Establishment of a Tissue Bank for Human Fetal Lungs of Congenital Diaphragmatic Hernia Patients

By

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ABSTRACT

Statement of problem:

Congenital Diaphragmatic Hernia (CDH) is a fatal disease associated with abnormal lung development. Our aim is to establish a clinical database coupled with a biobank for CDH patients to facilitate further research.

Methods:

Our database and tissue bank were formed from the Pathology autopsy database for the CDH non-survivors and control subjects. We compared our database with CDH survivors records from a separate REDCap clinical CDH database.

Results:

The study comprised a total of 155 subjects, of which, 99 were CDH survivors, 40 were CDH non-survivors, and 16 were deceased subjects without CDH used as controls. Seventeen lung tissue blocks with different stages of lung development were obtained. Male subjects (59.7%) and left-sided CDH (76.9%) were more common. Among non-survivors we found a lower lung-to-body-weight (mean value 0.008 ± 0.004) compared to controls (0.019 ± 0.006).

Conclusion:

This clinical database and tissue bank can help future research.

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DEDICATION

I dedicate this thesis to my family.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 3. RESULTS

LIST OF FIGURES

ABBREVIATIONS

%LH	percentage of liver herniation
$AaDO_2$	alveolar-arterial oxygenation difference
ABG	arterial blood gases
aCGH	array-based comparative genomic hybridization
AP	alkaline phosphatase
ASB	AIDS specimen Bank
ASD	atrial septal defect
ATR	abdomen to thorax ratio
CAPSNet	Canadian Pediatric Surgery Network
CDH	congenital diaphragmatic hernia
CHTNN	cooperative human tissue network
CMV	conventional mechanical ventilation
CTRNet	canadian repository network
CV	cardiovascular
ECMO	extracorporeal membrane oxygenation
FETO	fetal endoscopic tracheal occlusion
FISH	fluorescent in situ hybridization
GA	gestational age
GI	gastrointestinal
HFOV	high-frequency oscillatory ventilation

iNO	inhaled nitric oxide
IT	information technology
LBWR	lung-to-body-weight ratio
LHR	lung-to-head circumference ratio
LiTR	herniated liver volume to volume of thoracic cavity
LNA	locked nucleic acid
MGI	mcgoon index
MicroRNAs	miRNAs
MRI	magnetic resonance imaging
NICU	neonatal intensive care units
NRP	neonatal resuscitation program
O/E LHR	observed to expected lung-to-head circumference ratio
O_2	oxygen
OI	oxygenation index
$PaCO_2$	partial pressure of carbon dioxide
PaO_2	partial pressure of oxygen
PaO_2/FiO_2	partial pressure of arterial oxygen to a fractional inspired oxygen concentration ratio
PBS	phosphate buffered saline
PDA	patent ductus arteriosus
PDE	Phosphodiesterase
PEEP	positive end-expiratory pressure
PFA	paraformaldehyde

PGE_1	prostaglandin E_1
PGI_2	prostacyclin, prostaglandin I_2
PHTN	pulmonary hypertension
PIP	peak inspiratory pressures
PPF	pleuroperitoneal folds
PPHI	prenatal pulmonary hypertension index
PPHN	persistent pulmonary hypertension
PVR	pulmonary vascular resistance
REDCap	research electronic data capture
SB	stillborn
SD	standard deviation
SPSS	statistical package for the social sciences program
SSC	sodium chloride/sodium citrate buffer
SVR	systemic vascular resistance
TFLV	total fetal lung volume
TP	termination of pregnancy
UCSF	University of California, San Francisco
VSD	ventricular septal defect
WFCC	World Federation of Culture Collections

CHAPTER 1. GENERAL INTRODUCTION

1.1. Normal Lung Development

It is important to understand the normal development of the lung to find the underlying cause of lung diseases (1). During early development, the embryo orients into two dimensions- top-to-bottom (rostral-caudal) and front-to-back (anterior-posterior) (2). During embryogenesis, the embryo transforms into three different functional layers. The outermost layer is the ectoderm, which later forms the skin and nervous system. The middle layer is the mesoderm, which makes the blood, bone and muscle. The inner most layer is the endoderm, from which the respiratory system and alimentary tracts originate (2). The main function of the respiratory system is to supply oxygen and eliminate carbon dioxide from the body (3). For this function, lung development and pulmonary vascular development run parallel to each other to eventually form the air-blood barrier (4).

Normal lung development occurs in stages, which consist of complex regulated events (5). First, in the pre-embryonic stage, the lung along with the trachea develops from the ventral wall of the foregut endoderm. In this process, the endoderm cells of the foregut invade into the surrounding mesoderm to form two lung buds and the trachea (4). Though different stages of lung development overlap, it can be further categorized according to its histological appearance (6). These stages have been identified as embryonic (4-7 weeks in utero), pseudoglandular (5-17 weeks in utero), canalicular (16-25 weeks in utero), saccular (26-35 weeks in utero) and alveolar (36 weeks in utero-7 years after birth) (7).

Embryonic Stage (4-7 weeks in utero)

In the embryonic stage, separation of the trachea and the esophagus occurs (8). In addition, the main bronchi and the five lobes of the lung are formed. The 18 main lobules can also be

recognized at this stage (5). Towards the end of this stage, the pulmonary arteries are formed from the sixth aortic arches creating a vascular plexus (4).

Pseudoglandular Stage (5-17 weeks in utero)

The main characteristic of the pseudo-glandular stage is that the lungs undergo a regulated branching process (9,10). These include three types of branching known as domain branching, planar bifurcation, and orthogonal bifurcation (10). This results in the formation of the conducting airways, blood vessels, and terminal bronchioles (4). The airways are lined with columnar epithelial cells which make the lung look like an undifferentiated endocrine gland (9).

Canalicular Stage (16-25 weeks in utero)

The main hallmark of this stage is pulmonary vascularization to form a proponent capillary network (5). The distal airways develop into definitive primary acini, which consist of respiratory bronchioles, alveolar ducts, and rudimentary alveoli (11). Epithelial cells start to differentiate into Type I and II pneumocytes (5). Type I pneumocytes have an essential role in gas exchange, whereas, type II cells mainly secret surfactant, which prevents the lung from collapsing after birth (7).

Saccular Stage (26-35 weeks in utero)

During the saccular stage, the terminal branches of the airways form groups of alveolar sacs. These sacs, later on, develop into alveoli (11). This is a result of the mesenchymal layer undergoing differentiation as well as substantial apoptosis and the pneumocytes being completely differentiated. By the end of this stage, the lung can function to provide the body with gas exchange (7).

Alveolar Stage (36 weeks in utero -7 years after birth)

The final stage is the alveolar stage, wherein, the alveolar surface area is maximized for sufficient gas exchange through a thin air-blood barrier (12). Secondary alveolar septae are formed. These septae consist of connective tissue and a double capillary loop, which splits the saccules into terminal alveoli (4,6). This results in the formation of the air-blood barrier, which consists of the basement membrane of alveolar epithelial pneumocytes (type I) fused with capillary endothelial cells (6,12). As a consequence, complete alveolar formation and maturation take place (4).

1.2. Normal Pulmonary Vasculature Development

The development of the lungs is associated with development of the pulmonary vasculature (13). The first appearance of a vascular plexus is by a mechanism called vasculogenesis (14). It forms new vessels from differentiating mesenchymal cells around the lung buds (13,14). From the beginning of the canalicular stage of lung development all the way to the alveolar stage, the pulmonary vessels are formed from existing vessels, and this mechanism is called angiogenesis (14,15). The main function of the pulmonary arteries is to regulate the amount of blood flowing to the lungs. It regulates the radius of the pulmonary vessels, which subsequently sets the needed pulmonary vascular resistance (PVR). Therefore, the smaller the radius, the higher the resistance and less blood flow (13,14). The pulmonary artery pressure is determined by PVR and pulmonary blood flow (16).

During fetal life, the placenta serves as the organ of gas exchange, which is a low-pressure circulation. On the other hand, pulmonary vascular resistance remains high during this period. Therefore, only 5-10% of the combined ventricular output goes to the pulmonary vascular bed and the remaining blood bypasses the pulmonary circulation to reach the aorta through the ductus

arteriosus. Even though the surface area of the pulmonary vascular beds increases with the increasing maturity of the fetal lung, the PVR increases with gestational age, when corrected for lung or body weight. This suggests that pulmonary vascular tone increases during the later stages of the gestation. Due to this elevated PVR, pulmonary pressures are equivalent to systemic pressures in the fetal life. A series of events occur during birth leading to the cardiopulmonary transition of gas exchange function from the placenta to the lungs. As soon as the placenta is cut at the time of birth, the low resistance placental circulation is lost leading to a marked rise in the systemic vascular resistance (SVR). Simultaneously, a rapid drop in PVR as well as pulmonary artery pressure leads to a 10-fold rise in pulmonary blood flow. As PVR becomes less than SVR after birth, blood flow reverses through the ductus arteriosus directing more blood to the pulmonary circulation. The most essential stimuli to initiate these transitional changes are mechanical distension of the lung, a drop in carbon dioxide tension, and the rise in oxygen tension in the lungs (17). In the first few days of life, the mean pulmonary arterial pressure in a full term infant is half of the systematic arterial pressure (16,18).

1.3. Normal Diaphragm Development

The diaphragm functions as the primary muscle of respiration. It is the muscle that separates the chest and the abdominal organs (19). It is mainly composed of the peripheral muscular part and to a lesser extent a central fibrous tendon (20). During embryogenesis, the diaphragm develops around 4 through 12 weeks of gestation through the closure of the pleuroperitoneal canal (19,21). It originates from the fusion of four embryonic structures; the septum transversum, pleuroperitoneal folds (PPF), esophageal mesentery, and muscular body wall (19). The septum transversum is made of mesodermal tissue, which initially separates the cardiac muscle from the liver, this later gives rise to the central tendon (14,16). The PPFs are known to develop the main

precursor cells of the diaphragm (22). Fusion of the PPFs to the septum transversum and dorsal mesentery of the esophagus forms the posterolateral segments of the diaphragm (20). However, the anterolateral portion of the diaphragm is formed by the fusion of the transverse septum and the muscular body wall (19). Around 11 weeks of gestation, movement of the diaphragm is visible on prenatal ultrasound (23).

1.4. Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia (CDH) in newborns is a fatal condition, which is defined as a spectrum of developmental defects present in the diaphragm (24). This defect allows herniation of the abdominal viscera/contents into the chest. Despite standardized postnatal treatment of CDH newborns, substantial morbidity is close to 100% with a mortality rate of 30-50%. In most of the neonates with CDH, the morbidity and mortality depend on the severity of pulmonary hypoplasia and abnormal development of the pulmonary vasculature (25,26). In contrast, CDH presenting in adults is not associated with lung developmental abnormalities and thus was not a focus of the work presented in this thesis. The disease features, presentation and mortality rate differ from CDH in infants (27,28).

1.4.1. Prevalence of CDH

It is estimated that the incidence rate of CDH is 1 in every 2500 to 3000 live births worldwide (26). The Canadian Pediatric Surgery Network (CAPSNet) collaborated with 17 perinatal canters across Canada including the Health Science Center in Winnipeg. They have shown that each year, 90 neonates with CDH are born in Canada. Even after providing maximum medical management, nearly 20% of these babies die and the remaining survive with long-term complications of CDH. Yearly $10 million is spent in Canadian hospitals on care for CDH babies (29).

1.4.2. Etiology of CDH

Although most of the cases of CDH have an unknown cause, genetic and epigenetic factors are a possible explanation for the etiology of the disease (30). Epigenetics is defined as heritable alterations in gene expression without any changes in the DNA sequence. Two main areas of epigenetics affecting gene regulation, development, and oncology are DNA-methylation and histone modification (31).

MicroRNAs (miRNAs) are small noncoding RNA molecules, which are encoded in the genome and usually transcribed by RNA polymerase II. These molecules are about 22 nucleotides long and can have a significant effect on gene expression. miRNAs negatively control target gene expression. In humans, at present, there are more than 1000 known miRNAs. The expression of miRNAs has been linked to many diseases, including lung disease (31). During embryonic development, microRNAs are essential for normal organogenesis. Studies have shown the differential miRNA expression that takes place between various stages of lung development. It is yet to be confirmed whether specific miRNAs play a role in the pathogenesis of congenital lung diseases. However, isolated CDH, which is characterized by abnormal lung development and a normal karyotyping, can serve as an example to study the molecular mechanisms facilitating lung growth and differentiation (31).

MicroRNA-200b is a member of the miR-200 family. This microRNA family also includes miR-200a, miR-200c, miR-141, and miR-429. These miRNAs have similar sequences. Furthermore, they are transcribed in 2 clusters: miR-200b, miR-200a, and miR-429 form a cluster on chromosome 1, whereas miR-200c and miR-141 are transcribed as a cluster on chromosome 12. Our studies have shown that miR-200 expression is up-regulated in epithelial lung tissues. Moreover, the high miR-200b expression is also seen in bronchial and alveolar epithelial cells

(26).CDH can be associated with chromosomal abnormalities (32); one study, showed that one of two identical twins presented with CDH, supporting the presence of multifactorial causes (33). It is also thought that disordered embryogenesis is one of the main causes of CDH (24).There are many theories for the hypothesis of CDH pathogenesis due to the several types of CDH with the wide variations of the defect size and location in addition to the severity of lung hypoplasia and pulmonary hypertension (PHTN) that is usually associated with the disease (34).

1.4.3. Types of CDH

There are different types of CDH according to the site where the defect is present. Abnormal diaphragm development is an outcome of the partial fusion of segments forming the diaphragm leaving a hole or weaker muscle fibers in the deficient part (24,35).

The hole can be unilateral or bilateral. The most typical neonatal presentation is a left-sided CDH, which is six times more prevalent than right-sided CDH (24). In 14% of the cases it occurs bilaterally, this could be due to diaphragm agenesis (24,36). In 80% of the cases the CDH is in the postero-lateral part, which is known as a Bochdalek hernia. If the defect is present in the anterio-medial part, (in 9% of the CDH patients) this is known as a Morgagni hernia. It is uncommon for the hernia to be in the central part of the diaphragm and this only occurs in 2-7% of the cases(24,30,37).

In some cases, the defect is covered by a thinner muscle of the diaphragm, and this forms a pocket for the herniated organs into the chest, which is known as a hernia sac (35). However, if the diaphragm is amuscular, this is named an eventration (30,38). In a study, authors linked the larger the size of the hernia to the more severe lung abnormality (36).

1.4.4. Isolated and Non-Isolated CDH

If CDH exists as a part of a chromosomal disease or syndrome, this is termed ‘non-isolated CDH'. On the other hand, an ‘isolated CDH' is referred to a case where CDH is the only congenital anomaly. It can also be seen in association with common cardiac anomalies, for example, a patent ductus arteriosus (PDA) and cardiac septal defects (34,39). The incidence of isolated CDH is 60-70%, which is much more frequent than non-isolated (39). This could be due to the variability in the reporting methods of each study that can affect this percentage (40,41).

Non-isolated CDH occurs secondary to other associated syndromes. These include Pallister-Killian and Fryns syndrome, Ghersoni-Baruch syndrome, Brachman-De Lange syndrome, and Wolf-Hirschhorn syndrome (32).

Chromosomal abnormalities are also associated with 5-30% of CDH patients (24). The most common chromosomal anomaly associated with CDH is trisomy of chromosome number 13, 18, 21 and 45X. Structural chromosomal abnormalities include duplications, inversions, deletions, and translocations (32,39). Some common ones include duplication of 1q25q31.2, deletion of 1q41-42, deletion of 3q22, duplication of 5p15, and deletion of 15q26, which are among the most commonly reported structural chromosomal abnormalities. CDH patients with 15q26 deletion present with a severe phenotype such as cardiac anomalies, limb anomalies, and dysmorphic features (32).

There is growing evidence that specific pathways play a role in the development of CDH (32). These distinct pathways can be identified from the candidate genes present in regions where commonly genes are deleted and/or duplicated in CDH patients. Several genetic animal models also help in recognizing these specific pathways. Some common pathways include *COUP-TFII, FOG2, GATA4, WT1,* and *SLIT3* (32).

1.4.5. Pathophysiology of Lung Abnormalities in CDH

It is often assumed that the ipsilateral pulmonary hypoplasia and hypertension associated with CDH are a consequence of compression by the abdominal organs herniated into the chest (42). However, the underlying process of the pulmonary hypoplasia in the contralateral lung of CDH is still unknown. Studies suggest that the lung abnormalities occur before the diaphragmatic defect and are further worsened by the herniating abdominal organs (42,43). This hypothesis is called the ‘dual-hit hypothesis,' according to which both pulmonary hypoplasia and PHTN play key roles. As per this hypothesis, the developing lungs have reduced bronchial branching due to an unidentified genetic/environmental "hit" during the early gestation period (5-16 weeks), which results in decreased bronchial branching. As time progresses, there is compression of the developing lung due to the herniation of the abdominal organs into the chest through the diaphragmatic defect ("second hit"). This causes a reduction in the size and number of alveoli, increased interstitial tissue and reduced alveolar airspace and gas exchange surface (43,44). This, in turn, interferes with the fetal breathing movement, which is vital for expansion of the growing lung. During this period, vascular changes also occur such as a decreased number of blood vessels and abnormal extension of the muscular layer into the small intra-acinary arterioles. As a result of this, the pulmonary capillary blood flow is reduced, and the blood flow may be further reduced in the case of abnormal pulmonary vasoconstriction. Postnatally, these changes become evident as pulmonary hypoplasia and PHTN (44).

1.5. Effect of CDH on the Lungs

In CDH, the structure of the lungs in utero is fundamentally altered. Both the structure of the lungs and the pulmonary vasculature are affected. This is the main cause of the high mortality

and morbidity associated with CDH (45). As a result, the severity of the lung hypoplasia and PHTN are the cardinal features of the outcome and survival rate of CDH(24).

1.5.1. Pulmonary Hypoplasia Associated with CDH

The small hypoplastic lungs have a reduced number of bronchi, alveoli and a thickened mesenchymal cell layer (46–49). With the growth of the infant, though the number of alveoli may increase, the numbers of larger airways do not increase. This is because at around 16 weeks of gestation bronchial development is complete and it is affected by the presence of the abdominal viscera in the thoracic cage (46,47).

1.5.2. Pulmonary Hypertension Associated with CDH

Persistent pulmonary hypertension of the newborn (PPHN) results from the failure of the normal cardiopulmonary transition to occur after birth (17). PPHN is a cause of cardiorespiratory failure in the term, post-term and even late pre-term neonates (>34 weeks) (50). The main developmental types of PPHN are under development, mal-development, and mal-adaptation of the underlying pulmonary vasculature. CDH falls in the category of underdevelopment cause of PPHN (51). The pulmonary vessels are primarily characterized by a hypertrophic endothelial layer of the pulmonary vessels as well as smooth muscle proliferation (46,47). In summary, the structural defects of pulmonary vasculature abnormalities includes the abnormal lung vasculature pattern, decrease in the number of pulmonary vessels, excessive vascular remodeling, that in turn results in augmented thickness of the pulmonary artery muscularization and decrease in the size of the pulmonary vascular bed (52,53). As a consequence, the pulmonary vascular resistance and arterial pressure increases causing a decrease in the pulmonary blood flow for oxygenation in the lungs (54).

After birth, inadequate oxygen and carbon dioxide exchange in the small lungs decrease the

oxygen and increase the carbon dioxide levels in the blood. This stimulates the pulmonary vessels to constrict, and PHTN occurs (25,47). This in turn decreases the blood flow to the lungs resulting in less oxygenation of the blood and thus a vicious positive cycle sets in. In addition, PPHN and pulmonary hyper-reactivity will prevent the normal transition of the fetal circulation to the neonatal circulation. This results in the blood flow through the foramen ovale and ductus arteriosus bypassing the lungs, forming a right-to-left shunt. Continuation of this circulation after birth worsens the condition, as the neonate needs high blood flow to the lungs for adequate oxygenation (24).

1.6. Diagnosis of CDH

Early detection of CDH in utero assists in a better understanding of the etiology and to better prepare for delivery allowing better treatment strategies to reduce morbidity and mortality (32,55). Differential diagnosis of CDH includes bronchopulmonary sequestration, bronchogenic cyst, teratoma, bronchial atresia and congenital cystic pulmonary airway malformation (56–58). Presence of abdominal organs in the lower thoracic regions makes a definitive diagnosis of CDH. In most of the cases, CDH is detected between the 22-24th week of gestation during routine prenatal anomaly ultrasound scanning of the baby (57). In some of the cases, CDH remains undetected and presents at birth, or even later in life. In these cases, the diaphragmatic defect is very small, the diagnosis of CDH can be an incidental finding (28,59).

1.6.1 Prenatal Diagnosis of CDH

The antenatal diagnosis rate of CDH has increased over the past few years (60). The detection rate of CDH is different according to various regions (61). The results of an extensive retrospective study conducted in Western Australia indicated a prenatal CDH diagnosis in 53% pregnancies compared to 47% post-natal CDH diagnosis rate (62). A wide variation of 10-90%

in the prenatal diagnosis of CDH was mostly dependent on the US screening protocol (63). CDH can be detected as early as the 16th week of gestation (64). Almost 50% of the CDH cases can be recognized at a mean gestational age of 24 or more weeks (58,61,65). Researchers have reported a variable mean gestational age (29 weeks (range, 16-37 weeks (66), 24.2 weeks (range 11-38 weeks (61), 24.5 weeks (range 18-36 weeks)(67).

CDH detection rates increase with advanced gestational age (47).The diagnosis of left-sided CDH can be made by the presence of a stomach bubble and left lobe of the liver at the level of the fetal heart along with a right mediastinal shift (37). On the other hand, recognition of an isolated right-sided CDH can be quite difficult as the herniated liver and lungs of the fetus have the same echotexture (68). Presence of pleural and pericardial effusion helps in a better visualization by further separating the organs (69). Color Doppler may assist in identifying intra-hepatic ducts in the thoracic cavity (70,71).

Accurate prenatal diagnosis of the severity of the CDH and degree of lung hypoplasia is essential for selecting treatment options (72). In addition to two-dimensional ultrasound, nowadays, three dimensional US, fetal echocardiography, magnetic resonance imaging (MRI) and amniocentesis analysis are techniques used in clinical practice for identifying the severity of CDH (73–75).

1.7. Antenatal Management

Medical Management: Administration of corticosteroids to the mother of a CDH baby is controversial. While it is believed it would benefit their immature lungs, a study has shown it had no benefit (76). Still, some researchers think that the use of antenatal steroids should be routine in all neonates born prematurely (77).

Tracheal occlusion: Fetal endoscopic tracheal occlusion (FETO) has been shown to improve survival rates in patients with severe CDH (78). Tracheal occlusion accelerates the lung growth

by stretching lung tissues secondary to accumulation of lung fluid while the balloon release is linked with lung maturation (79). It has been reported that the use of FETO is associated with an increase in pre-term births and premature rupture of the membranes (65). Therefore, the use of FETO for CDH should at present only be considered within the scope of a randomized clinical trial.

1.8. Prognosis of CDH

No single factor or marker can accurately predict the prognosis of CDH (80). Many factors have been recognized to play a role in the prognosis of CDH. However, the most critical factors are:

1. The severity of the lung hypoplasia.
2. The position of the liver.
3. The presence or absence of malformations of other organs or abnormal chromosomes.

For the isolated CDH cases, the most commonly accepted parameter to assess the severity of pulmonary hypoplasia is the sonographic lung-to-head circumference ratio (LHR) (81,82). However, as the rate of growth of the lung and head is not the same in a developing fetus, these measurements are affected by the gestational age. To correct for the gestational age, (observed [O]/expected [E]) LHR is calculated to assess the fetal lung volume (57,65). The percentage of predicted lung volumes may provide a better assessment of the fetal survival rate (83,84). Fetal MRI has also been used in calculating a prenatal pulmonary hypertension index (PPHI), modified McGoon index (MGI) (85), and total fetal lung volume (TFLV) (86,87).

Another important prognostic factor is the position of the liver and measurements of the size of the herniated liver (81,88,89). Many methods have been used to assess the degree of liver herniation; these include the percentage of liver herniation (%LH), linear (liver to diaphragm) and volumetric measurements (herniated liver volume to volume of thoracic cavity or total liver

volume). It was mainly interpreted as a ratio (liver-to-diaphragm, LiTR)(90,91). Liver herniation decreases survival rate in CDH patients (100% with liver down vs. 56% with liver up position) (92).

Herniation of the liver in the chest cavity along with the low O/E LHR values indicates increased mortality and morbidity(81).

The patients with non-isolated CDH have a worse prognosis compared to the isolated type of CDH (65,93). Fetal echocardiogram and MRI can provide additional information on cardiac and other systems associated anomalies (74,90). Array-based comparative genomic hybridization (aCGH), fluorescent in situ hybridization (FISH) and chromosomal analysis is used to screen for chromosomal abnormalities in CDH (32,94,95). The use of advanced techniques, i.e., exome sequencing, amniocentesis and chromosomal microarrays have also led to a better understanding of the genetic defects associated with CDH (81,96).

Other factors affecting fetal outcome should be considered as well. Abdomen to thorax ratio (ATR) may be used for the prediction of neonatal survival in CDH fetuses (82). The position of the stomach in the chest on ultrasound is also being evaluated as a prognostic marker in cases with CDH as intra-thoracic position of the stomach has been shown to be associated with a poor prognosis. (57,65). However, Mektus et al. found no significant value of stomach position, abdominal circumference, and polyhydramnios in prediction of the survival rate (97). The result of another study also indicated no role for polyhydramnios and gestational age at the time of CDH detection (93,98). Other studies found mortality rates decrease with advancing gestational age in CDH patients, so delivering the baby after 39 weeks is recommended to avoid complications associated with pre-term delivery (57,65)

1.9. Clinical presentation

CDH most commonly presents with respiratory distress of the newborn. While later-presention in children and adults can vary from asymptomatic to non-specific respiratory or gastrointestinal (vomiting) symptoms (59,99,100).

In neonates, clinical presentation may vary depending on the gestational age at the time of birth, type of defect, the degree of liver herniation and associated anomalies with CDH (65,101).

Respiratory distress in the first few hours or days of life is variable, and it is usually associated with cyanosis, feeding intolerance, and tachycardia. In severe cases, it can present as circulatory insufficiency, requiring aggressive resuscitative measures. On physical examination, a scaphoid abdomen and a barrel-shaped chest can be seen in the presence of significant visceral herniation (47). Respiratory system examination may reveal tachypnea, grunting, and retractions. Upon auscultation, breath sounds are diminished due to poor lung development, bowel sounds due to herniation of intestines may be heard in the chest, and heart sounds are distant or displaced. All of which are consequences of the lung, vascular and cardiovascular abnormalities produced by the herniation of the organs caused by the CDH (102). If the examination shows dysmorphic features, microarray analysis should be carried to rule out chromosomal abnormalities (65). Other associated anomalies include dysmorphisms such as craniofacial abnormalities, limb abnormalities, or spinal dysmorphism (103) may be found in non-isolated CDH (104). In these cases, cranial sonography followed by MRI should be done. MRI is also useful to diagnose intraventricular bleeding and hypoxic-ischemic changes. Renal ultrasonography should be considered in CDH to rule out associated genitourinary anomalies (37).

1.10. CDH Postnatal Management

The management protocol of CDH is continuously evaluated and updated due to advances in surgical and non-surgical techniques (105). Researchers have agreed on the fact that delaying delivery to near-term in suspected or confirmed cases of fetal CDH may give better results (106). Delivery of such patients should be conducted at centers having facilities, i.e., neonatal intensive care units (NICU) to manage these babies (107,108). Prompt initiation of support and resuscitation is needed which should be according to neonatal resuscitation program (NRP) guidelines (109). Bag-mask ventilation should be avoided which may cause intestinal insufflation (110). A nasogastric tube with continuous suction helps prevent lung compression and bowel distension (109). An emergency chest x-ray can assess the initial condition of the lungs and the position of the tube (65,111). The opposite mediastinal shift and bowel loops can also be seen on chest x-ray (112).

Effective management of CDH in infants focuses on proper oxygenation, perfusion, and blood pressure monitoring. Central or peripheral venous access should be secured for rapid administration of fluids and medications. Similarly, an arterial line (preferably right radial or ulnar artery) is needed to draw blood for arterial gases and for monitoring blood pressure (65). Tissue perfusion is the aim of hemodynamic and systemic blood pressures monitoring (113). Blood pressure can be supported by the use of inotropic agents, vasopressors, and volume expansion fluids (114). Serum lactate, capillary refill time, and kidney output are used to detect the circulatory insufficiency or severe hypoxemia (65,114).

Catecholamine, dopamine, dobutamine, and vasopressin can be used to maintain the systemic blood pressure at normal levels for gestational age which minimizes the right-to-left shunting

(65,114). Low-dose hydrocortisone can be tried for rapid normalization of cardio-vascular status if shock is not responding to vasopressors (115).

Oxygen is titrated using a pulse oximeter probe or arterial blood gases (ABG) to maintain pre-ductal oxygen saturation >70% (65). It is useful to make a diagnosis of PPHN in cases with CDH by recording the difference in oxygen saturation between pre-ductal (right-hand) and post-ductal (either foot or left hand) sites, which is secondary to a right-to-left shunt at the ductus arteriosus level. Pre- and post-ductal saturation difference on pulse oximetry or ABG can estimate the severity of PPHN in these cases (116). Frequent ABG measurements should be done to assess for pH, partial pressure of carbon dioxide ($PaCO_2$), and partial pressure of oxygen (PaO_2)(116).

An echocardiogram is considered as an ideal diagnostic test to assess pulmonary and cardiac pressures in neonates. It is usually done within the first 24 hours of life and is a good indicator of survival in CDH patients (75). It is also important to rule out structural heart diseases, i.e., tetralogy of Fallot, septal defects, transposition of the great vessels, hypoplastic left heart and aortic coarctation (117).

Inhaled Nitric Oxide (iNO), is an endothelial-derived selective pulmonary vasodilator and is the treatment of choice for management of PPHN in infants >34 weeks' gestation (118,119). The criteria to initiate iNO is based on the severity of PHTN assessed by the oxygenation index (OI) and alveolar-arterial oxygenation difference ($AaDO_2$) (120). An oxygenation index of ≥20 is an acceptable cut-off for initiation of iNO with a starting dose of 20 parts per million iNO for term and near-term infants (50), although other studies have reported variable starting doses (65,121). An increase in the partial pressure of arterial oxygen to a fractional inspired oxygen concentration (PaO_2/FiO_2) ratio by ≥20 mmHg after starting iNO therapy indicates a good

response to iNO (65). The results of the neonatal iNO study group showed a short-term effect of iNO in treating PPHN and hypoxemia, however; no effect on mortality was reported (118,121).

Mechanical ventilation does not reduce PVR, though it is aimed at synchronizing the efforts of the infant to breath and avoid peak inspiratory pressures (PIP) of more than 25 cm H_20. The optimal ventilation mode is not known and current evidence shows that it is reasonable to start with conventional mechanical ventilation (CMV). High-frequency oscillatory ventilation (HFOV), as the initial mode of ventilation, has not shown any additional benefit. Gentle ventilation is preferred to prevent iatrogenic lung injury. Usual initial settings are PIP <25 cm H_2O and positive end-expiratory pressure (PEEP) ≤5 cm H_2O on CMV mode to maintain pre-ductal oxygen saturation of >85%, post-ductal saturations of >70% and $PaCO_2$ of 45–60 mmHg. (57,65,122).

Extracorporeal membrane oxygenation (ECMO) has been used as the last treatment option for patients with a poor response to maximum ventilator support and medical treatment (105,123). Increased survival rates have been reported by the researchers after successful use of ECMO in right-sided CDH patients (124). Criteria for the use of ECMO in a patient vary from center to center depending on their guidelines. Most of the selection criteria include the inability to maintain a pre-ductal O_2 saturation above 85%, systemic hypotension (systolic blood pressure < 90mmHg) or respiratory/metabolic acidosis (pH < 7.15) refractory to fluid and inotropic agents, an oxygenation index of more than or equal to 40 and low cardiac index (cardiac index less 2 $L/min/m^2$) (123,125). Veno-arterial ECMO is preferred in patients with cardiovascular compromise. However, studies have reported similar survival rates in patients treated with venoarterial and venovenous ECMO (126,127). Duration of treatment on ECMO is also variable

across the centers and is still debatable, yet studies have reported increased mortality rates with a prolonged period of ECMO (65,128).

Surfactant therapy is routinely used in pre-term infants for maturation of lungs. However, studies have failed to prove beneficial effects of surfactant administration in term or pre-term infants (129,130). Prospective trials are needed to evaluate the useful role of surfactant therapy in alleviating respiratory symptoms in premature CDH infants (65).

Many other pharmacological therapies are tested in clinical trials, but still can be considered in treating PHTN in patients with CDH. Examples of medications under research include prostaglandin E_1 (PGE_1) (131), prostacyclin (PGI_2) (132,133), sildenafil (134), milrinone and bosentan. PGE_1 has been reported to reduce the load on the right ventricle by reopening the ductus when used in infants with CDH have right heart failure or a duct-dependent circulation (131). PGI_2 may be useful as a non-selective pulmonary vasodilator in the management of late PHTN developing after CDH repair (65,132). Sildenafil, a phosphodiesterase (PDE) 5 inhibitor, is a potent pulmonary vasodilator, improves oxygenation and reduces mortality in PPHN (135). Milrinone, a PDE 3 inhibitor, improves the myocardial function as well as systemic and pulmonary hemodynamics that can be used as a synergetic drug with iNO (136–138). Oral bosentan (endothelin (ET-1) receptor blocker), is used as an adjunctive therapy to manage chronic PHTN in CDH cases (139).

1.11. Diaphragm Surgical Repair

Surgical repair of the diaphragmatic hernia dates back to 1902, when the first successful surgery of a CDH infant was performed (140). Initially, surgical repair of the diaphragmatic hernia was done immediately after birth, but the mortality rates were high. Immediate surgical management after birth is no longer the first-line treatment. Thus, it is crucial to emphasize the importance of

proper neonatal management on decreasing the high mortality rate (102). Advanced treatment strategies, e.g., iNO, ECMO, surfactant administration and HFOV and improved management in intensive care units have enabled surgeons to use a delayed surgical approach (108,141–143). However, prolonged delay in surgery may lead to strangulation, volvulus or obstruction of the herniated intestines (144). During surgical repair, herniated organs are reduced into the abdomen, and the hernia defect is closed (65). The hernia may be closed either by using minimally invasive surgery (laparoscopy or thoracoscopy) or by open surgery through a thoracic or abdominal approach (102). Studies have reported higher recurrence rates of the hernia after the thoracoscopic approach compared to open surgery (145). Small hernia defects are primarily repaired by approximation of the margins of the hernia, whereas large defects are closed using a patch (65).

1.12. Long-Term Complications

Patients who develop short-term complications after treatment are more prone to develop long-term complications as well (146). A multidisciplinary approach is required for CDH infants to detect and manage hernia recurrence. Structured long-term follow up of CDH patients has discovered developmental, metabolic, gastrointestinal, cardiovascular, pulmonary, and musculoskeletal problems in these patients (147).

1.13. Animal Models of CDH

Researchers have used various surgical, pharmacological, molecular and transgenic animal models to gain a better insight into the pathogenesis and molecular mechanisms of the disease (148–150). Fetal lamb and rodent models have enabled scientists and researchers to create and identify new treatment options (151).

1.13.1. Teratogenic Model

The most common teratogenic model used for CDH is the nitrofen model. Nitrofen is a 2, 4-dichloro-1- (4-nitrophenoxy) benzene, a herbicide of the diphenyl ether class. It is known to have carcinogenic effects on rodents and is considered a Group 2B class carcinogen. Individual studies have identified nitrofen as a teratogen (152). The teratogenic effect of nitrofen is exerted mainly during the period of organogenesis. As a result, the herbicide is orally fed to dams, between the gestational age of E8–9 for mice and E8–12 for rats, which results in hypoplastic lungs and a number of diaphragmatic defects similar to CDH in humans is seen (152). Through the nitrofen teratogenic model, multiple developmental pathways have been studied in the mechanism of CDH (104). It has also been used to demonstrate the effectiveness of tracheal occlusion (153,154).

1.13.2. Surgical Model

Initially, surgical models of CDH were widely used. A diaphragmatic hernia was surgically created in sheep, rabbits, and dogs (155–157). Surgical models, do not examine the etiology of CDH, they are used to evaluate pulmonary consequences attributed to CDH. This includes the effects of compression of the herniated organs on lung development. These models have also helped to assess treatment options for CDH (158,159). Surgical models have also been used for fetal tracheal occlusion treatment (160).

1.14. Biobank and CDH

Biobanks, also known as tissue banks, are organized collections of biological samples of humans, animals, and plants. Human biobanks usually comprise of associated health information of individuals, along with, their biological specimens (161). Biobanks accept, process, store, and distribute bio-specimens and their related information for use in biomedical research and clinical

care. The evolution of biobanks dates back several decades. Over the years, several types of biobanks have evolved including population-based, disease-centric, genetic, commercial, and virtual biobanks. The evolution of biobanking has seen tremendous change in the last 30 years, growing from University-based repositories to Institutional and Government based repositories (162).

Due to the different types of biobanks, many have been labeled as the first in history. In 1949, the first tissue bank was created by Dr. George Hayatt (163). Later in 1970, The World Federation of Culture Collections (WFCC) was recognized as the world's first biobank network (164). The organization deals with the collection, authentication, maintenance, and distribution of micro-organisms. For human tissues, one of the first biobank networks was the US Cooperative Human Tissue Network (CHTNN) founded in 1987 (164). The CHTN is also funded by the US National Cancer Institute and provides human tumor tissues for biomedical research. In Canada, one of the first biobank networks was the Canadian Repository Network (CTRNet), which, besides collecting and maintaining human biological specimens, also provides solutions for biobank management (164).

Lung tissue biobanks exist in several countries. In Canada, the Alberta Cancer Research Biobank allows open access to an extensive collection of samples from different types of cancer. Though disease-centric biobanks exist, such as the University of California, San Francisco (UCSF) AIDS Specimen Bank (ASB), there are not any disease-centric lung biobanks for CDH (161).

Goal of Our Study

Our overall goal is to establish a database of CDH autopsy cases, along with a lung tissue bank for future validation of animal studies.

The Main Objectives of the Project

1. To establish a comprehensive clinical database and high-quality human tissue bank of all CDH autopsy cases (non-survivors) at our hospital since 1980.
2. To match CDH patients with the appropriate controls.
3. To compare our database to a database with clinical information on CDH survivors.

CHAPTER 2. MATERIALS & METHODS

2.1. Establishment of CDH Database

Ethical approval was obtained with REB number HS15293 (H2012:134), from the Health Research Ethics Board, Bannatyne Campus, University of Manitoba. This retrospective study was conducted in the Health Sciences Center, Winnipeg, Manitoba. After obtaining approval from the institutional ethics committee, we reviewed the pathology records of all the CDH autopsy cases in our hospital from 1980 to 2016. The autopsy cases included CDH infants of terminated pregnancies (TP), stillbirth (SB) and patients who died after birth (neonatal death- subdivided into preoperative and postoperative death). Autopsy cases with available lung tissue blocks, were matched with appropriate controls. Our data was compared to the data collected by the Pediatric Surgery Section for patients who were born with CDH, survived surgery and discharged from our hospital.

The Department of Pathology autopsy database at the Health Sciences Centre consists of all pediatric autopsies since 1980 and all adult autopsies since 1996 (both medico-legal and family permission). It is amenable to full-text search using DocFetcher software (SourceForge.net; open source software license from Eclipse Public License). Candidate cases were identified (by Dr. M. Del Bigio) through whole text search using a range of key words related to "congenital diaphragmatic hernia". The initial search retrieved the records of 76 patients diagnosed with CDH. Out of these 76 patients, 13 patients were excluded, because the final pathological diagnosis was different from the initial clinical diagnosis of the CDH. Out of the remaining 63 CDH patients, 21 patients survived after a successful surgical intervention while 42 patients died of the complications of CDH. The surviving patients' pathological reports had the description of tissue collection different than the lungs and limited information about the patient CDH details.

There was an overlap of 10 patients presented in this group and from the patients collected from the clinical database for CDH survivors. Therefore, the surgical reports (*n=21*) were excluded.

In the unfortunate event of a patient's demise- (CDH non-survivors)- TP (*n=6*), SB (*n=4*), preoperative neonatal death (*n=22*), postoperative neonatal death (*n=8*), over 12 years old aged death (*n=2*) - an autopsy report (*n=42*) with lung tissue specimen (*n=17*) was available in most of the cases.

The tissue slides for the 42 patients were retrieved from the pathology system to evaluate the tissue quality and to choose the appropriate tissue blocks. The tissue blocks included tissues collected from various organs and multiple locations. The tissues were processed and embedded in paraffin in the Department of Pathology. We focused on tissue blocks for the lungs of the ipsilateral side of the diaphragmatic hernia. Evaluation of each tissue block was performed by Dr. Camelia Stefanovici, MD, FRCPC, Anatomic Pathologist through assessment of its representative tissue slide. The lung tissue slides were sectioned and stained (Hematoxylin & Eosin) in the pathology department at the time of death for each patient. Part of choosing a better-quality tissue block would be the one with less meconium staining compared to the rest of blocks. In addition to, selecting the block with less post mortem histological changes such as epithelial tissue detachment. Therefore, we recorded the post mortem interval from the date of pronounced dead and the date of autopsy.

A further stratification of the autopsy reports into different groups was performed according to the patient's year of death and the associated anomalies.

2.2. Establishment of Appropriate Controls for Autopsy Cases of CDH

The following demographic data were collected for each CDH autopsy case: the gestational age (GA), the sex, the side of the diaphragmatic defect, whether the infant died in utero (TP, SB) or

was born alive (live birth). In cases with live births, the duration of survival after delivery, and if the diaphragm was surgically repaired was also added. The presence of other disorders or syndromes (cardiovascular, gastrointestinal, renal, neurological, metabolic, musculoskeletal and dermatological anomalies) was recorded. We included the details of each patient's prenatal diagnosis, date, body and lung weight, type of cardiovascular anomalies (Atrial Septal Defect (ASD), Ventricular Septal Defect (VSD) and other cardiovascular anomalies). We considered the main cause of death in the reports and categorized them into due to pulmonary complication, pulmonary and associated anomalies, in utero death (SB, TP) and unknown. The presence of hyaline membrane disease and pneumothorax was noted. The details of CDH information available, was the side of CDH and organs herniated into the chest (liver, stomach, small intestine, colon, spleen, kidneys, gonads and other organs). The post-mortem to autopsy duration interval was collected in days.

The details of the CDH patients with available lung tissue blocks, were given to the Pathology department, Information Technology team (IT) to search the Pathology record system to identify matched controls. A total of 1777 autopsy reports were screened for all the pediatric autopsy cases (aged < one-year) between 2006 till 2017. We went through the details for each report to identify the best control for the each CDH patient. Our matching criteria were gestational age, sex, and the pregnancy outcome (stillborn (SB)/termination of pregnancy (TP) or live born (± three days of the age at death). Lung tissues of the control group were obtained from the same side as the lung tissue specimens of the CDH patients. Processing of the tissue blocks collection was similar between patients and matched controls.

2.3. Comparing CDH Survivors Database to Our CDH Autopsy Cases Database

The information of the CDH survivors was extracted and collected by the Section of Pediatric Surgery. They retrospectively recruited the records for CDH survivors from the clinical charts and imported it into an electronic clinical database for the time interval between 1990 till 2016. CDH Survivors were patients surviving surgery and discharged from our hospital.

CDH Survivors clinical electronic charts were exported from a secure web database program REDCap (Research Electronic Data Capture) into an excel sheet. These included the details of each patient's prenatal diagnosis, gestational age, birthplace, date and weight, Apgar score at 1 minute and 5 minutes, sex. The presence of associated anomalies (cardiovascular, gastrointestinal, renal, neurological, metabolic, musculoskeletal and dermatological), type of cardiovascular anomalies (PDA, Atrial Septal Defect (ASD), Ventricular Septal Defect (VSD) and other cardiovascular anomalies) were also collected. The details of CDH information available, was the side and type of CDH, herniated organs into the chest, associated type of PHTN. The available maternal information was limited to the age and the use of smoking and alcohol during pregnancy.

For appropriate comparison between these data and our data, we manually imported it into the same statistical analysis program. Then, we further categorized the available data for 99 CDH patients into different variables. These included isolated CDH for patients having only cardiovascular anomalies associated with CDH. However, we did not include patients with PDA as this is a consequence of PHTN in CDH. As a result, the presence of cardiovascular anomaly was edited in the data. All other patients with other congenital anomalies were under the non-isolated CDH group. Another variable added was recoding the gestational age into two categories, one according to term pregnancy (preterm <37 weeks) and the other according to the

lung development stage at the time of birth (Embryonal 1-4 weeks, pseudo-glandular 5–17 weeks, canalicular 16–25 weeks, terminal saccular 26-35, and alveolar >36 weeks (7)).

2.4. Lung Morphology Assessment Using Hematoxylin & Eosin Staining

After obtaining the lung specimens, the paraffin lung tissue blocks were sectioned using a microtome into 5µm sections on a SuperFrost microscope slide (Fisher Scientific, Hampton, NH, USA) (Figure 1).

Figure 1. Left lung tissue (5µm section) of left CDH on a microscope slide for further experiments

To melt the paraffin wax, slides were heated at 58°C for 30 minutes. Further deparaffinization was achieved through dipping the slides in different dilutions (100% to 70 %) of xylene and ethanol. The tissue was washed with filtered water and then stained with Haemotoxylin for 3 minutes. The slides were then dipped in 1% acid alcohol, ammonia water and 70% ethanol washed in between with filtered water. Tissues were then stained with Eosin Y and rinsed in 70% ethanol. Dehydration of the tissue slides was performed by dipping the slides in an ascending gradient of ethanol and xylene. The slides were fixed using Permount (Fisher

Scientific), and coverslipped. Images were taken using a light microscope (Carl Zeiss, GmbH 37081 Gottingen, Germany) at a power of 5X, 10X, 20X, 40X magnification. Images were used to assess lung histology.

2.5. In Situ Hybridization for MiR-200b

An established protocol has been adapted from previous different protocols (165,166). All trays and instruments were decontaminated using RNAase AWAY solution (Ambion, Burlington, ON, Canada) or by heating to 180°C for 8 hours. Human tissue blocks were sectioned by microtome into 5µm sections transferred to a SuperFrost microscope slide (Fisher Scientific, Hampton, NH, USA). The tissue slides were heated to 58°C for 30 minutes and dipped in xylene to melt the wax. Tissue sections were rehydrated using different gradients of ethanol concentrations (100% to 70%). Then tissue slides were immersed in phosphate buffered saline (PBS) with 0.1% Tween. Each section was circled with an ImmEdge pen (Vector Laboratories, Burlington, ON, Canada) that provides a hydrophobic barrier. A 20µm/ml of proteinase K was applied to the tissue to partially digest it at 37°C for 10 minutes. This step allows a proper tissue exposure to the miRNA probe. The reaction was stopped by 0.2% glycine and post-fixation in 4% paraformaldehyde (PFA) for 10 minutes. Followed by washing the tissue with PBS preparing it for acetylation (66 mM HCl, 0.66% acetic anhydride and 1.5% triethanolamine). This step eliminated non-specific protein-RNA interactions. Followed by frequent washes of PBS and prehybridization by 1X sodium chloride/sodium citrate (SSC) buffer at 50°C for 30 minutes in a sealed humidified chamber. Tissue hybridization was carried out using 50% formamide, 5X SSC, 500 µg/ml yeast t-RNA and 1X Denhardt's solution. This hybridization solution is also used for diluting the locked nucleic acid (LNA) human (has-miR-200b) probe to 100nM at 52°C for 60 minutes. The probe will not be added to the negative control tissue section for future

comparison. Washing of the tissue slides with different concentrations of SSC (5X, 1X and 0.2X) at 52°C. Using blocking solution, consisting of 1X Roche Blocking Reagent (Roche, Mannheim, Germany), Tris-NaCl buffer (PH 7.5) and 0.1% Tween on the tissue. A sheep alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche) (Exiqon, Vedbaek, Denmark) was diluted in the previous blocking solution and applied on the tissue overnight at 4°C. The second day starts by washing the tissue sections with Tris-NaCl buffer and NMT buffer (0.1 M NaCl, 0.1 M Tris-HCl, pH 9-9.5 and 0.05 M $Mgcl_2$). The next step was applying NBT/BCIP solution (Thermo Scientific, Waltham, MA, USA) to the tissue slide in the dark at 30°C. Final steps included washing the tissue with distilled water, counterstaining with methyl green, dehydration in different concentrations of alcohol, xylene and coverslip with Permount (Fisher Scientific).

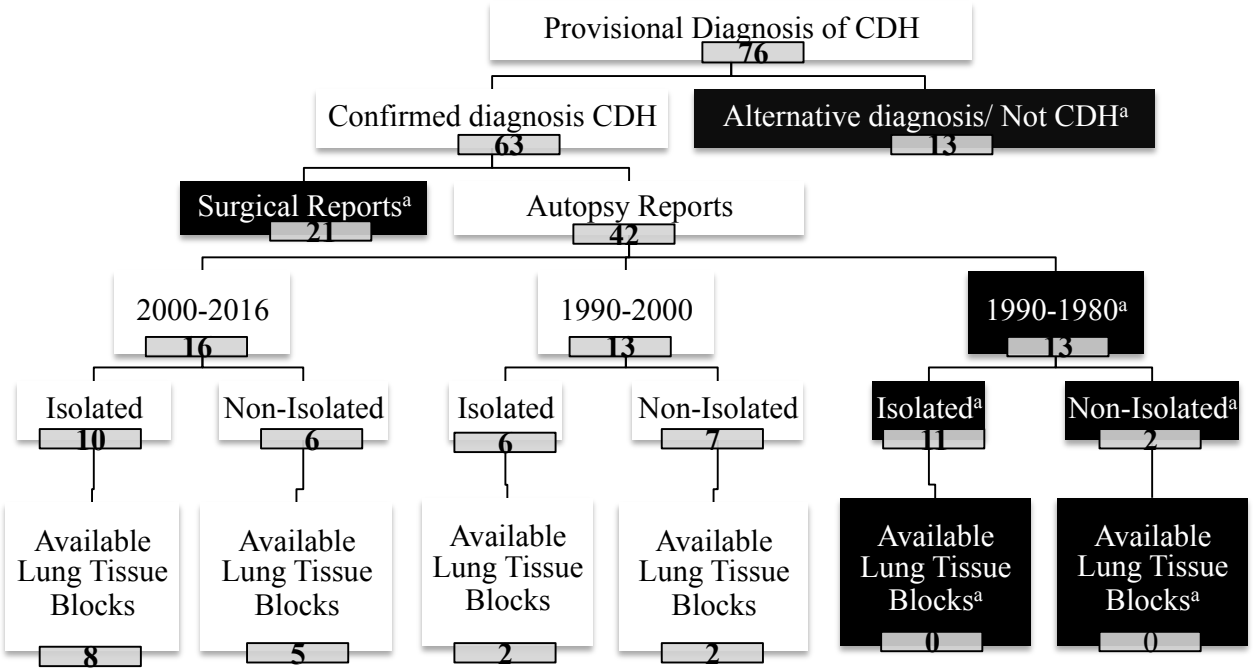
2.6. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS program version 16, Chicago, IL, USA) was used for data analysis. Data were screened for any entry mistakes using frequencies. Continuous data are presented as mean (Standard Deviation), and categorical variables are presented as frequency (percentage).

CHAPTER 3. RESULTS

3.1. Database Results

A total of 155 subjects were included in the current study and were divided them into three groups. The first two groups contained 40 deceased subjects of CDH (non-survivors) while 16 deceased subjects who were not having CDH were included in the control group, these were collected from the pathology autopsy database. The third group was formed by the clinical REDCAPs database including 99 live CDH patients who underwent surgical repair (survivors). We compared our two group (non-survivors and controls) to the REDCAP database group (survivors). The CDH study subjects were categorized in isolated and non-isolated type of CDH. The stratification of CDH autopsy reports (Figure 1) revealed 13 cases between 1980 and 1990, 13 cases between 1990 and 2000, and 16 cases between 2000 and 2016. Furthermore, we found that between 2000 to 2016, there were 10 cases of isolated CDH and 6 cases of non-isolated CDH, of which we could obtain 8 lung tissue blocks of isolated CDH and 5 lung tissue blocks of non-isolated CDH. Between 1990 and 2000, there were 6 cases of isolated CDH and 7 cases of non-isolated CDH. We could only obtain 2 blocks of lung tissue from each of these groups. However, from 1980 and 1990, we were not able to retrieve any lung tissue blocks with CDH. These lung tissue blocks were either disposed, or a full autopsy was not permitted by the guardians.



[a]Represents patients excluded from the study (query or non–CDH cases, surgical reports, CDH patients without tissue blocks)

Abbreviations: CDH: congenital diaphragmatic hernia.

Figure 1. Study flowchart (the total number of lung tissue blocks available for the study were limited to 17 as compared to 76 reports of CDH obtained in the initial search of the autopsy database)

To sum up, of the 42 autopsy cases, only 17 lung tissue blocks were obtained, representing 10 tissue blocks of isolated and seven lung tissue blocks of non-isolated CDH patients. We excluded

2 autopsy cases (14 and 16-year-old) who developed an acute diaphragmatic hernia, as they did not have the pulmonary developmental abnormalities observed in the congenital form.

Of the total 40 autopsy cases, most patients (n=30) died in the neonatal period, while there were a higher number of TP (n=6) compared to SB (n=4). The range of post mortem to autopsy interval was (0-2 days) with a mean ± SD (1.37 ± 0.61 days).

Most of CDH survivors 75.7% were delivered at full term, while 50% of CDH non-survivors were preterm with 15% of TP and 10% of SB. Though our records have limited maternal information, among survivors, there was a higher use of smoking and alcohol in (16.7%) non-isolated CDH compared to (11.1%) in the isolated CDH. Mean gestational age (in weeks), birth weight (in grams) and lung-to-body-weight ratio (LBWR) were comparable in the study subjects. LBWR in non-survivors was less than in the control group. Details of general characteristics and parameters of the study subjects are shown in Table 1.

Parameter	CDH n=139				Controls n=16
	Survivors n=99		Autopsy cases (non-surviovors) n=40		
	Isolated CDH n=81	Non-Isolated CDH n=18	Isolated CDH n= 24	Non-Isolated CDH n=16	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Maternal age (years)	28.3 ± 5.8	28.0 ± 5.7	N/A	N/A	N/A
Gestational age (weeks)	38.2 ± 2.3	37.1 ± 2.4	36.5 ± 5.8	31.3 ± 6.9	33.2 ± 7
Birth weight (grams)	3358.5 ± 660.1	2998.0 ± 748.6	3240 ± 1368.6	1805 ± 1287.6	2289.4 ± 1219.4
Apgar score (1 minute)	5.7 ± 2.5	4.89 ± 2.1	N/A	N/A	N/A
Apgar score (5 minutes)	7.5 ± 1.6	6.72 ± 2.0	N/A	N/A	N/A
LBWR	N/A	N/A	0.008 ± 0.004	0.008 ±0.004	0.019 ±0.006

Table 1. General characteristics of the study subjects

It was noted that nearly 80.8% of CDH survivors have reached the alveolar stage of lung development, while, 10 % had reached the saccular stage. None of the CDH survivors were born in the canalicular stage. On the contrary, 62.5% of CDH non-survivors reached the alveolar stage, whereas, 15% and 17.5% had reached saccular and canalicular stages respectively. Detailed comparison between various study groups according to the stage of lung development is shown in Table 2.

		CDH n=139		Controls
		Survivors n=99	Non-Survivors n=40	n=16
Stage of lung development	**Parameter**	Mean ± SD	Mean ± SD	Mean ± SD
Canalicular stage*	Gestational age (weeks)	0	21.7 ± 1.4	21.3 ± 0.5
	Weight (grams)	0	410.4 ± 246.8	470.3 ± 49.0
	LBWR	N/A	0.010 ± 0.003	0.018 ± 0.005
Saccular stage*	Gestational age (weeks)	32.9 ± 2.5	32.3 ± 2.4	33 ± 3.3
	Weight (grams)	2174.1 ± 559.6	2023.5 ± 809.4	2184.6 ± 483.8
	LBWR	N/A	0.011 ± 0.002	0.019 ± 0.007
Alveolar stage*	Gestational age (weeks)	38.6 ± 1.3	38.3 ± 2.0	38.5 ± 1.6
	Birth Weight (grams)	3427.3 ± 569.0	3517.7 ± 914.7	3144 ± 892.8
	LBWR	N/A	0.006 ± 0.003	0.019 ± 0.007

Table 2. Comparison of gestational age, birth weight and lung-to-body-weight ratio in various study groups according to the stage of lung development

*Lung development stages according to the gestational age: Embryonal 1-4 weeks, pseudo-glandular 5–17 weeks, canalicular 16–25 weeks, terminal saccular 26-35, and alveolar >36 weeks (7).

Male subjects were more common in all study groups compared to female subjects. In the CDH survivor group, 59.6% of the subjects were male, 60% in the CDH non-survivor group while 62.5% of the controls subjects. Distribution of male and female subjects in various groups is depicted in (Figure 2).

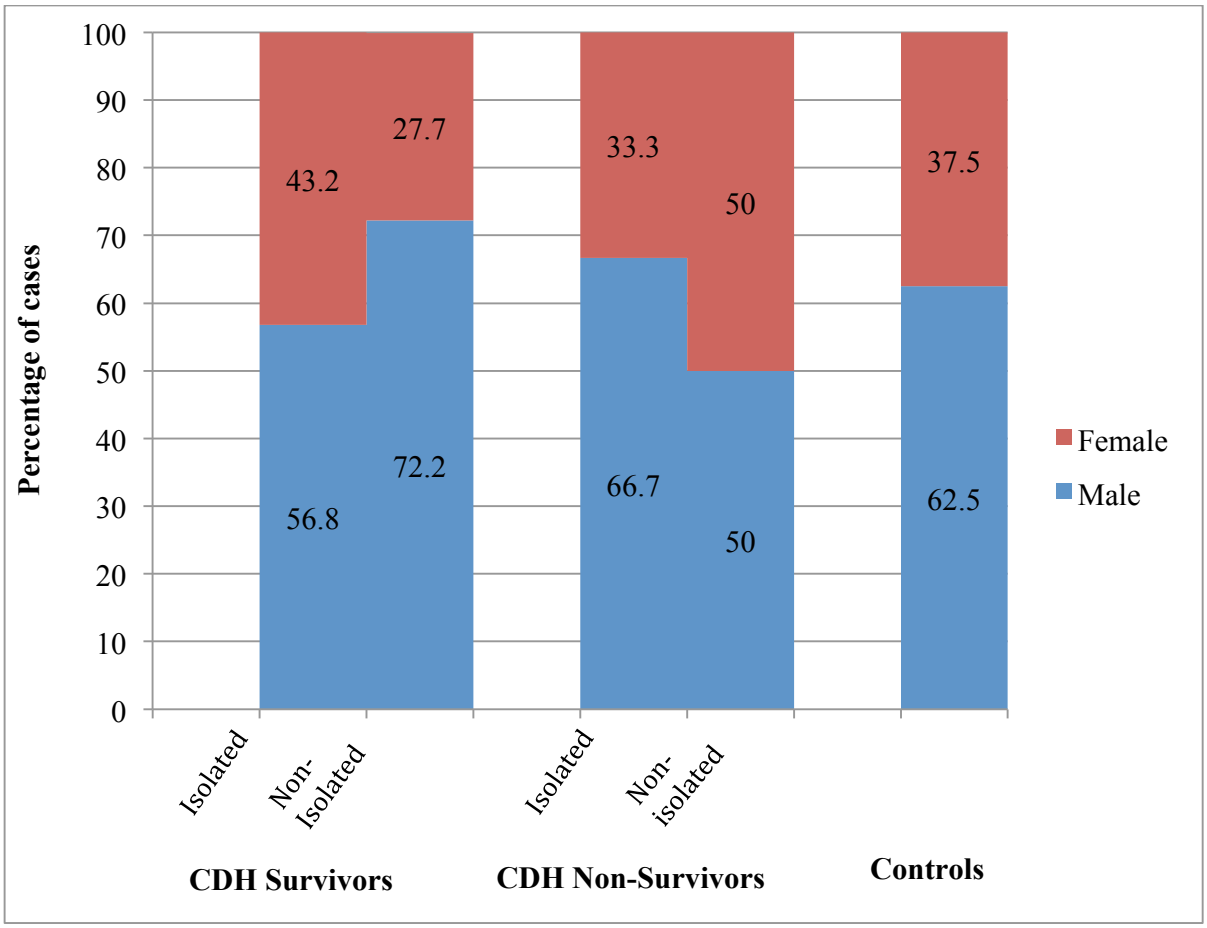


Figure 2. Frequency distribution of male and female subjects in study groups

The study subjects were taken from the year 1980 to 2016. The time period was divided into 4 different eras. The subjects included in CDH Survivors were taken from 1990 while the CDH non-survivors were taken from the year 1980. Details of the study subjects according to their CDH status and time period is shown in Figure 3.

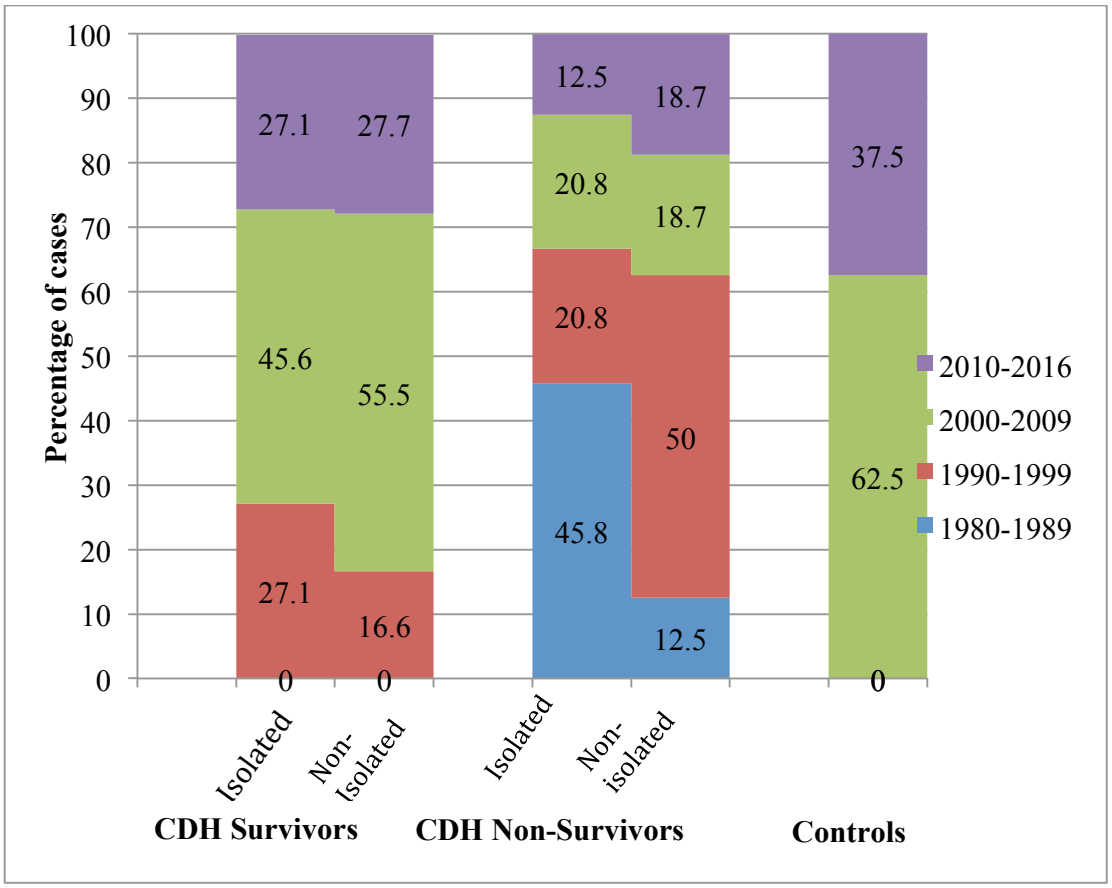


Figure 3. Categorization of study subjects into groups according to the year of birth

Antenatal diagnosis of CDH has increased over the decades. The rate of prenatal diagnosis of CDH non-survivors cases was only 15.3% between 1980-1989. In all CDH groups, the prenatal diagnosis rate increased to 39.4% in decade 1990-1999 and to a higher rate of 52.7% in the decade 2000-09 and further raised to 69.7% in the current decade. A substantial increase can be seen in the detection rates till the present times in Figure 4.

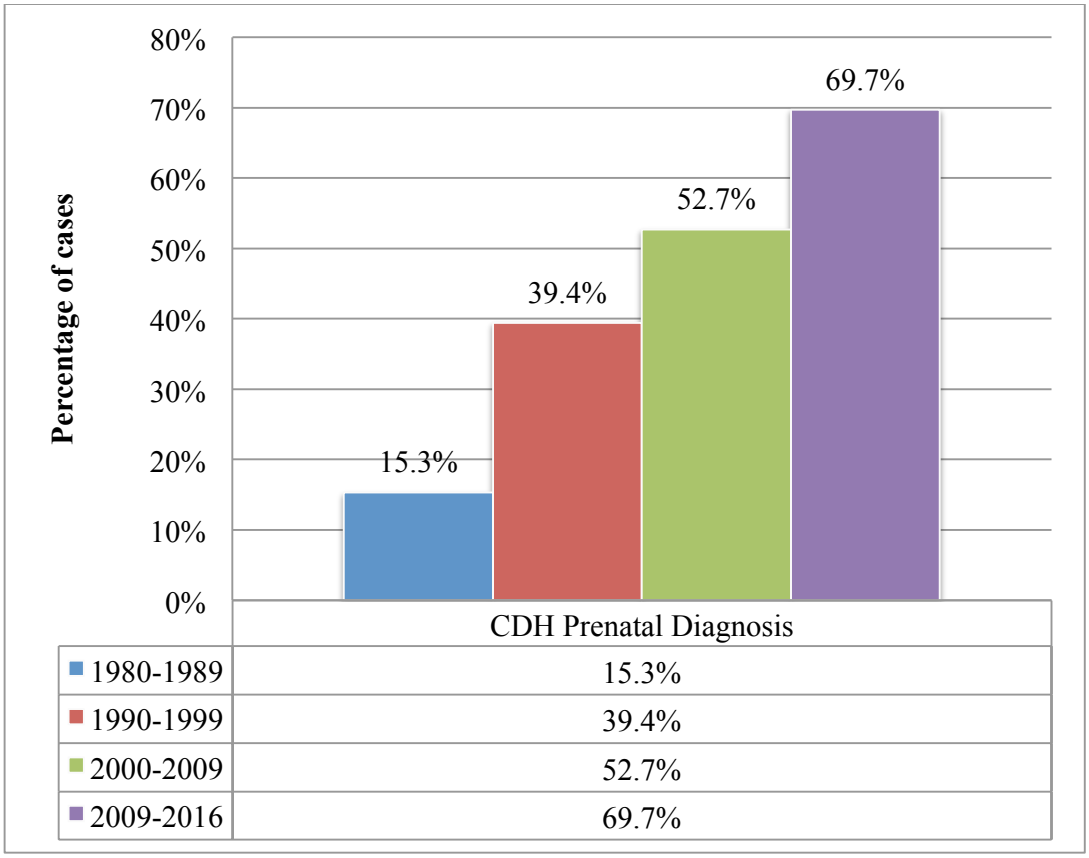


Figure 4. The antenatal diagnosis of CDH over the decades

Among all CDH groups, the most frequently occurring anomalies reported were cardiac anomalies (42.6%), followed by neurological (12.2%) and muscular abnormalities (12.2%). Gastro-intestinal anomalies were present in (11.5%) of the subjects and renal in (10%) of the CDH subjects, whereas; lower association with genetic (5.7%), dermatological (3.6%) and metabolic abnormalities (0.7%) were present in only a few subjects. Group-wise detail of various abnormalities is shown in Figure 5.

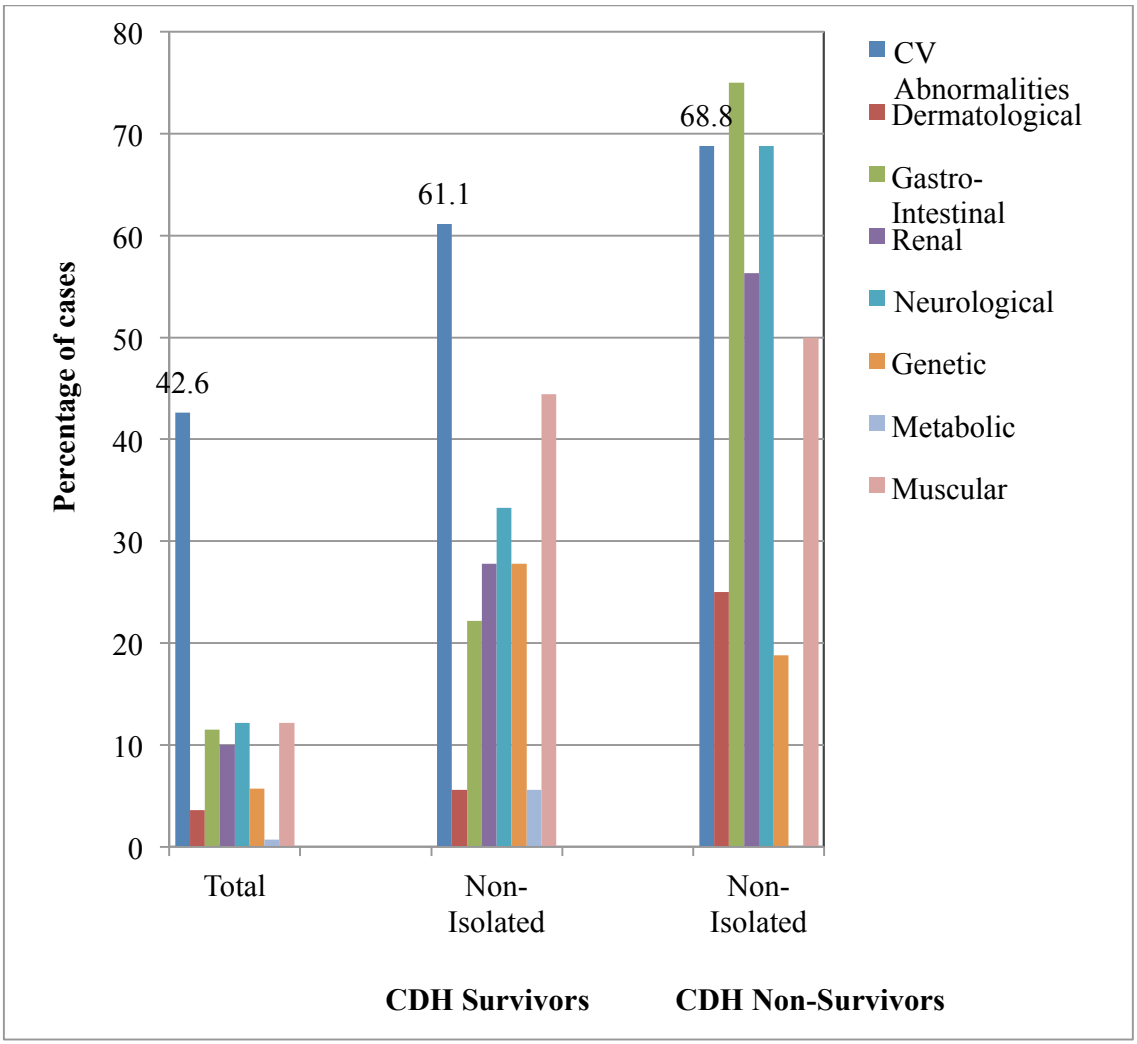


Figure 5. Categorization of associated abnormalities of the systems in CDH subjects

More than half of the CDH subjects (n=66) had some type of cardiac abnormality. The most frequent occurring cardiac anomaly in CDH groups was ASD followed by VSDs. The details of the various cardiac anomalies in groups are shown in Figure 6.

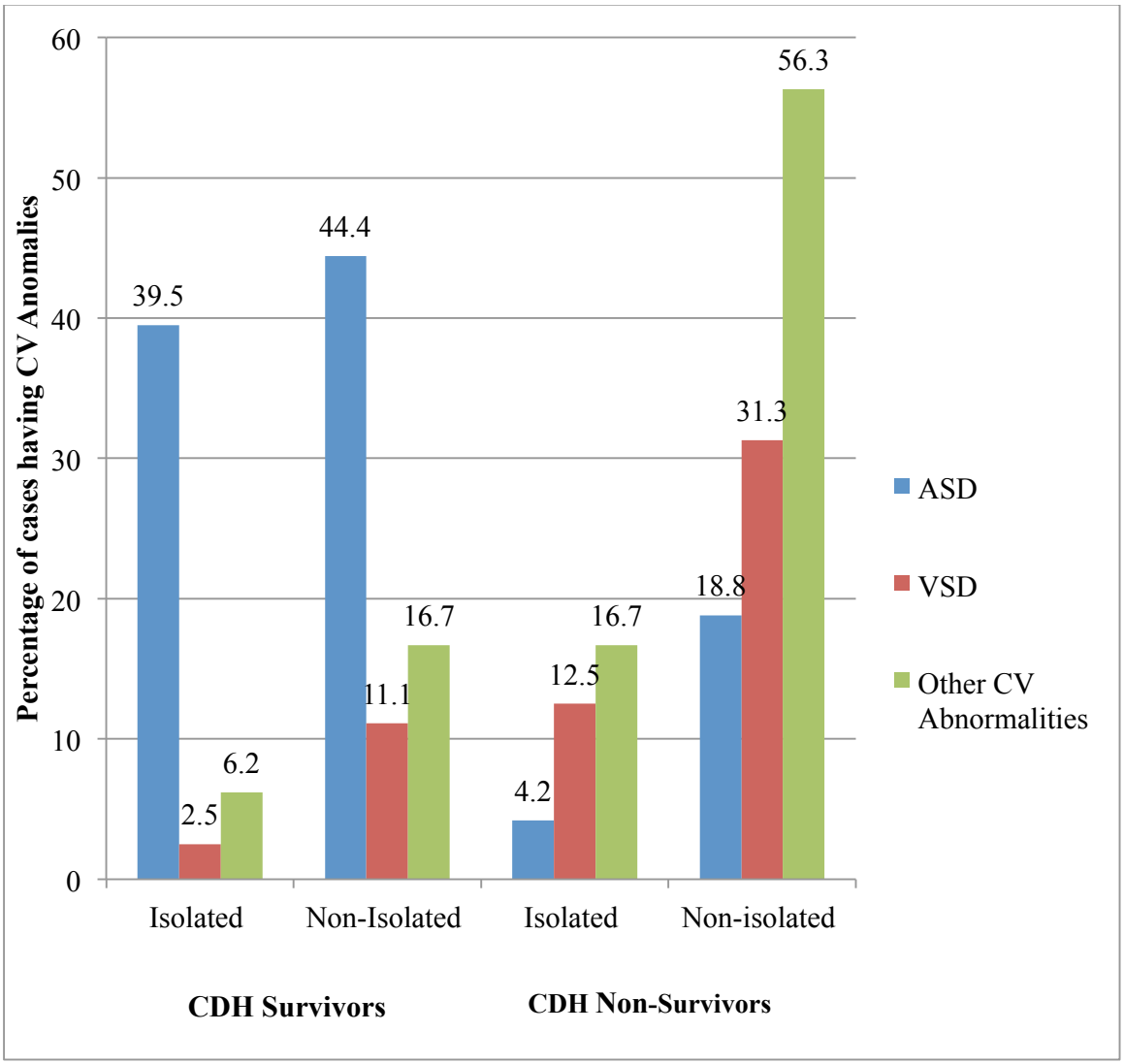


Figure 6. Details of various cardiac anomalies in study groups according to their CDH status

*CV: Cardiovascular

Among all CDH subjects, isolated CDH (75.5%) was the predominant anomaly compared to non-isolated type of CDH (24.4%). Left sided CDH was much more common (76.9%) compared to right sided CDH (17.2%). Bilateral CDH was found in only 2 subjects while status of the 6 study subjects could not be verified from the records (Figure 7).

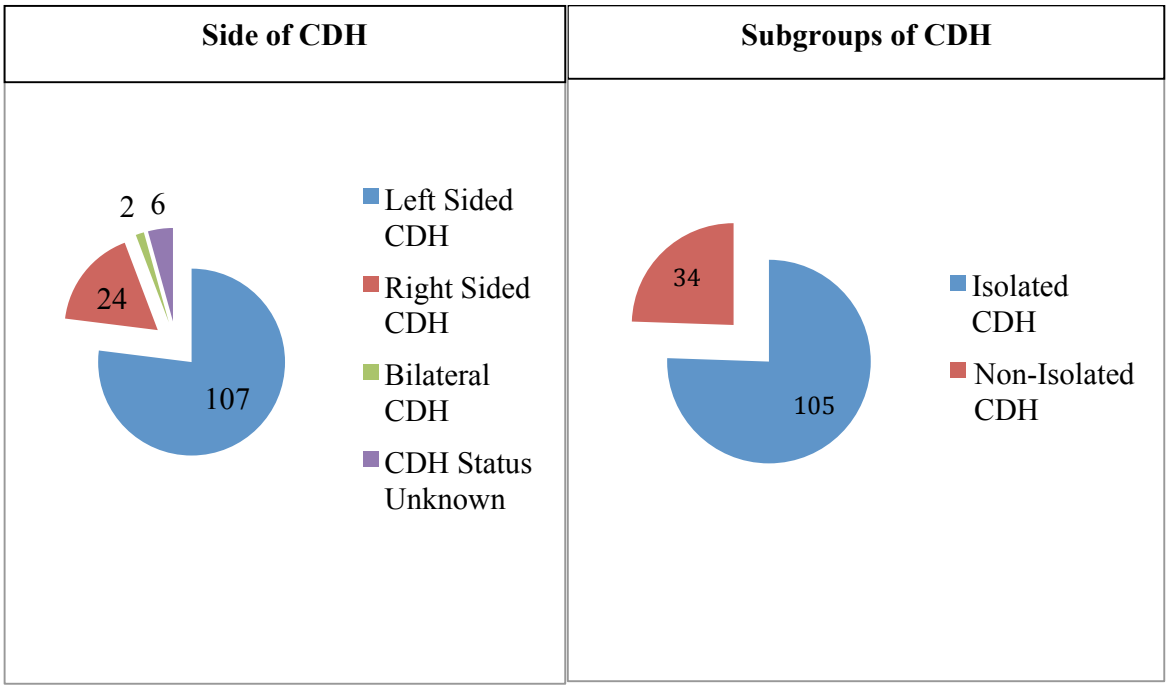


Figure 7. Pie chart showing side and type of CDH in CDH study subjects

Type of hernia was identified in 98 of the survivors' subjects. Among all CDH survivors, the most common type of CDH was Bochdalek (posterolateral) (51.6%), followed by Morgagni (7.7%). A hernia sac was present in 30% of the CDH survivor subjects. Among all CDH groups, the most frequently herniated organ into the chest cavity was small intestine (75.5%), followed by colon 61.8% and spleen 53.2%. Stomach 46% and liver 37.4% were less commonly herniated compared to intestine. Kidney was herniated only in 3.6% subjects while none of the subjects were having gonads shift into chest cavity (Figure 8 & 9). Hyaline membrane disease and pneumothorax was seen in less than half of the study subjects in the CDH deceased group. Data regarding presence of hyaline membrane disease and pneumothorax was not available for the CDH survivors group.

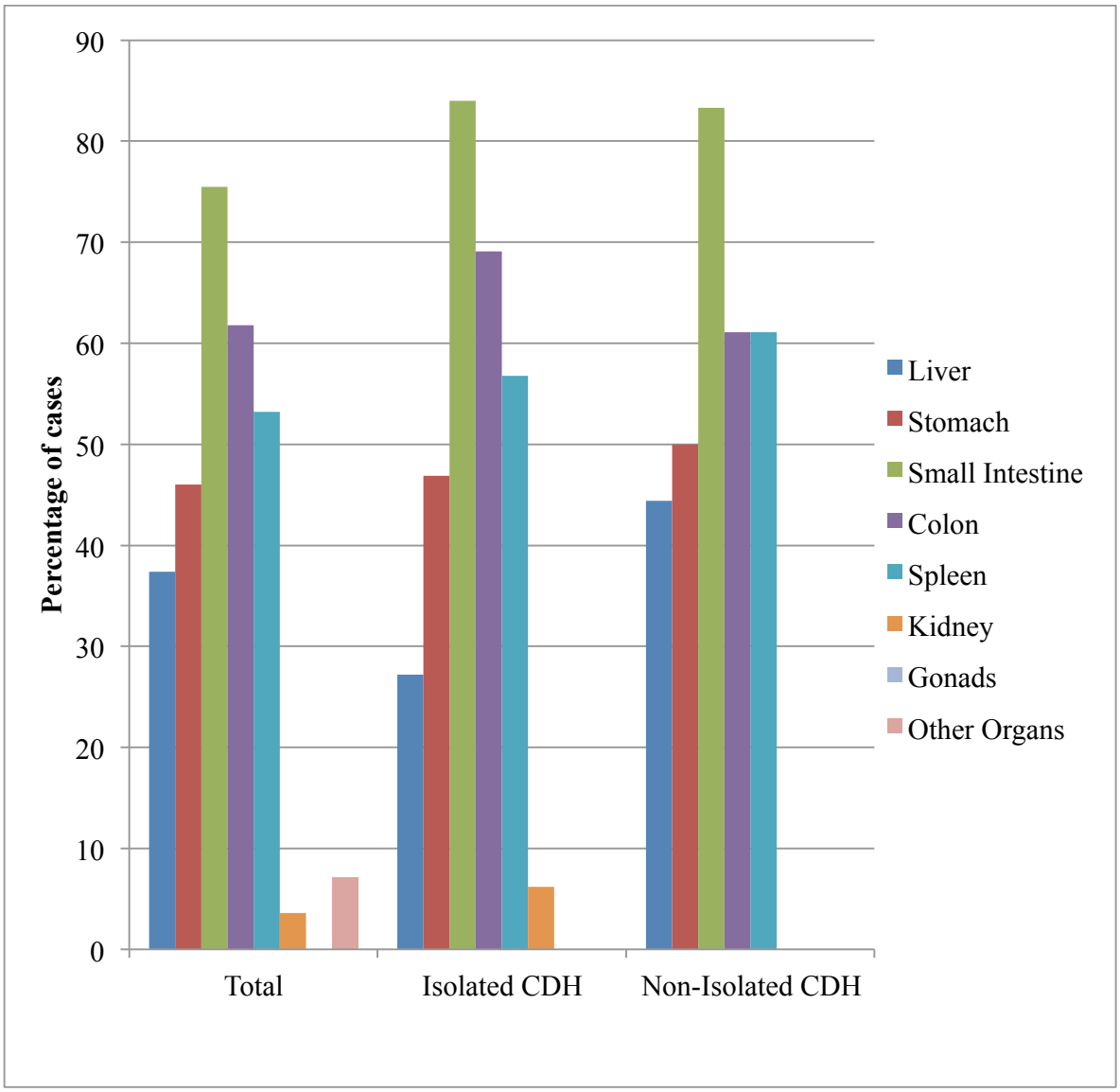


Figure 8. Status of various abdominal organs in chest cavity in various CDH survivor groups

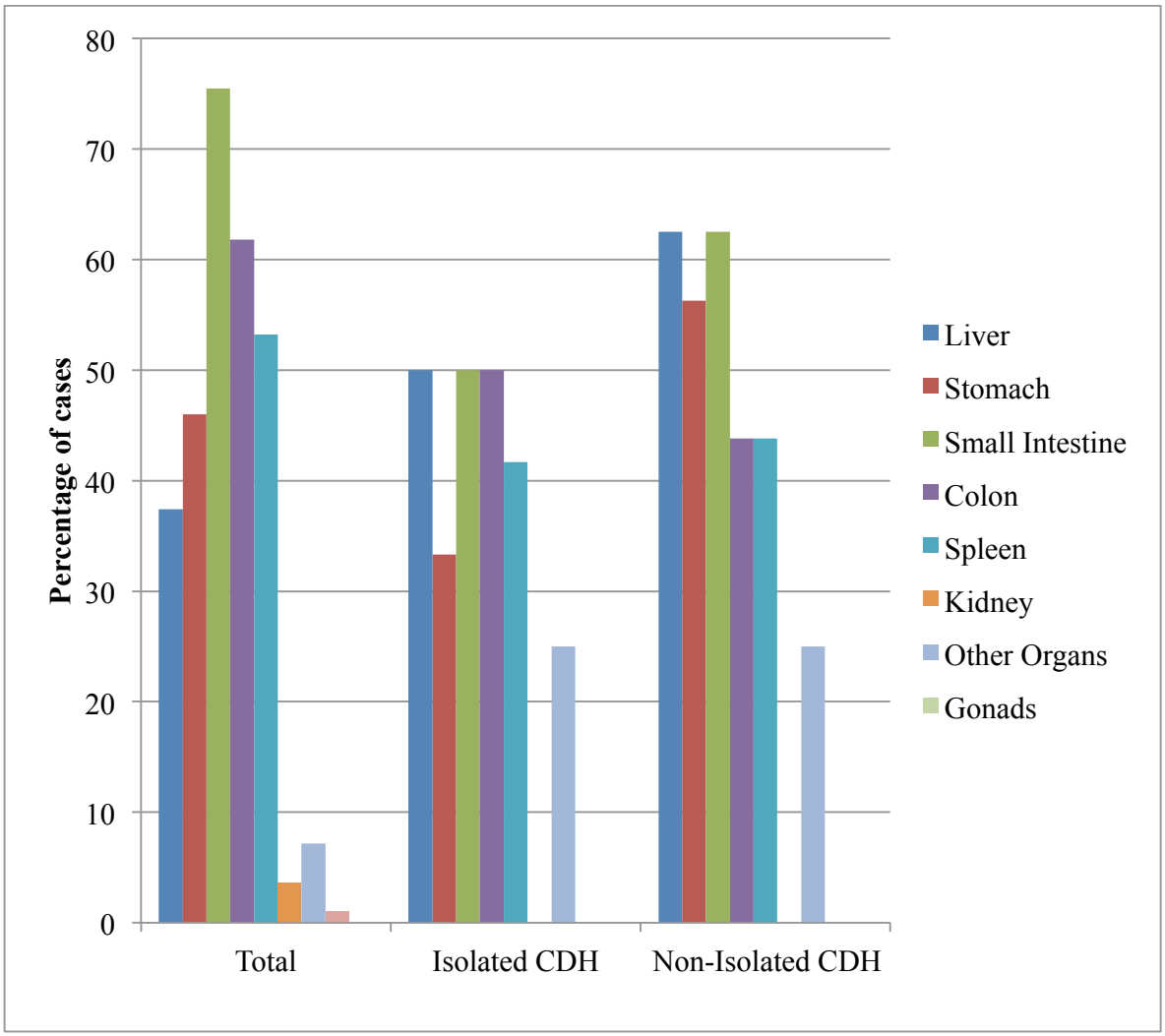


Figure 9. Status of various abdominal organs in chest cavity in various CDH non-survivor groups

More than half of the CDH survivors subjects (55.4%) were diagnosed as having some form of PHTN. Organ-wise comparison of form of hypertension is shown in Table 3.

Abdominal organs present in the chest	Mild PHTN n=22 (15.83%)	Systemic PHTN n=24 (17.27%)	Supra-systemic/ severe PHTN n= 14 (10.07%)
Liver	8 (36.4%)	9 (37.5%)	6 (42.8%)
Stomach	6 (27.8%)	16 (66.7%)	9 (64.2%)
Small Intestine	21 (95.5%)	23 (95.8%)	11 (78.5%)
Colon	14 (63.6%)	20 (83.3%)	9 (64.2%)
Spleen	11 (50%)	18 (75.0%)	12 (85.7%)
Kidney	2 (9.1%)	1 (4.2%)	0 (0%)
Gonads	0 (0%)	0 (0%)	0 (0%)
Other organs	0 (0%)	0 (0%)	0 (0%)

Table 3. Status of pulmonary hypertension in subjects according to herniated abdominal viscera

The most frequent cause of death in the non-survivor isolated CDH group was pulmonary complications 83.33%. There were 6 cases (2 isolated, 4 non-isolated) of TP while 4 (1 isolated, 3 non-isolated) were of SB. The mean post-partum to autopsy interval for isolated non-survivor group was 1.44 ± 0.53 days (range 1-2 days) while for non-isolated group it was 1.29 ± 0.76 days (Range 0-2 days). The detail of cause of deaths in non-survival group is shown in Figure 10.

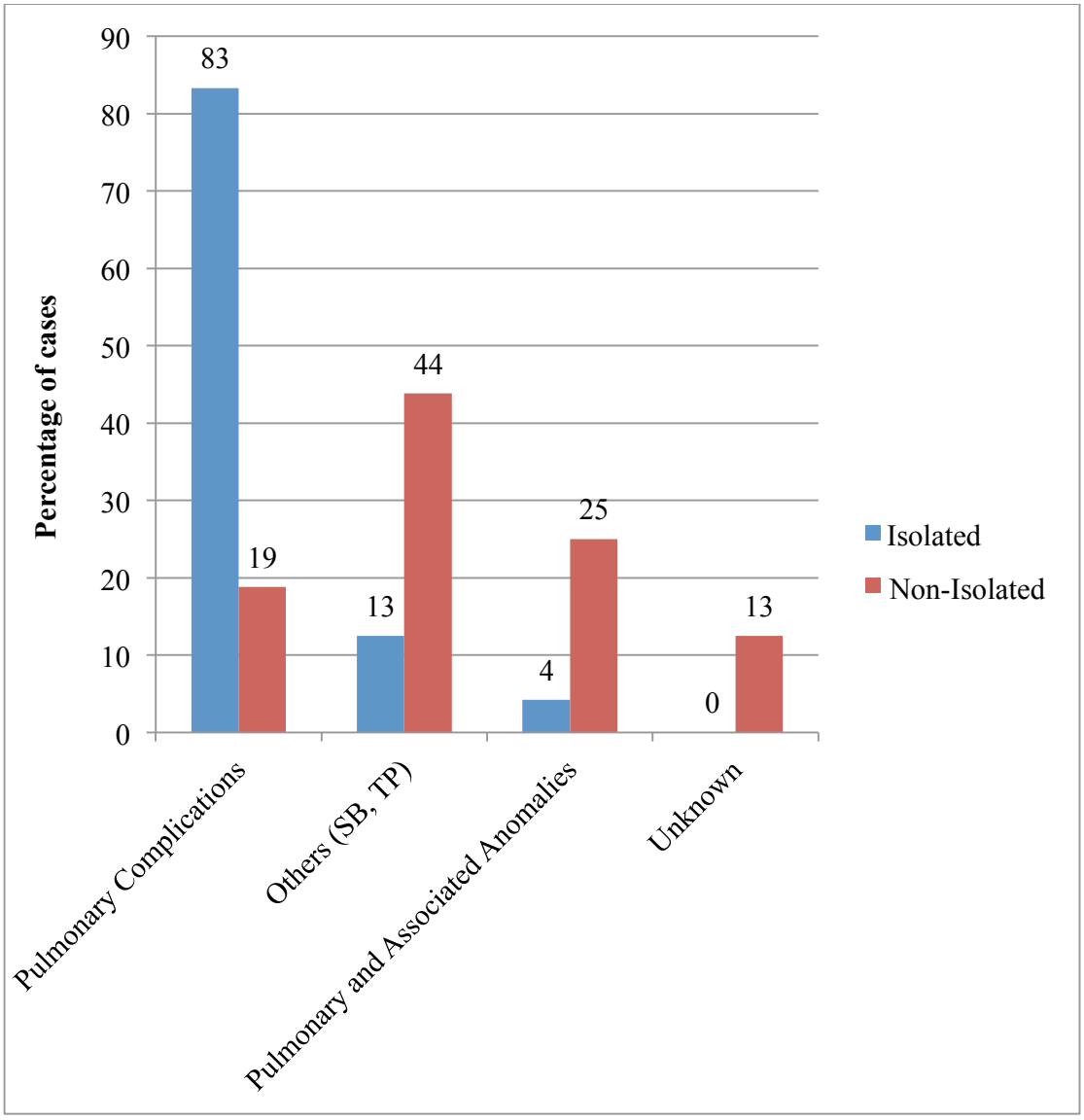


Figure 10. Cause of death in the non-survivor CDH groups

As illustrated in Figure 11, of the total number of CDH autopsy cases, 25% were SB and TP. Of the remaining 75% live births, 62.5% died within the first three days of life, and 10% died after the 3rd day of life. Of the total deceased within the first three days of life, 24% had a surgical repair attempted.

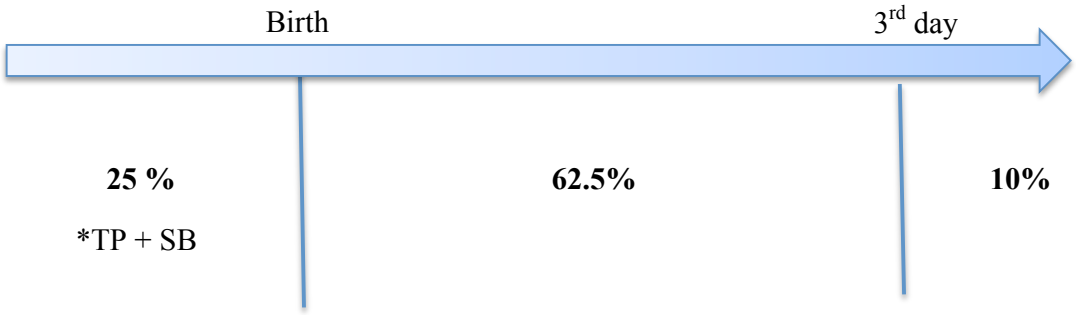


Figure 11. The outcome of pregnancies of women with CDH babies

* TP: Terminated Pregnancy, SB: Stillbirth**.**

3.2. Experimental Results

Lung Morphology Assessment Using Hematoxylin & Eosin Staining

Hematoxylin & Eosin staining of a lung section for a full term, left sided, isolated CDH at the age of 2 days old, aimed to assess the quality of the tissue block chosen. The staining of the contralateral lung was also performed for comparison purposes. Figure 12, shows a section of the right lung (contralateral side of CDH), whereas, Figure 13 and 14, show sections of different blocks from the ipsilateral lung of a CDH neonate. Comparing the lung tissue sections of these blocks, the chosen tissue block was A3 as we can see an early epithelial tissue detachment of an airway in A4.

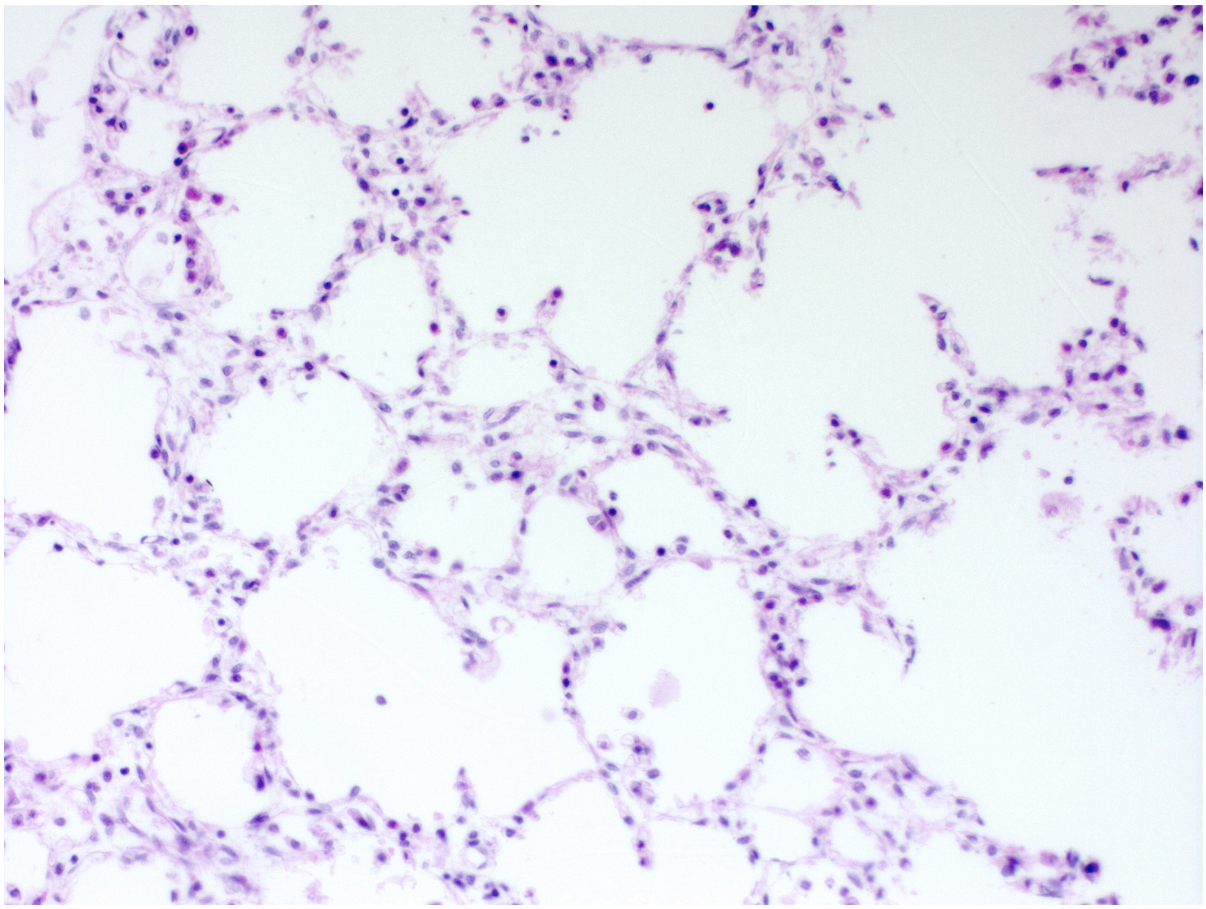


Figure 12. Hematoxylin & Eosin stain of contralateral lung for CDH human neonate, alveolar stage of lung development (20X magnification)

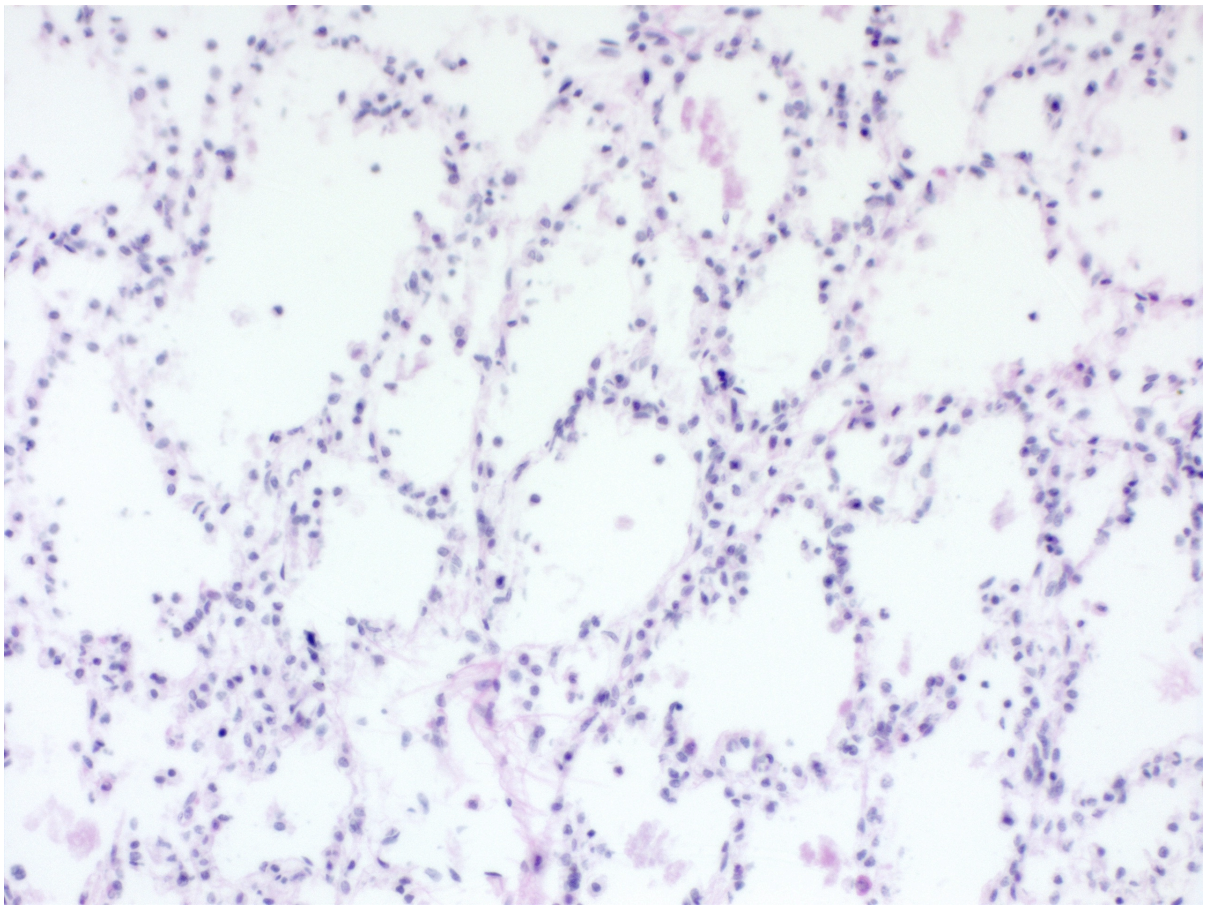


Figure 13. Hematoxylin & Eosin stain of ipsilateral lung for CDH human neonate, alveolar stage of lung development (block A3) (20X magnification)

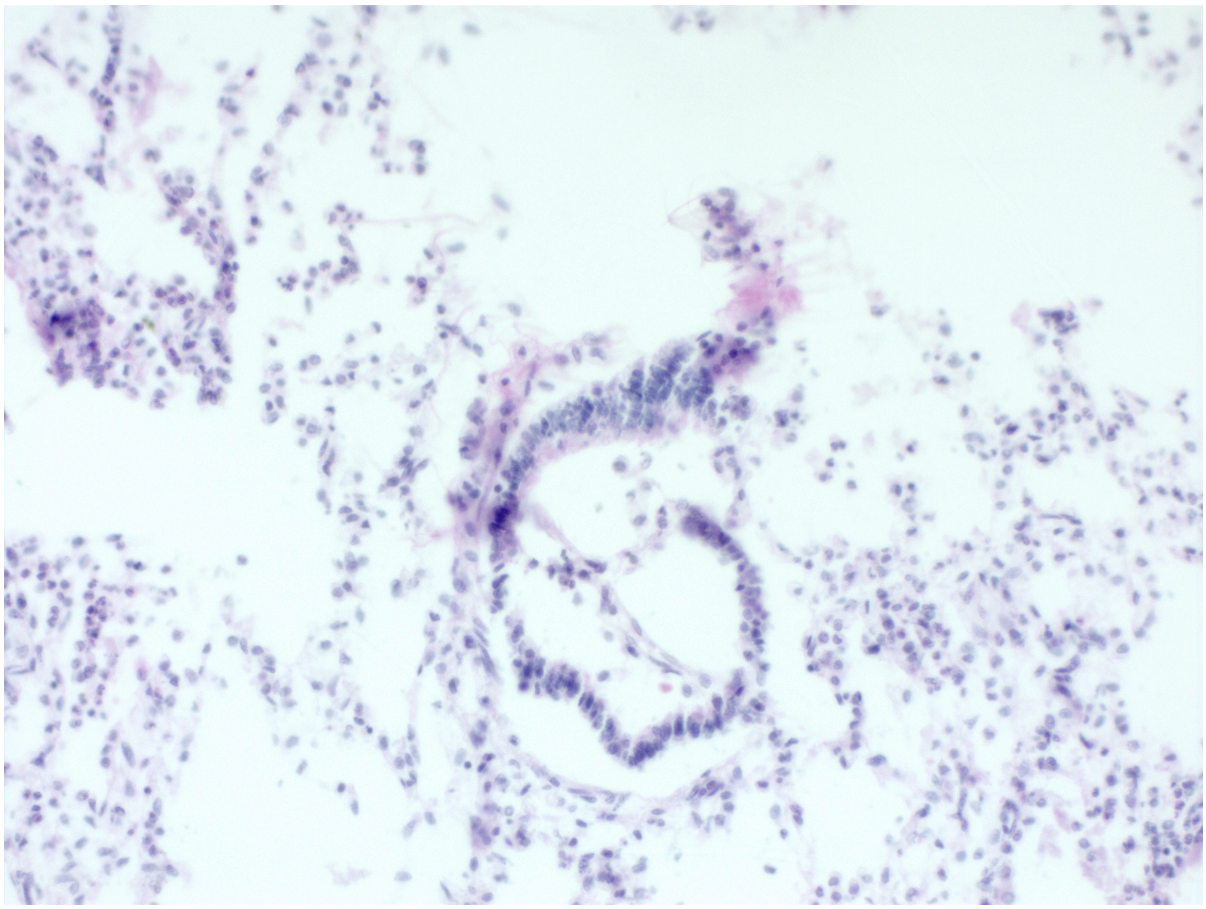


Figure 14. Hematoxylin & Eosin stain of ipsilateral lung for CDH human neonate, alveolar stage of lung development (block A4) (20X magnification)

To compare different stages of lung development we looked at the H&E stained lung sections for a 22-weeks, non-isolated, still born CDH neonate (Figure 15).

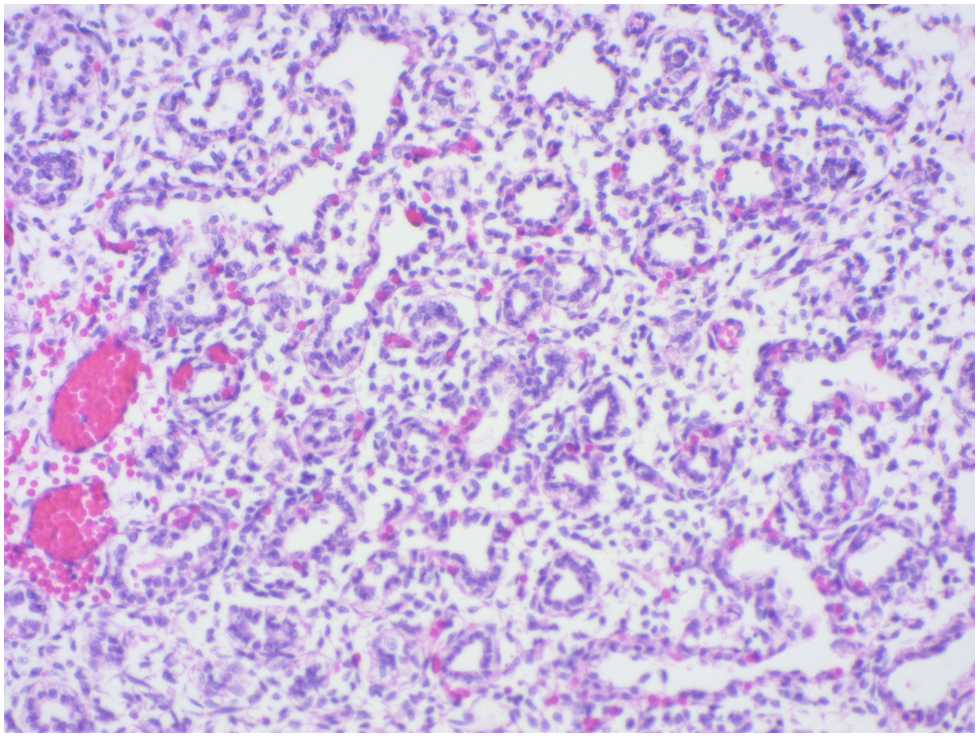


Figure 15. Hematoxylin & Eosin stain of ipsilateral lung for CDH human neonate, canalicular stage of lung development (20X magnification)

In Situ Hybridization for MiR-200b

We performed in situ hybridization for miR-200b expression in CDH human neonate lung (canalicular stage) sections to set up the proper conditions for future experiments. The infant was part of non-isolated CDH group, deceased in utero. The positive staining of the miR-200b is blue in color (Figure 16). No signal was observed in the negative control (Figure 17).

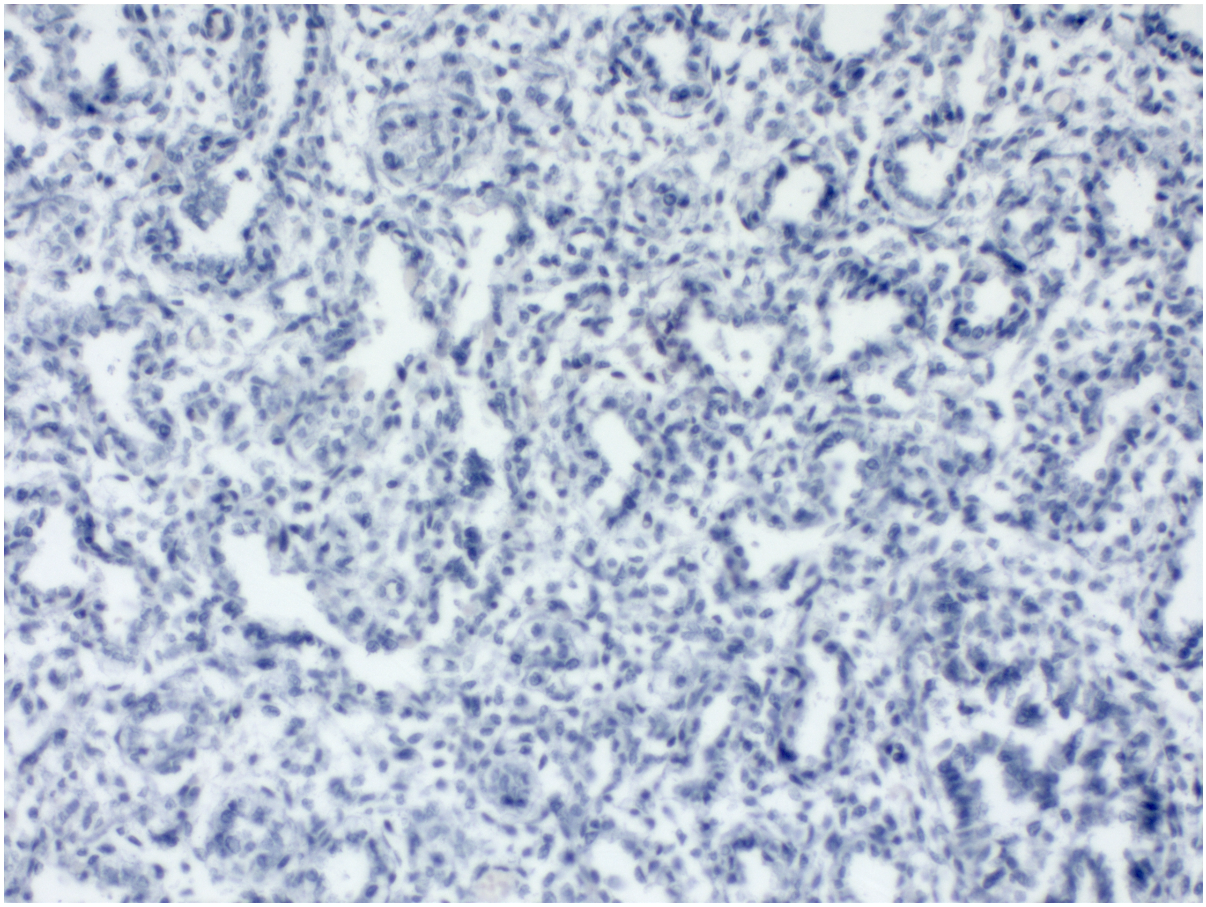


Figure 16. Expression of miR-200b in CDH human neonate, canalicular stage of lung development (20X magnification)

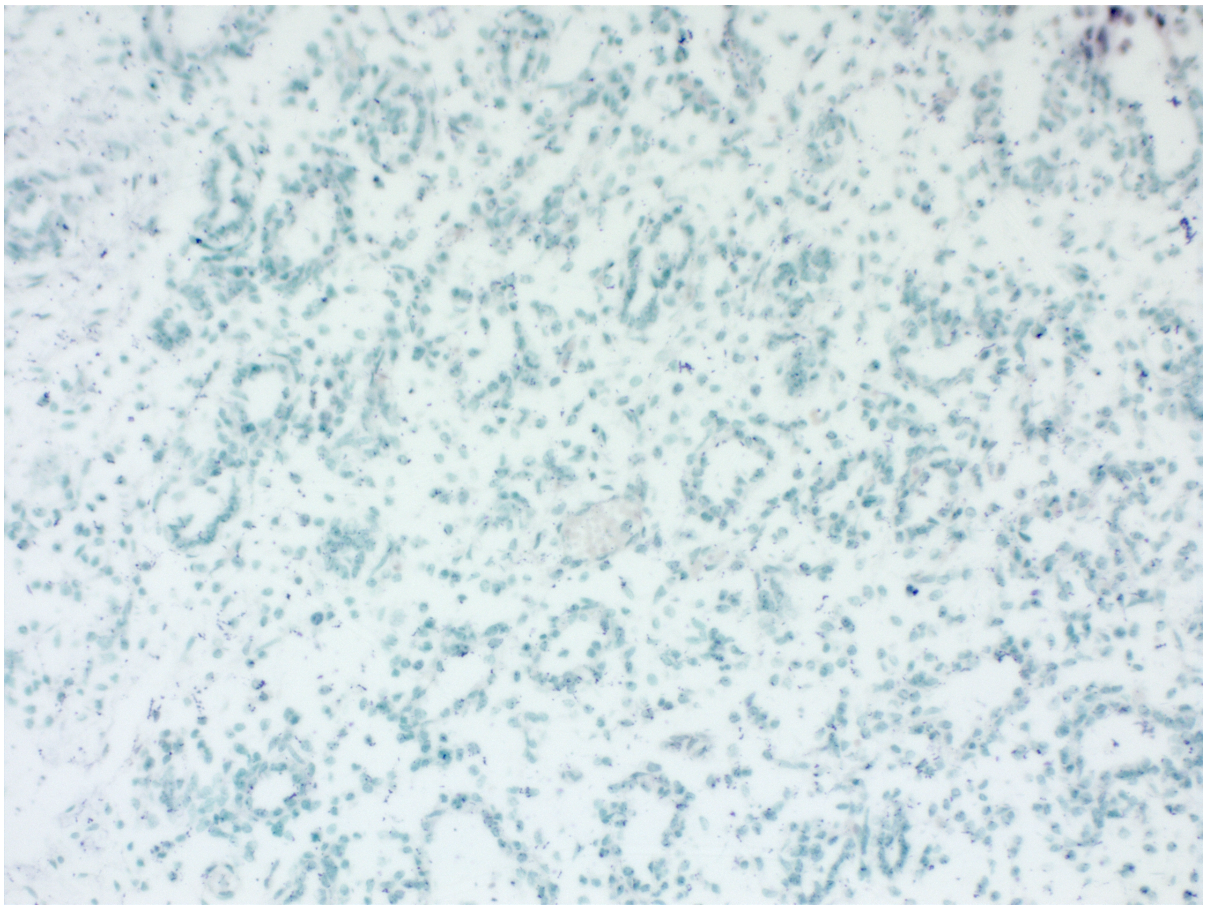


Figure 17: Negative control in CDH human neonate, canalicular stage of lung development (20X magnification).

CHAPTER 4. DISCUSSION

4.1. Overview of the Results

CDH in newborns is a disease with a high mortality, and its etiology remains largely unknown (34,65,110). The availability of the clinical information for each CDH patient along with tissue blocks can help in understanding the molecular mechanisms underlying the pathophysiology of the disease. Our study is the 1st in Manitoba that provides a database of the clinical characteristics coupled with lung tissue blocks for CDH babies. In the current study, we included a total of 155 subjects out of which 139 were CDH patients while the remaining 16 were controls. Of all CDH cases, 99 were survivors after surgery while the remaining 40 were deceased CDH subjects. Survivors and deceased were further categorized into isolated (75.5%) and non-isolated (24.4%) CDH subjects.

Among all CDH groups, antenatal diagnosis of CDH increased from 39.3% in (1990-1999) to 69.7% in (2009-2016). Male subjects (59.7%) and left-sided CDH (76.9%) were more commonly affected by CDH. The most frequently herniated organ into the chest cavity was the small intestine (75.5%). The principal associated congenital anomaly was congenital cardiovascular defects (42.6%). The most frequent cause of death in CDH subjects was pulmonary complications (83.3%).

4.2. Human Lung Fetal CDH Tissue Bank

The use of a paraffin embedded lung tissue bank has been established by others in chronic obstructive lung disease and lung cancer. It helped in understanding the pathophysiology of the disease and the treatment options (167,168). A study of 38 human fetal lung tissues (frozen samples) emphasizes the molecular profile of gene expression in different stages of normal lung development (169). Another study has also used 23 human fetal lung tissues (frozen samples)

with a postmortem interval of less than 2 hours (170). A tissue biobank (US Biomax) provides both frozen and paraffin embedded human lung tissue (171). However, for lung tissues in CDH neonates, there are a few studies that used frozen lung tissue or paraffin embedded tissues (172,173). Up to our knowledge, there are limited studies of human paraffin embedded fetal lung tissue bank for CDH. Our tissue bank (paraffin embedded) provided different stages of lung development in CDH cases with >20 weeks of pregnancy. We could not obtain CDH lung tissue before 20 weeks of gestation, as it is difficult to diagnose CDH early in pregnancy. A study conducted by Garne et al., (61) reported that the mean gestational age for the diagnosis of CDH is 24.2 weeks. Therefore, women opting for termination of pregnancy can do so only after completion of 20 weeks of gestation.

Morbidity and mortality in CDH are usually due to lung hypoplasia and PPHN. Reduced lung volume and impaired alveolarization of both lungs have been observed in CDH patients (174). In the present study, lung hypoplasia was seen in 100% of the CDH–autopsy subjects. The combined lung weight (20.86 ± 16.67gm) in the CDH deceased group was lower than the lung weight (45.5 ± 33gm) in the control group. The lung samples were not obtained from the survivor's group as it is unethical to obtain lung tissue from hypoplastic lungs thereby compromising the patient’s clinical outcome.

Due to a wide gestational age range (20-42 weeks), subjects were divided into groups according to their lung development stage. A study on normal fetal lung development has used the same subgrouping with similar gestational ages(175). In our study, the highest number of CDH subjects was in the alveolar (75.5%) stage, followed by the saccular (11.5%) and the canalicular (5%) stages respectively. Among CDH non-survivors, 62.5% reached an alveolar stage, whereas, 15% and 17.5% had reached saccular and canalicular stages respectively. Subjects in non-

survivor groups, who were in the canalicular stage were having a lower combined lung weight of 4.5 ± 2.2 compared to combined lung weight of the controls it 8.4 ± 2.5. Values increased the lungs were at the saccular stage and further rose at the alveolar stage.

4.3. Lung Hypoplasia and LBWR Measurements in CDH

Researchers have used lung volume to body weight and LBWR in animal models as well as in human studies as a valuable tool to assess the degree of lung hypoplasia in study subjects (176–179). Among non-survivors we found lower LBWR values (0.008 ± 0.004) compared to controls (0.019 ± 0.006). Our results are in accordance with other studies whereby researchers have reported similar trend in CDH study subjects. Runao and his co-workers reported significantly low ratio in a non-survivor group [median 0.009 (range 0.004-0.021)] compared to surviving subjects [median 0.011(range 0.008-0.020)](180). Wu et al. reported a progressively lower LBWR between CDH in a rabbit model (0.014 ± 0.0040) and control animals (0.030 ± 0.04)(181). Subjects in non-survivor groups, who were in the canalicular stage were having a lower lung-to-body-weight ratio of 0.010 ± 0.003 while for controls it was 0.018 ± 0.005 respectively. Whereas the values for mean birth weight rose in saccular and alveolar stage, the values for LBWR rose in the saccular stage but then decreased in the alveolar stage in both non-survivors and control groups. The unexpected decrease could be due to the missing data for some subjects for the birth weight and/or lung weight.

4.4. Comparison Between Different Types of CDH

Studies have shown that isolated CDH (60-70%) is more common than the non-isolated type (39). In our study, 75.5% cases were of isolated CDH, while, 24.4% were non-isolated cases of CDH. This was also supported by Waag et al. which reported 70% of the isolated type as well as Fauza and Wilson et al. which recorded 60% (182,183). While conducting a Hospital-Based

Malformation Surveillance Program in Boston, Pober et al. (33) reported 55% and 45% incidence of an isolated and non-isolated type of CDH. However, in a study conducted in a tertiary center showed a lower incidence of isolated CDH (49%)(184). Variation in the percentages could be due to different definition criteria of isolated vs. non-isolated CDH and the type of the hospital the patients were recruited.

Of all types of CDH, left-sided hernias are more common accounting for approximately 72 to 90% of the cases while right-sided hernias usually account for only 9-24% of the cases (24,40,185). This was also seen in our study where 76.9% cases were left-sided CDH, and just a few cases consisted of right-sided CDH. Similar percentages for left-sided CDH (72%) were reported by Coughlin et al. (2015) (185). McHoney in his review article stated an incidence of left and right sided CDH with a ratio of 6 to 1 (24). In a 20-year review article, Brownlee and his co-workers also reported a 75% incidence of left-sided CDH which is in accordance with our study (40).

Anatomically, CDH can be classified into various types depending upon the location of the defect. In our research findings, the most common type of CDH defect was Bochdalek (80.8%) followed by Morgagni (12.1%). Our research findings are in accordance with the studies conducted in 1992 by Torfs et al. (186) and a recent study by Veenma at al. (30) whereby authors reported 70-75% prevalence of the Bochdalek type of CDH. Taylor et al. also reported Bochdalek as the most prevalent type of CDH defect followed by Moragnai CDH accounting for 9-12% of diaphragmatic hernias (37). Researchers from another study reported 70% incidence of Bochdalek, 27% for Morgagni and the remaining types accounting for 2-3% of the subjects only (68). The most likely reason for this high incidence of Bochdalek type is an improper fusion of the PPFs to the septum transversum and dorsal mesentery of the esophagus, which form the

posterolateral segments of the diaphragm (20). Researchers have postulated that presence of a hernia sac may delay the presentation of CDH (110,187). In our study, a hernia sac was present in 30% of the CDH survivor subjects. Skarsgard and Harrison reported a 20% incidence of hernia sac in CDH subjects (188).

4.5. Other Factors Associated with CDH

Studies have reported a varying degree of mortality rates in CDH patients (189). In addition to pulmonary hypoplasia, due to pre-term delivery and low birth weight, these infants are at an increased risk for infections and have a high mortality compared to term delivered babies (62,189–192). In our study, CDH non-survivors had a higher incidence of being born preterm (<37 weeks) (50%) compared to survivors (24.5%). The mean gestational age (34.32 ± 6.73 weeks) and birth weight (2.68 ± 1.50 kg) in CDH non-survivors were less compared to CDH survivors (38.03 ± 2.38 weeks, 3.28 ± 0.69 kg). The findings of our study also corroborate the results from a study by Colvin et al. (2005) (62) whereby the researchers reported a higher mean birth weight (3.2 ± 0.8 kg vs. 2.9 ± 0.9 kg) and gestational age (37.5 ± 2.4 weeks vs. 37.0 ± 4.4 weeks) in CDH survivors compared to CDH non-survivors subjects. Similar findings were reported by Sweed and Puri from a study conducted in Ireland whereby mean gestational age (36.1 ± 4.5 weeks vs. 39.0 ±+ 2.4 weeks) and birth weight (2415 ± 906 gms vs. 3140 ± 563 gms) in the deceased group were lower than survivors (193). Mean birth weight for CDH survivors was 3283.9 ± 690.6 gms which are in accordance with the study Muratore et al. where they reported a mean birth weight of 3.16 ± 0.7 kg was reported in 100 CDH patients (194). Another study reported lower birth weight (<2500g) in CDH infants and increased chances to be born before term (190).

Development of CDH has been linked with male gender, increasing maternal age >40 years old, smoking and use of alcohol during pregnancy (38,195). The mean maternal age of CDH survivors was 28 ± 5 years, with a higher use of smoking and alcohol in the non-isolated group (16.7%) compared to isolated group (11.1%). Apgar scores were available only for the CDH survivor group. Studies have reported higher mean Apgar scores in a CDH survival group compared to CDH non-survivors (196). In the CDH isolated group, the mean Apgar score at 1 minute was 5.7 ± 2.5 and at 5 minutes was 7.5 ± 1.6. A study reported by Muratore et al. had similar results of Apgar scores of 6.8 ± 2 at 5 minutes (197). He also reported in another study a mean Apgar score of 7 ± 2 at 5 minutes in 100 CDH survived subjects (194).

4.6. Associated Anomalies with CDH

The incidence of associated anomalies with CDH varies between 20% to 50% depending on the patient selection and definition of the anomaly (198). In our study, the most common associated congenital anomaly was congenital cardiovascular defects (42.6%). A similar percentage was reported by Bedoyan et al. (2004) (199) with only 43% of the cases having associated congenital anomalies. Brownlee et al. in their review article reported cardiac anomalies (30.9%) as the most prevalent associated anomaly followed by GI abnormalities (28.8%) (40).Another recent study reported 32.9% of the CDH subjects were having cardiac anomalies (200)**.** Fauza and Wilson also reported s higher percentage of cardiac anomalies (63%) as the most frequently associated anomaly with CDH (183). This high variation in percentages of various parameters may be explained by differences in subject selection, available information and maternal and environmental factors.

In the present study, the most frequently occurring cardiac anomaly was ASD (31.6%) followed by VSDs (8.6%). Similar findings were reported in another study, where they also found a higher

percentage of patients having ASD (56%) compared to VSD (46%) (117). In another study, researchers reported ventricular septal defect (42.2%) as the most prevalent type followed by aortic arch obstruction (15%) and tetralogy of Fallot (11.1%) (201). This difference in values can probably be explained by the fact that the researchers excluded the patients having PDA and ASD. Other than cardiac anomalies, an association of other organs anomalies is also frequent (110). The survival rate of a neonate with CDH is primarily influenced by prematurity and major associated anomalies. This explains the lower mortality rate observed in isolated CDH infants compared to infants born with associated anomalies (202).

Studies have reported that persistent PHTN contributes significantly to mortality of CDH babies(185). In the current study, PHTN was detected in 40.2% of the CDH subjects. Among CDH survivors, mild PHTN was diagnosed in 15.5% of the subjects, 17.2% with systemic PHTN while 10% were having severe PHTN. Other researchers have reported the association of intra-thoracic herniation with the degree of PHTN (203,204). Some studies have shown an increased death rate (37% vs. 2%) and incidence of chronic lung disease in infants with an intrathoracic liver (65,205).

In our study, the most frequently herniated organ into the chest cavity was the small intestine (75.5%), followed by the colon (61.8%) and spleen (53.2%). Herniation of the liver has far greater effect on ultimate survival of the infant compared to stomach position (206). However, increasing stomach herniation has also led to poor prognosis in the infants (205). In our study, we found a higher percentage of liver herniation (55% vs. 30.3%) among non-survivors compared to survivors. On the other hand, herniation of the stomach was less common in non-survivors 35% compared to 47.7% of survivors. Chandrasekharan et al. suggested that presence of intra-abdominal fetal stomach is associated with a favorable prognosis in comparison to the

intra-thoracic position during the fetal or neonatal period (65). Mullassery and his co-workers reported lower survival rates in liver up subjects (45.4%) compared to 73.9% in subjects with abdominal liver (92). Similar findings were reported by Mayer et al. with a 52.7% survival rate in liver up infants (207). Studies on animal models have also demonstrated similar findings and a direct effect of herniated organs on the developing fetal lungs (188).

Hyaline membrane disease and pneumothorax could be assessed only in the deceased group due to the availability of the lung tissue blocks. Hyaline membrane disease and pneumothorax was found in 27.5% and 25% of the CDH deceased subjects respectively. A very high percentage of hyaline membrane disease (91%) and pneumothorax (65%) has been reported by Sakurai et al.(208). In another study, others reported the development of pre/post-operative pneumothorax in 69 of 510 neonates (209).

4.7. Survival Rate and Prenatal Diagnosis

The survival rate of infants with CDH has increased in the past decade owing to appropriate antenatal planning with advances in ultrasound and fetal MRI scans (125,210). The decrease in mortality rate is also considerably influenced by advanced medical treatment following delivery (110,142). Another contributing factor to the improved survival rates is the better prenatal diagnosis of CDH, because of which, patients seek to TP. In this way, over the last decade, the overall mortality rate of CDH patients has decreased (211–213). In our study, prenatal diagnosis of CDH was made in 57.5% of the CDH deceased subjects with total of 15% that terminated their pregnancies. On the other hand, prenatal diagnosis in CDH survivors was lower (44.4%), this can be explained by possibly a smaller defect size that can be missed during antenatal diagnosis and have a better prognosis. Chandrasekharan et al. also reported the detection of more than 50% of CDH cases at an average of 24 weeks gestational age (65).

The highest mortality rate of CDH patients is reported in the first week after birth (62,189–192). The results of our study revealed that SB and TP comprised 25% of the cases. Out of the remaining 72.5% live births, 62.5% of the neonates died within the first three days after delivery, and 10% died afterward. A recent study by Malowitz et al. in 2015 (214) reported 40% neonatal mortality rate in CDH subjects within first 24 hours of life. A similar finding was also reported in a study conducted by Aly et al. (215) where 21.1% of the neonates with CDH died within 0-2 days after birth, and 30% died within 3 to 7 days. The researchers also found that 59% of the infants were males. This finding is validated by our study where the incidence of CDH is higher in males (59.7%) than females (40.3%). Colvin et al. (62) reported 44% mortality among CDH children within the first week of life.

4.8. Sample Size in CDH Studies

Our study was a single centre study with a sample size higher compared to a study including all CDH babies (*n=77*) detected in utero (210). Another study that compared both survivors and non-survivors had a total of 32 CDH neonates (179). Whereas, a study that focuses on only the deceased CDH subjects had a total of 30 neonates (216). However, larger sample sizes are possible in studies with registries such as the study performed by the CAPSNet included over 500 CDH infants (29). An even bigger sample size of 3123 CDH newborns was established in a 10 year multicenter study (217). The variation in the sample size can be due to the inclusion criteria, the duration of the study and whether the data was collected from multicenter or one center.

A unique feature of our study is the establishment of a disease database and a tissue bank for CDH patients. As per our knowledge, our CDH tissue bank and database is the first of its kind providing information about both the clinical data of CDH patients and the pathologic

information of the tissue specimens. We achieved our goal in obtaining both CDH and matched controls tissue bank. The establishment of a similar database and tissue bank with extensive details on CDH patient's clinical data and pathologic features in different countries can be immensely useful to collaborate with in the future.

4.9. Study Limitations

Limitations of our study were that we could not calculate the LHR as this information was not collected during the autopsy. This is used to calculate the prognosis of CDH during prenatal ultrasound. However, a study that compared LHR to the LBWR found it correlates well with left-sided CDH but not with right-sided CDH (216). Similarly, we did not collect information about the size of the defect, because again, this information is not collected during autopsy. Furthermore, due to the nature of the retrospective data collection some of patient's data were missing.

4.10. Conclusion

Our study highlights several important clinical features of CDH patients. The establishment of this database with the tissue bank, will give us a better interpretation of experimental results and eventually will validate our findings from animal studies**.**

APPINDIX

5.1. References

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