q deletions co-occurring with CDH usually are usually located towards the terminal part of chromosome 15q. Based on the deletion we found in our study (chapter 8) on 15q26.1-q26.2, we concluded that at least in our cases RALDH2 can not be the candidate gene. However, this 15q24-26 region harbors other genes involved in retinoic acid metabolism, i.e. CRABP1 (Cellular Retinoic Acid Binding Protein-1)(15q24), and RLBP1 (Retinaldehyde-binding protein 1)(15q26). These genes can be candidate genes for CDH, although our deletion only points to RLBP1, which will be inestigated in the next future.

Besides these mouse models, the spontaneous occurrence of CDH in other animals can be used for both environmental and genetic studies. In golden lion tamarins (a monkey) CDH has been observed and a genetic factor suggested (Bush, Montali et al. 1980).
The fact that different strains of animals have different incidences of CDH suggests differences in genetic susceptibility. We realize that animal data on either induced or spontaneous CDH, can never be completely extrapolated to humans. For example, Andersen observed isolated CDH in the offspring of Long Evans strains of rats administered a vitamin A deficient diet In contrast, Warkeny observed CDH associated with cardiovascular, urogenital, ocular and respiratory malformations in the offspring of rats of the Sprague-Dawley and Albino Farms strains (Andersen 1941; Warkany and Roth 1948; Wilson, Roth et al. 1953)

Epidemiological studies

Case ascertainment may be of importance for the evaluation of the phenotypes of CDH. In chapter 2 we studied a population of post-mortem CDH cases. The number of associated anomalies in our study was much higher (59%), than in a study of all CDH cases in the population of births (43%) (Torfs, Curry et al. 1992). A high incidence of MCA of 73% in fetuses at prenatal diagnosis has been reported by others (Bollmann, Kalache et al. 1995) The incidence of chromosomal anomalies in a group of prenatally diagnosed cases is also higher than the incidence in the total CDH population (Manni, Heydanus et al. 1994). These differences in outcome of these results, show that the choice of the study group can affect the outcome of a study. To obtain an evaluation of the phenotypic expression of CDH in an area, cases should include those diagnosed prenatally, by autopsy and at birth. Based on our findings in chapter 3 a post-mortem examination of a deceased infant with CDH should be performed, after permission of the parents.

Databases

In chapter 3 and 4, the registry of the California Birth Defects Monitoring Program (CBDMP) is given as an example of the usefulness and importance of such databases in epidemiological studies. In the northern part of the Netherlands, there is a registry of birth defects that sends their data to both EUROCAT (European Registration of Congenital Anomalies and Twins registry) and the International Clearinghouse for Birth Defects, which registers birth defects on a voluntary base since 1981. These two large databases are used to investigate possible environmental etiologies, to evaluate the results of prevention strategies and prenatal diagnoses, and to conduct various research projects. Unfortunately, a birth defects registry exists only in the northern part of our country, not nationally as in a few other European countries such as Sweden.

Since 1999, in the Erasmus MC Sophia hospital, we have used a database integrating all CDH patients admitted after birth, most of the CDH cases from prenatal diagnosis services, and cases from the pediatric pathology and genetic clinics. This database was created to register also patients with congenital defects, in particular diaphragmatic hernia, with a focus on associated birth defects, genetic information, obstetrical history and surgical procedures. For almost every CDH patient identified, with or without associated anomalies, a clinical geneticist with experience in dysmorphology was consulted throughout the whole study period. This database registers at this moment only patients with CDH and EA but it is desirable that all patients with a congenital anomaly –including prenatally diagnosed cases, terminations of pregnancy, and stillborns– should be registered in this database.

Internationally, a CDH Study Group has been set up that collects data on CDH live born cases from collaborators from 80 centers worldwide with 2268 registered to date. Detailed information on genetic diagnoses, if any, prenatal diagnosis, treatment modalities and outcome are collected on special forms. IRB permission is given in all centers.
This database can be used to perform larger studies, for instance, to evaluate the occurrence of syndromes or associated anomalies, or to identify rare syndromal cases. Through use of this database, Neville *et al* have identified 23 cases (1.3%) of Fryns syndrome in a group of 1833 CDH patients (Neville, Jaksic et al. 2002).

Teratology research

Searching for environmental causes of congenital malformations is very difficult. Only major outbreaks of teratogenic effects are usually recognized, as happened in the case of thalidomide. Other examples are the atomic bombs in Hiroshima and Nakasaki causing small-head size (microcephaly) in the children (Miller and Blot 1972), and also the incidence of heart and eye defects associated with an outbreak of German measles in Australia (Gregg

1941). According to Wilson (Wilson 1973), reviewed by Kalter (Kalter 2003) the principles of teratology are:

1. Susceptibility to teratogens depends on the genotype of the conceptus and its interaction with the teratogen.
2. Susceptibility to teratogens varies with the developmental stage of the fetus at the time of exposure.
3. Teratogens act by specific mechanisms on developing cells and tissues to initiate a sequence of abnormal developmental events (pathogenesis).
4. The access of teratogens to developing tissues depends on the nature of the teratogen.
5. The manifestations of abnormal development are death, malformations, growth retardation and functional deficits.
6. Manifestations of abnormal development increase in frequency and degree as the dosage of the teratogen increases, from the level with no effect of the teratogen to lethality.

In animals, several teratogens can cause diaphragmatic defects, nitrofen being the most studied one. Extrapolation of results obtained from animal studies to the human situation has to be done with a certain degree of reservation. For example, the exact mechanism of nitrofen teratogenesis remains unknown. Also, different rat and mice strains have different sensitivity to nitrofen's effect. In humans, although associations between teratogens and CDH have been suggested (Torfs, Curry et al. 1992; Tibboel and Gaag 1996; Enns, Cox et al. 1998), we did not identify an association between OH-PCBs and CDH in our pilot study (Chapter 10).

However, one cannot exclude the possibility that our hypothesis on the role of OH-PCBs is not correct. Others have recently suggested that vitamin A deficiency could play a role in the etiology of CDH. There is now sufficient circumstantial and direct experimental evidence to warrant further testing of the retinoid-CDH etiologic hypothesis in humans (Greer, Babiuk et al. 2003; Mey, Babiuk et al. 2003). An investigation on the effect of different degrees of vitamin A deficiency could be a possible research goal. It would require using a well-defined study-design for measuring levels of vitamin A and RALDH2 in the bloods of mothers and children. In our pilot study there was a relatively large difference between cases and controls in the time interval between blood sampling and the birth of the child, which may have added some confounding to our results although the compounds were very stable and will not change within a year. In future studies, it will be important to use standardized protocols for blood sampling and administration of questionnaires. In a planned epidemiological study investigating environmental factors for cardiac defects, blood sampling of the child and mother will take place when the child is 18 months old. Following this protocol, we shall investigate the relative contribution of several environmental factors for a variety of congenital anomalies occurring in the Southwestern part of the Netherlands. Studying

environmental factors will be quite difficult to perform in an international collaborative effort since regional but even more important intercontinental differences in exposure may exist; an example are pollutants such as PCBs.

Comments on clinical genetic research

In this thesis we showed the benefits that result from the evaluation of the affected child by a clinical geneticist, which may lead to a correct clinical genetic diagnosis. As a consequence, every CDH patient in our hospital is evaluated by a clinical geneticist. Even more importantly, when there is no clear diagnosis at the time of birth, it is of great value to have the clinical geneticist reviews the patient after a few years, as the phenotypes of many syndromes tend to become more explicit over the years.

For our study, we used a research protocol (introduction) that includes the collection of DNA from the CDH patients and their parents. We also started a prospective study for the chromosomal analysis of CDH patients. According to the rules of the health insurance companies karyotyping is only carried out when there are more then one congenital malformation. Nevertheless, we karyotyped all new CDH patients. Because of the increasing quality of conventional chromosome analysis and because of the development of new cytogenetic techniques (FISH, spectral karyotyping (SKY) and CGH) we expect to find more chromosomal anomalies which were difficult to detect with the old techniques. However, in the future it might be more profitable to screen only for chromosomal regions known to be involved in CDH (1q, 3q, 4p, 8p and q, 15q). For these purposes, storage of patients' material, such as DNA and cell-lines, is of major importance for future studies. In case of death of the child, permission for an autopsy should always be asked from the child's parents. In chapter 3 we showed that other birth defects, such as cardiac, urogenital and gastrointestinal anomalies, were often identified only during autopsy. At that time, patient material can also be obtained for DNA analysis, as it is now possible to accomplish from paraffin embedded tissue. However, there are limitations for the type of research that can be carried out with these fragmented DNA.

We believe that studying specific combinations of CDH with other congenital anomalies, such as limb, kidney and heart defects, can be worthwhile because such studies may uncover genes whose mutations affect the developmental pathway of several organs. It is important to realize that CDH can be a variable feature in several syndromes, as is clearly illustrated in cases of Fryns syndrome and Donnai-Barrow syndrome. The variable expression of CDH may then erroneously lead to splitting cases with the same gene mutation into different syndromes, whereas lumping those cases into one syndrome would be more accurate. The genes involved in these syndromes will potentially shed further light on the developmental pathways underlying CDH and may be useful in the understanding of the non-syndromal form of CDH as well.

What are the perspectives for future genetic studies of isolated CDH?

The study design of choice (family-based versus population-based, inbred versus outbred populations, linkage-based versus association-based) is primarily based on the type of families and the number of patients that are available.

Large multigenerational families with CDH are not available because mortality is high and the reproductive fitness for CDH-patients is low. Therefore classical linkage approaches are not possible. Affected sib pair analysis, an alternative to the linkage-based approach, studies series of affected siblings with a genomic wide screen might be instrumental for the identification of genes in six candidate areas of the genome(1q,3q, 4p, 8p and 8q and 15q).

The degree of sharing of marker alleles between affected siblings is compared; values exceeding the expected value of shared alleles are compatible with a disease locus nearby. The major advantages of this method are that multigenerational families are not needed and that a specific genetic model does not have to be defined. The disadvantages are that high numbers of siblings are often needed when the susceptibility loci confers a low relative risk and that the regions found to be linked are large (Risch and Merikangas 1996).Furthermore, since few sib pairs are available, this study design is of limited value for CDH.

CDH is a multifactorial congenital anomaly. This necessitates other approaches for genetic study design. Genome-wide or locus specific single and multi-locus association studies might be considered as the next step in mapping CDH susceptibility genes. Candidate genes include genes in the retinoic acid metabolism, such as the RAR alpha and beta, RLBP1, CRABP1 and Raldh2, and other genes such as *SLIT3* (5q35), and *FOG2* (8q22), and positional candidate genes on 15q26. These kinds of studies enable the identification of possible haplotypes associated to putative genes and ancestral disease mutations. Genetic association studies can be performed in a case-control design or in a trio design (an affected child and both parents). Distortion in transmissions of single alleles or haplotypes from heterozygous parents to their offspring (Transmission Disequilibrium Test) is then investigated (Ewens and Spielman 1995). A disease-associated allele or haplotype will more frequently than not be transmitted to the affected individual. This design can be used for further studies on CDH. We started suing this method by collecting DNA of more than 40 trios. Collaboration with members of the CDH study group is necessary in order to collect high enough numbers of trios necessary for this type of studies.

Overall conclusions from this thesis

1. In contrast to prevailing text books, the lung's vascular development occurs by formation of new capillaries from pre-existing vessels as the lung bud grows as a network (distal angiogenesis). (chapter 2)
2. Autopsy can detect additional structural cardiac defects, malformations of the urogenital system and digestive tract in children with CDH. (chapter 3)
3. The co-occurrence of LRDs and CDH in two populations suggests an early embryological insult affecting both precursor anlages , and suggests researching candidate genes or environmental factors in amimal and human studies
4. The co-occurrence of CDH with EA/TEF and of LH with EA/TEF is higher than expected and suggest shared development genes, including genes that affect the branching morphogenesis of both lung and kidney. (chapter 5)
5. The lung-head ratio (LHR) is shown to be a good predictor for fetal outcome, independent of gestational age at the time of the measurement. LHR > 1.4 is associated with 100 % survival and LHR < 1 is associated with 100% mortality (chapter 6).
6. CDH has a locus on chromosome 15q26.1-q26.2. This region harbors RLBP1, which can be a good candidate gene for certain forms of CDH (chapter 8). Further confirmation is necessary to come to definitive conclusions.
7. CDH can be part of rare monogenic syndromes, such as the craniofrontonasal syndrome or as a combination with a laterality defect, and therefore the differential diagnosis of CDH should be expanded. (chapter 9)
8. OH-PCBs levels in the maternal blood of CDH patients are not different from levels in mothers of controls and therefore they are not thought to be a risk factor for CDH (chapter 10)

Finally, we feel that every health professional involved in the care of CDH patients, may use this protocol:

Protocol for the CDH patient
1. Clinical genetic evaluation by a clinical geneticist (preferably experienced in dysmorphology)
2. Storage of DNA, and cell-lines
3. Karyotyping
4. Autopsy by a pediatric pathologist, with a clinical geneticist involved
5. Clinical pictures
6. Additional investigations, X-ray, MRI if an autopsy is refused by the parents
7. Registration in an (international) database
8. Long-term follow-up, re-evaluation by clinical geneticist to identify changes in phenotype

It is likely that interaction between basic and clinical research will continue to play an essential role in the study of the etiology, pathogenesis, prevention, diagnosis and treatment of CDH. Knowledge on teratogenic agents and CDH susceptibility genes and their role in developmental pathways will be forthcoming, eventually resulting in improvements of treatment of patients, risk estimates and of prevention of CDH.

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CHAPTER 13

SUMMARY / SAMENVATTING

SUMMARY

Congenital Diaphragmatic Hernia (CDH) is an important cause of mortality and morbidity in the Neonatal and Surgical Intensive Care Unit. It consists of a defect in the formation of the diaphragm, a variable amount of pulmonary hypoplasia and abnormal pulmonary vascular resistance after birth. The most common anatomical type of CDH is the posterolateral defect or Bochdalek hernia, which represents about 96% of the cases. Multifactorial inheritance is suggested but the etiology of CDH remains largely unknown. In 40-50% of the cases, multiple congenital anomalies (MCA) are observed, such as cardiac, urogenital and limb defects. Chromosomal aberrations are observed in some of the cases, in particular in CDH patients with multiple congenital anomalies (MCA). The most common observed genetic abnormalities among CDH patients with MCA are trisomy 18 and trisomy 13. Environmental factors have been suggested, following reports of an animal model for CDH in which the administration of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen) to pregnant rats was followed by the birth of offspring with a diaphragmatic hernia. Until now, there is no proof that teratogens cause CDH in humans.
In this thesis the focus is on genetic and environmental factors potentially involved in the etiology and pathogenesis of CDH.

This thesis consists of four parts. *Part 1 (Chapter 1* to *6)* contains the introduction and general aspects of CDH and includes epidemiological studies of CDH associated anomalies. *Part 2 (Chapter 7* to *9)* describes studies on genetic factors of CDH. *Part 3 (Chapter 10* and *11)* describes potential environmental factors in the etiology of CDH. *Part 4* contains the discussion and a summary or my research.

In Part 1 general aspects of CDH are discussed. *Chapter 1* is a general introduction that addresses the question: 'What is CDH?' We give a short review of points of view of various specialists involved in the care or study of patients with CDH. These specialists include embryologists, toxicologists, molecular geneticists, epidemiologists and clinical geneticists, pediatric pathologists and ultrasonographers. We conclude with the points of view of the attending physicians, including neonatologists, pediatricians and pediatric surgeons, who are involved in the postnatal care of the CDH-patient.

Chapter 2 describes the results of an observational study on the development of the lung vasculature in relation to the developing airways in mice, because opposing theories about this mechanism exist. With an ontogenic morphological analysis of lungs of Tie2-LacZ transgenic mice and wild type mice, we show that already at the earliest morphological signs of lung development the vasculature is a network connected to the embryonic circulation. We conclude that the vasculature is part of the embryonic circulation from the moment the lung starts to develop. The chapter ends with a new concept for early pulmonary vascular morphogenesis: Capillary networks surround the

terminal buds and expand by formation of new capillaries from pre-existing vessels as the lung bud grows, a process we called distal angiogenesis.

Chapter 3 describes a study on the post-mortem findings on a group of 39 CDH patients from a single center level III perinatal facility in the period 1988-2001. We compared the clinical data to the autopsy data for the prevalence of associated congenital anomalies. Clinically well established dysmorphic features, and skeletal and CNS anomalies were confirmed by autopsy. However, after autopsy, cardiac, urogenital and gastrointestinal anomalies were observed that were not diagnosed in the clinical setting. Therefore we concluded that post-mortem examination has a significant additional role in the detection of structural cardiac defects, anomalies of the urogenital system and the digestive tract in children with CDH, and recommend postmortem examination for every deceased CDH-patient.

Chapter 4 describes the results of an epidemiological descriptive study of CDH and limb defects, from data from a birth defects registry in California (n = 846) and from our hospital (n = 146). In the hospital group, 14 cases (10%) had an associated limb defect of which about one third were limb reduction defects (LRD), mostly of a non-severe type, such as hypoplasia of fingers. In the registry group, 162 (19%) had a limb defect of which 18 cases were severe LRD, usually of the upper extremities. Additional congenital anomalies were observed in all CDH-LRD cases in both groups. In the registry group, 78% of LRDs were either bilateral or ipsilateral, and mostly pre-axial, suggesting an early embryological insult affecting both precursor anlages. We suggested a developmental association between CDH and LRD, which has also been observed in knockout mice for the Wilms' Tumour 1 Gene (WT1) and for the c-met receptor gene. Analysis of candidate genes of patients with CDH and LRD for genes suspected to be involved in the pathogenesis of both organs may, in the future, elucidate this developmental association in humans Epidemiological studies suggest possible pathogeneses from the co-occurrence of defects or even from their distribution in the population.

Chapter 5 describes another epidemiological study in which we studied the co-occurrence of CDH, lung hypoplasia (LH) and esophageal atresia (EA) with or without tracheoesophageal fistula (TEF) in a large California birth registry with 3,318,966 live births and stillbirths, from 1983 to 1996. Traditionally these defects have been analyzed separately in epidemiological studies. LH, part of the CDH spectrum, is not usually associated with EA/TEF, yet both are foregut malformations. A Bochdalek type CDH (0.13 per 10,000 births) was found in 433 cases, whereas 893 had EA/TEF (0.27 per 10,000 births), and 646 had LH (0.19 per 10,000 births). Among them, 17 cases had CDH with EA/TEF (0.01 per 10,000 births), and 53 had EA/TEF and LH (0.02 per 10,000 births). Both prevalences are significantly higher than expected. Most infants had

additional severe congenital defects whose proportions were similar in both groups, except for recognized syndromes which were higher in the EA/TEF with LH group. We then reviewed published studies, mostly from experimental animal models, that have reported factors, either genetic or environmental, that affect the development of one or more of these organs. We then discussed the two prevailing hypotheses on the pathogenesis of CDH.

Chapter 6 contains the results of an analysis of the prognostic significance of the lung-to-head ratio (LHR) and other prenatal parameters on the outcome of fetuses with an isolated left-sided congenital diaphragmatic hernia (CDH) without chromosomal abnormalities. Survival was defined as discharge from the hospital. The overall survival rate was 50%. There was a statistically significant difference between the mean LHR of the survivors compared to the mean LHR of the non-survivors (1.8 vs. 1.0), whereas the mean gestational age of these two groups did not differ. LHR was not dependent on gestational age in the prediction of fetal outcome. The cut-off levels LHR < 1, 1-1.4, > 1.4 showed a good applicability in the prediction of fetal outcome within the present study population with a 100% survival if LHR > 1.4 and a 100% mortality if LHR < 1. An intrathoracic position of the stomach, mediastinal shift, and polyhydramnios, diagnosis before 25 weeks' gestation were shown to be poor sonographic predictors for fetal outcome. We concluded that LHR is a good predictor for fetal outcome, independent of gestational age at time of the measurement.

Part 2 deals with several genetic aspects of CDH. *Chapter 7 and 8* deal with chromosomal abnormalities associated with CDH. In *chapter 7* a patient is described with Wolf-Hirschhorn syndrome (WHS), a chromosomal disorder characterized by retarded mental and physical growth, microcephaly, Greek helmet appearance of the facies, and seizures/epilepsy. Closure defects of lip or palate, and cardiac septum defects occur in 30-50% of cases. Its cause is a deletion in the short arm of chromosome 4. Our patient showed unilateral cleft lip and palate, hypertelorism, a right-sided ear tag and mild epispadia. Due to acute respiratory distress and acute bowel obstruction requiring emergency laparotomy, a left-sided Bochdalek CDH was diagnosed. Regular karyotyping was normal. However, FISH analysis showed a microdeletion in the short arm of chromosome 4 (4p-), consistent with WHS. A combination of this syndrome with CDH has been rarely described. The life-threatening CDH, although not common in WHS, leads to the diagnosis of WHS relatively early in life. We strongly recommended a clinical genetic evaluation of each CDH patient with facial anomalies suggestive of the 4p- deletion syndrome.

In *chapter 8* we describe the co-occurrence of CDH with a chromosome 15q deletion. A survey of our cytogenetic data of over 150 karyotyped CDH patients revealed three CDH cases with a small deletion of chromosome 15 (15q24-26). Furthermore, we obtained

material of four additional, published CDH patients with a chromosome 15q24-26 deletion. In these seven cases we used FISH and CGH to investigate this region. We found a common small deletion of chromosome band 15q26.1-q26.2. RLBP1 (retinol binding protein1) is located in this part of the chromosome. If this gene is involved in the pathogenesis of CDH, it might support the hypothesis of a retinoic acid involvement in the formation of the diaphragm, as is implicated by teratogen-induced rodent mouse models of CDH. However, RALDH2 and MEF2a, both suggested as candidate genes for CDH by others, are not located in that region or chromosome 5. Further analysis is needed to come to definitive conclusions.

Many syndromes can be listed in the syndrome diagnosis for CDH. Accurate clinical (genetic) observation of every CDH patient can lead to prompt syndrome diagnosis. Two cases are reported in *chapter 9*.
In *chapter 9a,* a case report describes a female patient with CDH and craniofrontonasal syndrome or dysplasia (CFNS). This X-linked syndrome is characterized by craniosynostosis of the coronal sutures, hypertelorism, longitudinal grooves of the nails and various skeletal abnormalities (e.g. syndactyly of toes). Our patient is the first female described with the combination of CFNS and (left-sided) CDH, with clinical features such as hypertelorism, a broad nose, facial asymmetry, grooved nails and widely spaced nipples. X-rays showed craniosynostosis of the coronal suture on the left side. MRI showed an agenesis of the corpus callosum. Cytogenetic analysis was normal. This case report confirms that CFNS should be added to the expanding list of syndromes that include CDH as an infrequent feature.
In *chapter 9b*, we report for the first time a patient with a right-sided Bochdalek CDH and situs inversus totalis. No other anomalies or abnormal karyotype were found. We described the need for contralateral cannulation for the institution of ECMO. The individualized approach for ECMO cannulation resulted in an uneventful clinical course. We reviewed the literature and found only two case reports describing laterality abnormalities in combination with a right-sided diaphragmatic defect. However these concerned eventrations in combination with other midline defects such as a total laryngo-tracheo-esophageal cleft or a heart defect such as an atrial septum defect. We reviewed genes involved in laterality defects. This syndrome should be added to the differential diagnosis of CDH.

Part 3 discusses environmental factors involved in the etiology of CDH. In *chapter 10* a pilot study evaluated the role of specific teratogens in the development of CDH. We compared (OH) PCB levels in the blood of mothers of CDH patients and in mothers of controls. Hydroxylated polychlorinated biphenyls (OH-PCBs) resemble Nitrofen, which causes CDH in an animal model. Therefore we hypothesized that the concentration of PCBs would be higher in mothers of infants with CDH than in mothers of controls. We analyzed levels of several PCBs (p,p-DDE, PCB153, 4OH-PCB107, 4OH-PCB146,

4OH-PCB187 and pentachlorophenol) by gas chromatography using a ECD detector. Neither unadjusted analyses nor multiple regression analyses with adjustment for covariates revealed an association between high maternal serum PCB levels and an increased risk of getting a child with CDH. We concluded that these results do not suggest that high levels of PCBs in maternal blood are a risk factor for the development of CDH in the fetus.
In *chapter 11* we described the results of a unique finding of CDH in a male striped dolphin (*Stenella coeruleoalba*) which was stranded along the Dutch coast. The animal was estimated to be between two and three years old. The dolphin was still alive but died during rehabilitation in the Dolfinarium Harderwijk. Autopsy revealed both the stomachs and the intestines located in the left thoracic cavity, indicating a lef-sided CDH and left-sided lung hypoplasia. The animal had hypoplasia of the penis (less then 10% of a normal sized penis of an animal of this age and size). We discussed the occurrence of CDH in other animals and speculated on further environmental and genetic investigation in this dolphin since biologic material from this animal and from control animals are available.

In chapter 12 we discussed our findings. We also reviewed possible candidate genes for CDH such as Slit3, Fog2 and RALDH2. We discussed the approaches of epidemiological, teratogenic and clinical genetic research in the study of etiological factors for CDH. We ended with a recommendation for a protocol for future hospital based studies of CDH.

Overall conclusions:

1. In contrast to prevailing text books, the lung's vascular development occurs by formation of new capillaries from pre-existing vessels as the lung bud grows as a network (distal angiogenesis). (chapter 2)
2. Autopsy can detect additional structural cardiac defects, malformations of the urogenital system and digestive tract in children with CDH. (chapter 3)
3. The co-occurrence of LRDs and CDH in two populations suggests an early embryological insult affecting both precursor anlages , and suggests researching candidate genes or environmental factors in animal and human studies
4. The co-occurrence of CDH with EA/TEF and of LH with EA/TEF is higher than expected and suggest shared development genes, including genes that affect the branching morphogenesis of both lung and kidney. (chapter 5)
5. The lung-head ratio (LHR) is shown to be a good predictor for fetal outcome, independent of gestational age at the time of the measurement. LHR > 1.4 is associated with 100 % survival and LHR < 1 is associated with 100 % mortality (chapter 6).

6. CDH has a locus on chromosome 15q26.1-q26.2. This region harbors RLBP1, which can be a good candidate gene for certain forms of CDH (chapter 8). Further confirmation is necessary to come to definitive conclusions.
7. CDH can be part of rare monogenic syndromes, such as the craniofrontonasal syndrome or as a combination with a laterality defect, and therefore the differential diagnosis of CDH should be expanded. (chapter 9)
8. OH-PCBs levels in the maternal blood of CDH patients are not different from levels in mothers of controls and therefore they are not thought to be a risk factor for CDH (chapter 10)

SAMENVATTING

Congenitale hernia diafragmatica (CHD) is een ernstige aangeboren afwijking die voor een hoge mortaliteit zorgt op de neonatale en kinderchirurgische intensive care. CHD wordt gekenmerkt door onderontwikkeling van de longen en een verhoogde pulmonale vaatweerstand na de geboorte. De meest voorkomende vorm van CHD (96%) is het Bochdalek type, dit is een defect aan de posterolaterale zijde van het diafragma. Er wordt gedacht dat CHD een multi-factoriële aandoening is, echter is er nog zeer weinig bekend over de etiologie. In 40-50% worden geassocieerde aangeboren afwijkingen gezien, zoals hart-, urogenitale- en ledemaatafwijkingen. Chromosoomafwijkingen worden in een aantal CHD casus gezien, meestal bij patiënten met meerdere aangeboren afwijkingen. De meest voorkomende zijn trisomie 18 en trisomie 13. Naast genetische factoren, wordt er ook een rol toebedacht aan omgevingsfactoren. Het proefdier model voor CHD bestaat uit de toediening van het landbouwgif Nitrofen, 2,4-dichlorophenyl ether aan zwangere ratten, waardoor dit tot een hernia diafragmatica leidt bij het nageslacht. Er is tot nu toe echter geen bewijs dat toxische stoffen tot een CHD leiden bij de mens.

Dit proefschrift bestaat uit 4 delen. Deel 1 (hoofdstuk 1 t/m 6) bevat de introductie en algemene aspecten van CHD. Deel 2 (hoofdstuk 7 t/m 9) beschrijft de studies over genetische aspecten van CHD. Deel 3 (hoofdstuk 10 en 11) bespreekt het belang van omgevingsfactoren voor het ontstaan van CHD en deel 4 bevat de discussie en de Engelse en Nederlandse samenvatting.

In deel 1 worden algemene aspecten besproken van CHD. Hoofdstuk 1 is een algemene introductie met de algemene vraag: 'Wat is CHD?'. We geven een kort overzicht van de benadering van verschillende soorten specialisten die betrokken zijn in de zorg van CHD-patiënten. Basale wetenschappers zoals embryologen, toxicologen, moleculair genetici en epidemiologen, gaven hun inzichten, gevolgd door de visie van klinisch genetici, kinderpathologen en prenatale echo-artsen. Dit overzicht eindigt met een beschrijving van de artsen die betrokken zijn bij de behandeling van het kind met een CHD, zoals de kinderartsen, neonatologen en kinderchirurgen.

Omdat CHD samengaat met onderontwikkeling van de long, en met vaatproblemen van de longen, en er theorieën bestaan dat longhypoplasie ten grondslag ligt aan CHD, hebben wij de vaatontwikkeling in de vroege longrijping onderzocht. Hoofdstuk 2 beschrijft de resultaten van een observationele studie naar vaatontwikkeling in de long in relatie met de zich ontwikkelende luchtwegen in een muizenstudie. Er bestaan tegengestelde theorieën over longvaatontwikkeling en de longcirculatie. Met een morfologische analyse van Tie2-LacZ transgene muizen en 'wild-type' muizen, lieten we zien dat al op het moment van eerste morfologische tekenen van longontwikkeling, een

netwerk van vaten zich ontwikkelt, verbonden met de embryonale circulatie. We concludeerden dat het vasculaire netwerk zich ontwikkelt vanaf het eerste begin van longontwikkeling. Een nieuw concept voor pulmonale vaatontwikkeling wordt gelanceerd met de term 'distale angiogenese'. Capillaire netwerken omgeven de terminale vertakkingen van de luchtwegen en breiden zich tijdens de groei van de long, uit door de vorming van nieuwe capillairen uit al bestaande vaten.

Hoofdstuk 3 beschrijft een studie van de obductiegegevens van 39 overleden CHD-patiënten uit ons ziekenhuis in de periode 1988-2001. We hebben de klinische gegevens met betrekking tot het voorkomen van aangeboren afwijkingen vergeleken met de uitkomsten van postmortem-onderzoek. Klinisch vastgestelde dysmorfe kenmerken, afwijkingen aan het skelet en aan het centraal zenuwstelsel werden bevestigd, zonder nieuwe en/ of andere bevindingen, na postmortem-onderzoek. Echter, autopsie liet wel nieuwe bevindingen zien van hartafwijkingen en van afwijkingen aan de tractus digestivus en urogenitalis. Hieruit concludeerden wij dat postmortem-onderzoek een toevoegende waarde heeft in het opsporen van deze afwijkingen en dat bij het overlijden van een patiënt met CHD gedacht moet worden aan autopsie.

De resultaten van een epidemiologische beschrijvende studie over CHD en ledemaatafwijkingen in een geboorteregistratie in Californië (n = 846) en in ons ziekenhuis (n = 146) worden beschreven in hoofdstuk 4. In de ziekenhuis groep, hadden 14 kinderen (10%) een geassocieerde ledemaatafwijking, waarvan ongeveer een derde een verkorting van een deel van de ledematen heeft, meestal niet ernstig zoals hypoplasie van de vingers. In de registratie groep hadden 162 kinderen (19%) een ledemaatafwijking, waarvan 18 een ernstige vorm hadden van een ledemaatverkorting, meestal van de bovenste extremiteiten. In beide groepen werden andere aangeboren afwijkingen gezien. 78% van de ledemaat-reductie-afwijkingen kwamen bilateraal of unilateraal voor, en dan vaak aan de kant van de CHD. Dit suggereert een gezamenlijke embryonale ontwikkelingsstoornis. We hebben gespeculeerd over geassocieerde ontwikkelingsgenen.

Hoofdstuk 5 beschrijft een epidemiologische studie over het voorkomen van CHD in combinatie met longhypoplasie (LH) en oesofagusatresie (EA) met/zonder tracheo-oesophageale fistel (TEF) in een geboorteregistratie met 3.318.966 levend- en doodgeborenen in Californië, van 1983 tot 1996. Deze afwijkingen zijn tot nu toe alleen geïsoleerd onderzocht. Longhypoplasie, als onderdeel van het spectrum CHD, is meestal niet geassocieerd met EA/TEF. Een Bochdalek hernia werd gevonden bij 433, EA/TEF bij 893 en longhypoplasie bij 646 kinderen. Er waren 18 gevallen met zowel CHD als EA/TEF en 53 gevallen met EA/TEF en longhypoplasie. Deze prevalentie is hoger dan verondersteld, waardoor gesuggereerd kan worden dat er mogelijk een associatie bestaat. In de meeste gevallen werden meerdere afwijkingen gezien, vooral

de prevalentie van renale afwijkingen was hoog in de EA/TEF met longhypoplasie-groep. In de discussie is gezocht naar mogelijke genen betrokken bij beide afwijkingen.

Hoofdstuk 6 bevat de resultaten van een analyse naar het belang van de long-hoofd ratio (LHR) voor de prognose en tevens van andere prenatale parameters op de 'uitkomst' van foetussen met een geïsoleerde linkszijdige CHD zonder andere aangeboren afwijkingen of chromosoomafwijkingen. Overleving werd gedefinieerd als ontslag uit het ziekenhuis. Het overlevingscijfer was 50%. Er bestond een statistisch significant verschil tussen de gemiddelde LHR van de kinderen die overleefden en die niet overleefden (1.8 versus 1.0), terwijl er tussen de groepen geen verschil bestond in gemiddelde zwangerschapsduur. LHR was niet afhankelijk van de zwangerschapsduur in het voorspellen van de foetale uitkomst. De breekpunten < 1, 1-1.4 en > 1.4 bleken goed bruikbaar te zijn als voorspellende waarde voor de ernst van de CHD. In deze studiepopulatie betekende een LHR > 1.4 100% overleving tegenover een 100% kans op overlijden bij een LHR van < 1. Andere echografische voorspellende factoren voor een slechte uitkomst waren een intrathoracale maag, verschuiving van het medinastinum, polyhydramnion, individuele variabelen en een vroege diagnose (voor 25 weken). Uit deze studie concludeerden wij dat de LHR een goede voorspellende waarde heeft op de foetale uitkomst, onafhankelijk van zwangerschapsduur op het moment van de meting.

Deel 2 bevat studies over de genetische aspecten van CHD. Hoofdstuk 7 en 8 gaan over bepaalde chromosoomafwijkingen met CHD. In hoofdstuk 7 wordt een patiënt beschreven met Wolf-Hirsschorn syndroom (WHS). Dit is een chromosomale afwijking, met een deletie van de korte arm van chromosoom 4, die gepaard gaat met lichamelijke en geestelijke retardatie, microcefalie, Griekse helmschedel en epilepsie. Lip-/gehemeltespleten, hartafwijkingen treden op in 30-50%. Onze patiënt had een unilaterale lip-/gehemeltespleet, hypertelorisme, een rechtszijdige oor-tag en milde epispadie. Vanwege de respiratoire moeilijkheden en een acute buik, waardoor een operatie noodzakelijk was, werd een Bochdalek CHD gediagnosticeerd. Klinisch genetisch onderzoek liet de verdenking uitgaan naar een chromosoomafwijking. Normaal chromosoomonderzoek toonde geen afwijkingen. Bij FISH-onderzoek werd echter een microdeletie gezien van de korte arm van chromosoom 4. Een combinatie van WHS met CHD is niet vaak beschreven. We concludeerden dat ieder kind met een CHD onderzocht dient te worden door een klinisch geneticus en dat bij een CHD-patiënt met faciale afwijkingen gedacht moet worden aan WHS.

In hoofdstuk 8 wordt de combinatie van CHD met chromosoom 15 afwijkingen onderzocht. Analyse van de cytogenetische gegevens van meer dan 150 karyogrammen van CHD-patiënten, leverde 3 casus op met een deletie van chromosoom 15q24-26. Tevens hebben we materiaal verkregen van 4 gepubliceerde casus met CHD en 15q24-

26 deleties. Met FISH en CGH van deze 7 casus hebben we de deleties onderzocht. We vonden een gemeenschappelijke kleine deletie op 15q26.1-q26.2. RLBP1, een gen van belang bij vitamine A metabolisme, ligt in deze regio en kan belangrijk zijn voor de ontwikkeling van CHD, omdat er vanuit proefdiermodellen sterke aanwijzingen zijn dat vitamine A van belang is bij diafragma-ontwikkeling.
Andere kandidaat-genen, gesuggereerd in de literatuur, MEF2a en RALDH2 liggen niet in onze regio.

Hoofdstuk 9 beschrijft 2 casus met een syndromale vorm van CHD. In hoofdstuk 9a wordt een vrouwelijke patiënt beschreven met het craniofrontonasale syndroom (CFNS). Dit is een X-gebonden syndroom gekenmerkt door craniosynostose van de schedelnaden, hypertelorisme, longitudinale groeven van de nagels en verscheidene skeletafwijkingen (zoals syndactylie van de tenen). Dit is de eerste vrouwelijke patiënt die wordt beschreven met dit syndroom samen met een (linkszijdige) CHD, en bovengenoemde kenmerken. Röntgenonderzoek toonde craniosynstose van de linker kroonnaad. MRI toonde een agenese van het corpus callosum. Cytogenetisch onderzoek was normaal. Dit syndroom moet ook in overweging genomen worden bij een CHD-patiënt met geassocieerde afwijkingen.
Hoofdstuk 9b beschrijft een casus met een rechtszijdige CHD en een situs inversus totalis. Dit meisje had geen andere afwijkingen en een normaal karyogram. Extra-Corporale Mebraan-Oxygenatie was noodzakelijk. Door de situs inversus werden de lijnen aan de contralaterale zijde ingebracht. Alleen eventraties van het rechter diafragma zijn beschreven in combinatie met een situs inversus, met dan tevens andere middenlijnafwijkingen of een hartafwijking. Dit syndroom hoort ook in de differentiaal diagnose lijst van CHD.

In deel 3 worden aspecten besproken over omgevingsfactoren die een rol kunnen spelen in de etiologie van CHD. Hoofdstuk 3 beschrijft een studie naar de rol van (gehydroxyleerde) Polychloorbiphenylen (PCB´s) op het ontstaan van CHD. OH-PCB's lijken op het landbouwgif nitrofen dat CHD veroorzaakt in proefdieren. Daarom was onze hypothese dat moeders van kinderen met CHD hogere concentraties van deze stoffen zouden hebben in vergelijking met moeders van controles. We vergeleken p,p-DDE, PCB153, 4OH-PCB107, 4OH-PCB146, 4OH-PCB187 en pentachlorophenol tussen de verschillende groepen met gaschromatografie gebruikmakend van een ECD-detector. Noch enkelvoudige analyses noch multipele regressie modellen met correctie voor verschillende co-variabelen, lieten een associatie zien tussen een hoge concentratie van (OH)-PCB's en het optreden van CHD. Wij concludeerden dat deze resultaten niet suggereren dat hoge PCB-waarden in maternaal bloed een risicofactor zijn voor het ontwikkelen van CHD.

In hoofdstuk 11 beschreven we de unieke vondst van CHD bij een mannelijke gestreepte dolfijn (Stenella coeruleoalba) die is gestrand aan de Nederlandse kust. Het dier was ongeveer twee tot drie jaar oud en leefde nog bij de vondst, maar overleed in het Dolfinarium Harderwijk door ernstige respiratoire problemen. Autopsie toonde intrathoracaal gelegen magen en darmen aan de linker zijde en tevens linkszijdige longhypoplasie. Er was ook een micropenis zichtbaar. Lichaamsmateriaal is bewaard van dit dier zodat eventueel gezocht kan worden naar een oorzaak. We speculeerden over mogelijk oorzaken en bespraken het voorkomen van CHD bij andere diersoorten.

Hoofdstuk 12 bevat een discussie over verschillende aspecten van het bestuderen van oorzaken van CHD. Ten eerste wordt het belang aangegeven van dier-experimentele studies. Het belang van goede registratie en epidemiologische studies wordt besproken. Er wordt ingegaan op de moeilijkheden van het verrichten van toxicologisch onderzoek. Vervolgens worden mogelijkheden van toekomstige genetische studies besproken en we eindigen met een voorstel (protocol) waarin wordt aangegeven aan welke praktische aspecten gedacht moet worden in de kliniek om de etiologie van CHD te kunnen onderzoeken.

Samengevat kunnen uit dit proefschrift de volgende conclusies worden getrokken:

1. De vroege vaatontwikkeling van de long vindt plaats door de vorming van nieuwe capillairen uit al bestaande vaten rondom de zich vertakkende luchtwegen (distale angiogenese) (hoofdstuk 2).
2. Postmortem onderzoek is zinvol omdat afwijkingen aan hart, urogenitale systeem en afwijkingen aan de tractus digestivus worden gevonden die niet geobserveerd zijn in de kliniek (hoofdstuk 3).
3. De combinatie van CHD met ledemaatafwijkingen in twee onderzoekspopulaties suggereert dat aan deze afwijkingen een vroege gezamenlijke ontwikkelings-stoornis ten grondslag ligt (hoofdstuk 4).
4. De combinatie van CHD met EA/TEF en LH met EA/TEF wordt vaker gezien dan verwacht. Dit suggereert dat gezamenlijke ontwikkelingsgenen een rol spelen, inclusief genen die betrokken zijn bij het vroege vertakken van de luchtwegen en de nieren (hoofdstuk 5).
5. De long-hoofd ratio (LHR) is een goede voorspellende factor voor de klinische afloop van de foetus met CHD, die onafhankelijk is van zwangerschapsduur op het tijdstip van onderzoek. LHR > 1.4 is geassocieerd met 100% overleving en LHR < 1 met 100% mortaliteit. (hoofdstuk 6).
6. CHD heeft een locus op chromosoom 15q26.1-q26.2. Deze regio bevat RLBP1, een mogelijk kandidaat-gen. (hoofdstuk 8). Verder onderzoek vindt momenteel plaats om tot een conclusie te komen.

7. CHD kan een onderdeel zijn van zeldzame monogene syndromen, zoals van het craniofrontonasale syndroom of voorkomen samen met lateralisatie-defecten. De lijst met differentiaal diagnoses van CHD moet hiermee uitgebreid worden (hoofdstuk 9).
8. Concentraties van (gehydroxyleerde) PCB's in bloed van moeders van CHD patiënten verschillen niet van controles, en gehydroxyleerde PCB's worden daarom niet als risicofactor van CHD gezien (hoofdstuk 10).

CURRICULUM VITAE

Marieke van Dooren was born on July 3rd, 1972, in Gouda, the Netherlands. She attended the secondary school Emmauscollege in Rotterdam and passed the gymnasium exam in 1990. In the same year she started her medical training at the Faculty of Medicine and Health Sciences of the Erasmus University, Rotterdam. During her studies, she did practical work in hospitals in Semarang and Manado (Indonesia) at the Department of Pediatrics and Gynaecology. In August 1995 she resumed her medical studies and participated in a study on pre-eclampsia in the Erasmus MC Rotterdam. She obtained her medical degree in October 1997. From November 1997 until April 1999 she worked as a resident at the Pediatric Surgical Intensive Care Unit and the Pediatric Surgery of the Sophia Children's Hospital, Rotterdam. From May 1999 until November 1999 she worked as a resident at the Surgical Intensive Care of the Erasmus MC. In December 1999, she started working as a research physician for the project "Genetic and Environmental factors in CDH", which is presented in this thesis. The study was performed in the Sophia Children's Hospital and supervised by Prof. dr. D. Tibboel and Prof. dr. B.A. Oostra. For two months she worked in Berkeley on an epidemiological study on CDH as part of this thesis (supervisor C.P.Torfs). In 2001-2002 she worked in the laboratory of Cell Biology and Genetics, where she worked on the project "The role of FOG2 in lung development". From April 2003 she worked as a resident at the Department of Clinical Genetics at the Erasmus MC. In April 2004 she started her Clinical Genetics Training (AGIO) at the Erasmus MC, Rotterdam (Prof.dr. J.W. Wladimiroff and Dr. E.J. Meijers-Heijboer).

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