Vitamin A and its association with Congenital Diaphragmatic Hernia

By

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Abstract

Introduction: Congenital Diaphragmatic Hernia (CDH), is a birth defect that occurs in approximately 1 in every 3,000 births. It arises when the diaphragm fails to form properly during development leaving a hole in the muscle. *In utero*, the abdominal contents protrude through hole, taking up intrathoracic space, and, in so doing, impeding growth of the lungs. The consequent lung hypoplasia is the cause of significant perinatal morbidity and mortality. While the cause of CDH is poorly understood, the Retinoid Hypothesis has been suggested to explain the etiology and has been widely used. The hypothesis states that abnormal retinoic acid signaling, an active metabolite of dietary vitamin A, plays a role in the development of CDH. Two teratogens, Nitrofen (2,4-Dichlorophenyl 4-nitrophenyl ether) and Bisdiamine (N,N'-bis (dichloroacetyl)-1,8-octamethylenediamine) have been shown to induce CDH in offspring when given to pregnant dams. The two goals of this thesis were 1) to test the hypothesis that maternal Vitamin A status influences the development of teratogen induced CDH in a mouse model, and 2) to investigate whether CDH inducing teratogens alter the expression of retinoid metabolism-related genes in the developing embryo.

Methods: To achieve the first goal, a teratogenic mouse model of CDH was established by titrating the dose of Nitrofen and Bisdiamine until an adequate number of affected fetuses (with CDH) was observed without an excessive number of intrauterine deaths. Then, maternal vitamin A status was manipulated by feeding mice diets with deficient (0 IU vitamin A/g), sufficient (4 IU vitamin A/g) or excess (25 IU vitamin A/g) vitamin A. Vitamin A status was confirmed in the maternal and fetal tissues by HPLC. Lastly, we induced CDH in the offspring by treating timed pregnant mice on varied vitamin A diets with a combination of Nitrofen and Bisdiamine. Offspring were collected via dissection and the effect of the teratogen on the incidence and severity of CDH were recorded.

For the second goal, offspring of timed pregnant mice exposed to Nitrofen and Bisdiamine were collected 24 hours after administration of teratogen. Offspring expression of specific genes involved in retinoid metabolism and CDH were then measured using qPCR and compared to controls.

Results: We established a teratogenic mouse model of CDH by administering 0.5 mg/kg of Nitrofen and 0.125 mg/kg of Bisdiamine to pregnant dams. Also, we showed that manipulating dietary vitamin A content of female mice, changes their vitamin A status, and the status of their offspring. Mice fed a vitamin A deficient diet and their offspring had less vitamin A in the livers and plasma and those fed a vitamin A excess diet and their offspring had more vitamin A detected in their livers and plasma. Finally, for our first research goal, we demonstrated that marginal maternal vitamin A status, and, by extension, marginal fetal vitamin A status, is more susceptible to teratogen-induced fetal CDH, with increased incidence of CDH when compared to offspring with sufficient vitamin A status. Excess vitamin A status decreased the offspring's susceptibility to insult from teratogen exposure even further with an even lower incidence of CDH.

With investigating whether CDH inducing teratogens alter the expression of retinoid metabolism-related genes in the developing embryo, we observed a 50% decrease in gene expression of *Stra6* and *Rarb*. This showed that the teratogen affected the expression of these two genes which have been implicated in the formation of CDH previously in both human and animal studies.

Conclusion: This research helped support the Retinoid Hypothesis and highlighted the need for future studies on the role of vitamin A on diaphragm development.

Preface

This thesis is an original work by Ayanna W. Rocke. No part of this thesis has been previously published.

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Abbreviations

CDH	Congenital Diaphragmatic Hernia
cDNA	Complementary Deoxyribonucleic acid
CP	Cleft Palate
CRBP	Cellular Retinol Binding Protein
CYP	Cytochrome P450 Oxidase
ECMO	Extracorporeal Membrane Oxygenation
FETO	Fetoscopic Tracheal Occlusion
HEPA	High Efficiency Particulate Air
HPLC	High Performance Liquid Chromatography
LRAT	Lecithin Retinol Acyltrasnferase
OE	Oedema
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
PA	Pulmonary Artery
PDA	Photodiode Array Detector
PPF	Pleuroperitoneal Fold
RA	Retinoic Acid
RALDH2	Retinaldehyde Dehydrogenase 2
RARdn	Retinoic Acid Receptor dominant negative
RARE	Retinoic Acid Response Element
RAR	Retinoic Acid Receptor
RBP	Retinol Binding Protein
RNA	Ribonucleic Acid
VAD	Vitamin A Deficient
VAS	Vitamin A Sufficient
VAX	Vitamin A Excess
Wt1	Wilm's Tumor 1

Chapter 1: General introduction

1.1. Congenital Diaphragmatic Hernia

Congenital Diaphragmatic Hernia (CDH) is a common birth defect known to cause significant morbidity and perinatal mortality. The incidence of CDH is 1-5/10,000 births (5,26,27) with a 1-year mortality rate of ~50% (1). The diaphragm is the primary muscle of respiration and forms a physical barrier between the thoracic and abdominal cavities. CDH occurs when there is an incomplete formation of the diaphragm that creates a hole in the muscle. This hole allows the liver, intestines and stomach to herniate into the thoracic cavity, which take up intrathoracic space which results in inadequate space for the lungs and a consequent reduction in lung growth (Figure 1.1). Children born with CDH have severe respiratory issues leading to life-threatening respiratory failure (2,3). Treatment options for infants born with CDH focus primarily on the lung deficiencies and can include nitric oxide inhalation, high frequency oscillatory ventilation, extracorporeal membrane oxygenation, exogenous surfactant administration, and ultimately, if the neonate can be stabilized, surgical repair of the diaphragm (4). More recently, high risk fetal interventional procedures have evolved to try and improve growth of the lungs (123).

The etiology of CDH is complex and leads to a highly variable disease outcome. The pathogenesis is diverse and is known to be attributed to genetic mutations as well as environmental factors (4,5). One of the leading ideas to explain the developmental origins of CDH is the so-called "retinoid hypothesis". This hypothesis states that abnormal retinoid signaling contributes to incomplete formation of the diaphragm, leading to CDH. The purpose of this thesis is to further investigate the retinoid hypothesis as a plausible answer to the complex mechanisms which govern the formation of CDH.

1.2 Vitamin A

Vitamin A is a lipophilic micronutrient that is important for normal body function and development. This is especially true during gestation where retinoic acid (RA) has an important role in cell differentiation, proliferation and apoptosis (6,7). Vitamin A must be acquired from the diet either as preformed Vitamin A (primarily as dietary retinyl ester) or as provitamin A carotenoid (primarily as β-carotene) (8). Retinoids refer to metabolites of vitamin A, including retinol, retinal, RA and retinyl esters. RA is one of the active metabolites of dietary vitamin A, acting as a ligand for nuclear transcription factors called retinoic acid receptors (RARs),

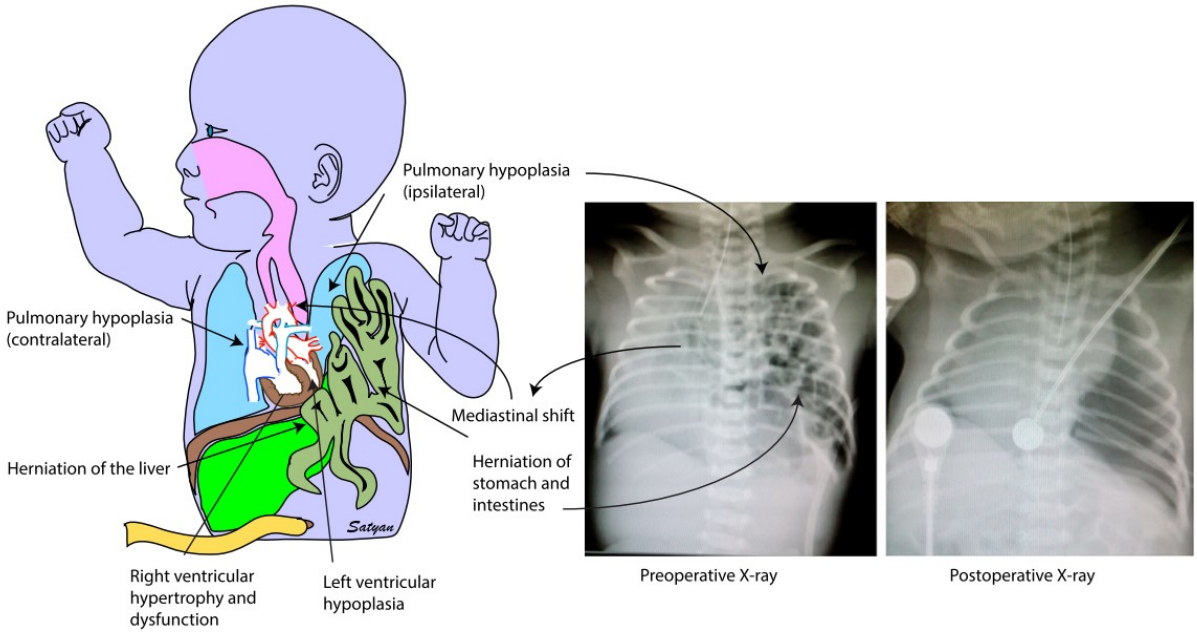

Figure 1.1 - Schematic and X-ray depicting physical insult and subsequent sequelae that occurs in CDH. In the picture on the left, the non-continuity of the diaphragm is depicted. As a result, there is stomach and intestinal herniation into the chest cavity causing mediastinal shift. The herniated bowel content impinges growth and expansion of the lung ipsilateral to the CDH, whereas the mediastinal shift of the heart may contribute to underdevelopment of the contralateral lung, resulting in bilateral lung hypoplasia. After birth, in addition to respiratory failure, the constant high arterial pressures result in right ventricular failure and inadequate cardiac output. In the chest X-ray, loops of the intestine, mediastinal shift and pulmonary hypoplasia are noted. Adapted from "Congenital Diaphragmatic Hernia- a review" Praveen Kumar Chandrasekharan *et al.* 2017 (9). *Copyright Satyan Lakshminrusimha*

signalling through which can control the expression level of >500 genes (10). The importance of vitamin A in development in general has been well-established in animal models (6). Previous experiments have shown that animals on a diet devoid of vitamin A give birth to offspring with abnormalities of the eye, urogenital tract, heart, lungs and diaphragm (11). These studies, amongst other investigations and observations, have laid the foundation for the importance of vitamin A during development. The impact of vitamin A on the formation of CDH is the basis of the Retinoid Hypothesis and the research questions of this thesis.

1.3 The retinoid hypothesis

As previously mentioned, the Retinoid Hypothesis states that abnormal retinoid signaling contributes to incomplete formation of the diaphragm, leading to CDH. It is based on several studies that highlight the link between altered vitamin A metabolism and signaling, and diaphragmatic defects. Examples of these include:

1) Animal studies showing litters of rats who were fed a Vitamin A deficient diet developed CDH (11), and that the incidence of herniation decreased when supraphysiological doses (i.e. 16,000 IU) of vitamin A were supplemented during gestation (11).

2) Studies showing that offspring of mice lacking RA receptor expression were born with CDH (12).

3) CDH has been identified in offspring of animals exposed to teratogens that affect the synthesis of retinoic acid (13). Two of these teratogens are Nitrofen (2,4-Dichlorophenyl 4-nitrophenyl ether) and Bisdiamine (N,N'- bis (dichloroacetyl)- 1,8-octamethylenediamine) which will be utilized in this thesis.

4) Plasma levels of retinol analyzed from the cord blood of infants born with CDH, were 50% lower when compared to control infants (14).

Despite ongoing research into the pathogenesis of CDH, the exact mechanism by which it occurs is still unknown. The molecular basis leading to altered retinoid signaling, its effect on diaphragm morphogenesis, and its relation to other known causes of CDH are poorly understood. It is these unknowns that make up the basis of this thesis.

1.4. Hypothesis and Research Questions

The central hypothesis of my thesis is that **abnormal retinoid signaling contributes to the development of CDH.**

There are two major research questions pertaining to this hypothesis:

1. Does maternal vitamin A (retinoid) status impact the susceptibility to teratogen induced CDH? This question aims to establish the importance of maternal vitamin A status as a risk factor for the subsequent development of CDH in offspring.
2. Do the CDH-inducing teratogens Nitrofen and Bisdiamine alter the expression of retinoid metabolism-related genes in the developing embryo? This question aims to address the link between an animal model of CDH and altered retinoid signaling.

1.5 Experimental Plan

1.5.1 Research Question 1- Impact of Maternal vitamin A status on teratogen induced CDH

The question of maternal vitamin A status in the context of CDH was developed with the knowledge of the importance of vitamin A during development and two previous studies highlighting the potential importance of maternal vitamin A levels in CDH. The first study was performed in humans and showed that low intake of dietary vitamin A during pregnancy is associated with an increased risk of infants developing CDH (15). The second study showed that when pregnant rats were given a supraphysiological dose of vitamin A following exposure to the CDH-inducing teratogen Nitrofen, the incidence of CDH decreased by 50-75% (18).With this knowledge we suggest that low maternal vitamin A levels increase offspring's susceptibility to insults causing CDH. Secondly, while previous work used a supraphysiological dose of vitamin A, we propose that an excess of dietary Vitamin A will have the same impact. Our suggestion is that like the supraphysiological dose, dietary vitamin A will provide an increase in substrate for conversion to RA thus offsetting the effect of Nitrofen on retinaldehyde dehydrogenase 2 and its role in converting retinal to RA (17).

1.5.2 Research Question 2- Impact of CDH-inducing teratogens on retinoic acid metabolism and signaling

As stated above, previous data shows that when pregnant rats are exposed to the teratogen Nitrofen, their offspring develop CDH (19). It has also been shown *in vitro* that CDH-inducing

teratogens have an inhibitory effect on RALDH2, which is needed to produce RA from retinal (13) and that teratogen-exposed embryos have decreased RA levels when compared to control embryos (20). In addition, teratogen exposure decreases markers of RA signaling (90). While these studies establish that CDH-inducing teratogens impact embryonic RA homeostasis, specific details regarding embryonic RA metabolism and downstream signaling is unknown. We propose that exposure to CDH-inducing teratogens will change the mRNA expression levels of genes involved in embryonic RA signaling. To explore this research question, timed-pregnant mice will receive a teratogenic combination of Nitrofen and Bisdiamine, 24 hours later, offspring will be harvested, and gene expression levels will be analyzed.

1.6 Rationale and Significance

While there is growing literature highlighting the important role of RA signalling in the developing diaphragm there are gaps in knowledge as the pathogenic mechanism is still incompletely understood. This thesis will further explore the mechanisms underlying the formation of CDH, as well as take an in depth look at the previous literature regarding CDH and vitamin A, including its connection with genetic perturbations and lung pathologies associated with CDH. The new knowledge generated by this research will be significant in three regards. It will: 1) advance our understanding of how altered retinoic acid signalling contributes to the etiology of CDH; 2) bridge the gap between observations made in the clinic and laboratory, allowing a more integrated understanding of CDH's etiology; and 3) establish a novel mouse model of CDH, as the majority of previous animal models utilized rats. In all regards, there is translational potential for improving CDH incidence, be it in the context of maternal vitamin A status and susceptibility to CDH or identification of high-risk individuals so they can receive the best prenatal care possible.

Chapter 2: Literature Review

2.1 Clinical Aspects of Congenital Diaphragmatic Hernia

2.1.1 Background

CDH is a life-threatening anomaly of the diaphragm that was first described in 1679 by Lazarus Riverius. It was later discussed by several scientists and in 1848 Dr. Victor Alexander Bochdalek went on further to define various types of CDH and today his name is synonymous with the most commonly clinically encountered form of this defect (21). In CDH, the diaphragm fails to completely form leaving a hole in the tissue. The diaphragm is a muscle that functions to aid in respiration and as a physical barrier between the abdominal and thoracic cavities (22). The defect in the diaphragm allows for herniation of the abdominal contents into the thoracic cavity, impeding growth and development of the lungs. The consequent pulmonary hypoplasia is a source of significant mortality and morbidity in patients. Figure 1.1 is a cartoon depicting the changes that occur in infants with CDH, specifically abdominal herniation into the chest and its effect on the lungs. In humans, the basic structure of the diaphragm is formed early in development during embryonic folding and separation of the body cavities around 4-6 weeks of gestation (23,24). During embryogenesis there is a joining of the pleuro-pericardial folds and the septum transversum which forms a structure called the pleuroperitoneal fold (PPF). It is from this transient structure that the diaphragm forms. Various studies in both animals and humans have shown that disturbances to PPF formation during development is the structural basis of the diaphragm defect in CDH (24, 25, 26, 23).

2.1.2 Incidence

The incidence of CDH is 1-5/10,000 births (5,26,27). The overall incidence may be underestimated as the number of associated fetal deaths and diagnosis of CDH are not accurately represented due to differing ascertainment and data-collection methods (28,29). In addition, in developing countries the lack of routine neonatal autopsies and birth defect registries may contribute to underreporting of affected neonates in these countries (30). When analyzing various countries, according to the International Clearinghouse for Birth Defects Surveillance and Research, in 2014 there was an incidence of 3.38 cases of CDH per 10,000 in Canada. In that same year, Alberta had an incidence of 4.40 cases per 10,000 births (26). From the same database, it was found that the incidence of CDH in Iran in 2014 was 6.50 per 10,000 births. In the United States of America, the incidence in 2013 was said to be 1.93 per 10,000 births, while the European

statistic found 2.38 cases of CDH per 10,000 births (5,27). With regards to differences in incidence between male and female infants, CDH is more commonly seen in male offspring with a male to female ratio of 1:0.64 (31).

2.1.3 Phenotypes

There are several phenotypes of CDH. In humans, three main types of CDH have been identified. The first and the most commonly seen is a posterolateral defect in the diaphragm known as Bochdalek hernia. It is seen in 70-75% of cases (32). While this type of CDH can occur either on the right side, left side or bilaterally, more than 80% of hernias are on the left side (34). The second type is known as Morgagni hernia which represents a defect in the anterior portion of the diaphragm. This type accounts for approximately ~27% of cases (33). The least common type involves a defect in the central tendon area of the diaphragm. It occurs on average in 2-7% of patients (32). Finally, a type of congenital diaphragmatic anomaly that is not as common as CDH is diaphragmatic eventration. It can be either congenital or acquired and is described as an 'abnormal elevation' in part of or the whole diaphragm secondary to a weakening of the muscle fibres in the diaphragm (105). Figure 2.1 depicts different CDH phenotypes. In addition to the type of diaphragmatic defects, patients with CDH can be divided into two groups, isolated and non-isolated CDH. Isolated CDH can be used to describe the defect if there are no other obvious malformations, while non-isolated occurs alongside other malformations (29). Up to 43% of CDH cases are non-isolated and some have distinct characteristics that can be classified as specific genetic syndromes (35,3). For example, Donnai-Barrow Syndrome is a syndrome where CDH has been identified in up to 50% of the cases. It is characterized by a mutation in the *LRP2* gene with additional clinical features such as ocular anomalies and sensorineural hearing loss (36,37).

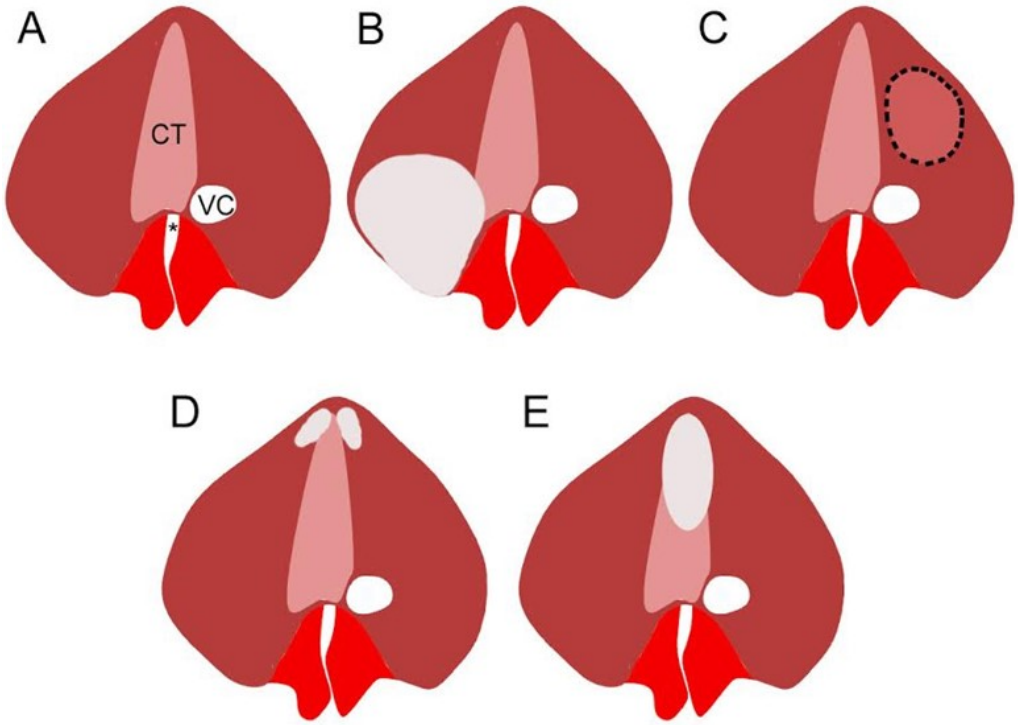

Figure 2.1 - Categorization of different phenotypes of CDH.
A) Normal diaphragm viewed from above, CT: central tendon, VC: natural opening through which the vena cava passes through the diaphragm, and * natural opening between the crural muscles of the diaphragm, through which the esophagus passes. B) Grey area represents hole in the diaphragm known as Bochdalek hernia. C) Dotted line represents an area of thinning or abnormal tissue/musculature development that leads to eventration of thoracic organs into the chest cavity. D) Grey areas represent holes in diaphragm typical of Morgagni herniation. E) Grey area represents a hole in the central tendon of the diaphragm. *Reproduced with permission of Robin Clugston*

2.1.4 Diagnosis

The diagnosis of CDH can occur both pre and postnatally. In prenatal diagnosis, ultrasonography is the primary tool used for diagnosis of CDH, with a detection rate of approximately 50-60% of all cases (39,40). Prenatal detection increases with increasing gestation and is more likely in the presence of associated pathology. Diagnosis of CDH during gestation can be made as early as the first trimester by observing primary defects like the stomach, liver or intestines in the chest cavity, or by secondary defects such as polyhydramnios. Once a diagnosis of CDH is suspected, patients are referred to centers with the expertise to manage serious congenital anomalies. Prenatal MRI may follow diagnosis by ultrasound to help in further visualizing the defect, its sequelae and any other anomalies that may be present (41). Postnatally, the diagnosis of CDH typically occurs when an affected newborn has respiratory distress leading to the acquisition of a chest X-ray. The finding of extracardiac pathology may also lead the clinician to obtain a chest X-ray in an infant without respiratory distress.

2.1.5 Management

CDH management begins from prenatal diagnosis and focuses primarily on the lung defects, as insults to pulmonary development are the major cause of mortality and long-term morbidity (4,42). Before delivery, corticosteroids have been given to mothers to mature the lungs of the infants. However, while animal studies have shown benefit of this approach, clinical studies have not been so promising (9,43). In addition to medical management, fetal intervention has evolved in an effort to improve lung growth in the most severely affected fetuses and those with very high risk of neonatal mortality. These approaches are still largely experimental and are not without significant risks including that of preterm delivery. One such intervention with promise is that of Fetoscopic Tracheal Occlusion (FETO) surgery, which after animal testing and clinical trials is just beginning to be implemented in clinical practice (44). FETO evolved following the observations that congenital high airway obstruction syndrome (CHAOS) in the fetus was associated with increased growth of the lungs. This led to initial external tracheal occlusion and eventually the creation of balloons inserted into the fetal trachea to provide the same effect but to allow for removal prior to delivery (45). While this procedure is relatively novel, there are promising results associated with fetal survival (46).

Post-natal management of CDH includes both medical and surgical interventions. Medical management includes mechanical ventilation, administration of surfactant, use of vasopressor/inotropic therapy and treatment of pulmonary hypertension. As a last resort, patients not responding to medical management are considered for extracorporeal membrane oxygenation (9, 51). Most of these treatments aim to support the patient until they are stable enough for surgical repair of their diaphragmatic defect (40). Surgical management mainly involves the reduction of the abdominal organs and closure of the diaphragmatic defect (9). For smaller defects, primary repair is performed while larger defects require use of a synthetic patch. Primary repair is closure of the hole using sutures to bring existing tissue together. According to a study including 4,112 patients, 51.7% of infants underwent primary repair, with the remainder requiring a patch repair (48).

2.1.6 Outcomes

Mortality and morbidity seen in CDH is primarily caused by the insult to pulmonary development (42). Researchers have investigated different aspects of lung injury in human CDH cases and animal models. In a study that focused on human cases of CDH, it was found that most patients with CDH also had some degree of pulmonary hypoplasia. While the study was not meant to directly measure pulmonary hypoplasia, it was noted in patients who did not even have major organ displacement. From these findings, it was hypothesised that there could be a primary defect in not only the diaphragm but also the lungs in these cases of CDH (49). It was not the first time this idea was presented. In 2000, Keijzer *et al.* suggested that pulmonary hypoplasia in CDH is caused by two separate insults during development, calling it the 'Dual- hit' hypothesis. The first hit being the same insult that affects the development of the diaphragm and the second being the mechanical obstruction caused by organ herniation (50). Previous to this line of thinking, it was said that the level of lung injury was directly proportional to the timing and severity of herniation into the thoracic cavity (46). In addition to pulmonary hypoplasia, there is significant pulmonary hypertension. With this knowledge, management options focus on treatment of lung sequelae.

The reported mortality rate of CDH varies considerably. In 2007, Lally *et al.* reported a survival rate of 70% in a study including 3,062 live births, while a study conducted in 2010 in England reported an overall survival rate of 42% (52,53). Another study conducted in Utah, examining births over a 10-year period noted the survival rate was 32.5% (54). However, similar

to Lally *et al*, a more recent study published in 2018, reported a survival rate of 71% (55). This variability in reported CDH mortality depends on multiple factors (10). It is thought that prenatally diagnosed CDH has a higher mortality rate than infants whose CDH was discovered at birth. This is attributed to the larger defects with more significant herniation of bowel contents and a greater shift in the heart position earlier in gestation and are thus more easily recognized at the time of early routine ultrasound screening. Survival rates in postnatally diagnosed infants were as high as 83% when compared to the 65% rate seen in prenatally diagnosed infants (55). Prenatal diagnosis is more common among fetuses as well with other pathology which contributes further to the risks (9,53). Indeed, isolated CDH has been shown to have a better prognosis than non-isolated cases (9). For example, in one study, after one-year isolated cases was said to have a survival rate of 77% while non-isolated CDH had a one year survival rate of 34% (53). Another factor noted in mortality is size at birth. Infants born small for gestational age have been shown to have a higher mortality rate with 41% dying before one year of age (57).

In terms of long-term sequelae, most infants born with CDH are discharged from the hospital with one or more major morbidities (58). Infants with right-sided CDH are said to have greater long-term morbidity than those with left sided CDH (59). Not only do CDH survivors show long-term pulmonary complications, many demonstrate ongoing gastroesophageal abnormalities, hearing loss, poor growth and developmental impairment among other comorbidities (60). Developmental impairment in CDH patients is said to be as high as 70% with one study showing that infants born with CDH have lower developmental scores at two years of age when compared to children born in good health (61). Fritz *et al.* discovered that one-third of the participants in their study required a home ventilator and/or feeding tube at school age (62).

2.1.7 Etiology

While the exact cause of CDH remains elusive, researchers have found that certain environmental and genetic factors contribute to its formation.

In terms of the environment during gestation, several maternal risk factors have been identified, including maternal age, smoking, alcohol use, and metabolic disease. For example, one study done in Colombia concluded that infants with CDH had a greater chance of having a mother whose age was >35 years (63). After their population study based on 32 million births in the United States, Balayla *et al.* concluded that maternal smoking and alcohol use during pregnancy were

statistically significant risk factors associated with CDH in offspring (1). Similar to this study, McAteer *et al.* showed an association between maternal alcohol use and increased risk for CDH. Further to this, they investigated maternal hypertension and pregestational diabetes as risk factors for CDH development. From their study it was shown that these factors were associated with an increased chance of CDH in offspring (64). In further relation to the presence of maternal diabetes, pre-pregnancy obesity has also been positively associated with the development of CDH (65).

While large-scale epidemiological studies have shed light on potential environmental contributions to CDH, there is also significant evidence for genetic factors contributing to its development. Indeed, although CDH has been largely classified as a 'sporadic' birth defect because the majority of the patients exhibiting the defect are the only members of their family to have it (29), an identifiable genetic etiology is thought to be responsible for approximately 30% of cases of CDH (36). There is much evidence for genetic contribution toward CDH development. Some of these include sporadic cases showing genetic mutations, animal models with mutations in single genes with CDH, and known monogenic syndromes associated with CDH (29). To date, over 60 loci in animals and humans have been associated with CDH (66), with another study identifying 218 genes that have been recognized to be involved in diaphragmatic defects in animals and humans, with the majority being found in humans (56%) (67). WAGR syndrome seen in humans is one such example of CDH being associated with a specific gene. WAGR syndrome occurs when there is a deletion of the gene *WT1.* In WAGR syndrome, patients usually present with Wilms tumor, aniridia, genitourinary anomalies, and mental retardation. One case study documenting WAGR syndrome also identified CDH in the patient supporting the correlation between *WT1* and CDH (68). Adding more evidence in support of *WT1*'s association with CDH, another syndrome, Denys Drash Syndrome is known to be caused by mutation of *WT1*, with documented individual cases that presented with CDH in addition to other expected anomalies (69). Another example supporting the genetic etiology of CDH, is the presence of CDH in at least 26 patients who had a deletion of the distal part of the long arm of Chromosome 15 (32). This region contains multiple genes, although subsequent genetic studies have identified the most-likely CDH-associated gene as *COUP-TFII*, mutations of which are known to cause CDH in mice (32,70,71). The significance of these gene mutations with respect to the Retinoid Hypothesis are discussed below.

2.1.8 Animal Models of CDH

There are many unknowns regarding the pathogenesis and etiology of CDH. Animal models of CDH are frequently used to provide a better understanding of how CDH develops and what causes it, as well as testing therapeutic interventions. As investigating embryogenesis/organogenesis early in the first trimester in humans is difficult, animal models are useful as they allow for a closer look into these early stages (56). There are three major types of animal models used to study CDH, genetic, teratogenic and surgical (71). Several animal models that have been genetically modified exhibit CDH (29). For example, in a mutant mouse model with deletion of *Wt1*, CDH was observed in addition to expected renal abnormalities (72). CDH has also been diagnosed in known genetic conditions such as Fryns' syndrome, Pallister–Killian, Wolf–Hirschhorn or Cornelia De Lange (36), providing a link between human cases of CDH and this animal model. In addition to genetically modified experimental animals, the teratogenic animal model has been used to investigate CDH. In 1981, the teratogen, Nitrofen, was first identified as causing CDH in offspring, similar to that in humans, when given to pregnant rats (19). Since then the Nitrofen model in animals has been used extensively to deepen our understanding of CDH and it's pathogenesis and etiology. In terms of its mechanism of action, nitrofen and other CDH-inducing teratogens have been found to affect the synthesis of RA by inhibiting RALDH2, an enzyme that aids in the production of RA from retinal (20). Another type of animal model is the surgical model. In larger animals such as rabbits and lambs, while *in utero*, a diaphragmatic hernia is created via surgical intervention. Subsequently, various effects are observed such as lung injury, effects of treatments and survival rates (101, 122). In this thesis, teratogenic and genetic small animal models of CDH will be utilized.

2.2 Vitamin A

2.2.1 Introduction

Vitamin A is a lipophilic micronutrient that can only be acquired from the diet (5). It is an essential nutrient needed in small amounts for the normal functioning of the visual system, it can maintain cell function for growth, normal immune function, hematopoietic system, epithelial integrity, red blood cell production, immunity and reproduction (73). The importance of vitamin A in human development and daily functioning has been well-established, however in low and middle-income countries, Vitamin A deficiency is a still a significant public health problem (74).

Vitamin A can be acquired in the diet from a number of different foods such as carrots, sweet potatoes, spinach and eggs (75). It can be found in two major forms from the diet: preformed retinoids and proretinoid carotenoids (76). Of the preformed retinoids, retinol and retinyl ester are those most abundantly found in the diet. Proretinoid carotenoids, for example β-carotene, have two fates when consumed. They can be either converted to retinoids or absorbed as proretinoid carotenoids and converted later on in tissues around the body (8). In an adult male the daily vitamin A requirement is approximately 4,000 IU (77). According to the World Health Organization, in the background of adequate nutrition, vitamin A supplementation in pregnancy is not needed. In populations where vitamin A deficiency is a severe public health concern, daily supplementation of up to 10,000 IU is recommended (77). Excess vitamin A should be monitored as it is known to have teratogenic effects to offspring (78).

2.2.2 Absorption and Metabolism

As previously mentioned, vitamin A is primarily consumed in the diet in the form of retinyl esters or β-carotene. Dietary retinoids are taken up from the intestinal lumen and metabolized within the enterocytes, summarized in Figure 2.2. Dietary retinyl esters are either hydrolyzed to retinol at the brush border of the intestine or hydrolyzed to retinol by pancreatic enzymes before being absorbed at the brush border. Once retinol is in the enterocyte, it is bound to Retinol Binding Protein 2 (RBP2) where it is then re-esterified to retinyl esters. These newly synthesized retinyl esters are then packaged into chylomicrons, which are secreted by the enterocyte into the lymphatic system and eventually to the rest of the body (76). Dietary β-carotene is directly taken up into enterocytes, where it is enzymatically cleaved to generate retinal, which is further reduced to retinol. Similar to the retinol derived from dietary retinyl esters, retinol liberated from dietary β-carotene is then esterified and packaged into chylomicrons. Retinyl ester in chylomicrons can be directly taken up from certain tissues or processed by the liver.

The liver is of central importance in whole-body vitamin A metabolism, acting as a source to meet the body's need for vitamin A. Hepatocytes secrete retinol bound to retinol binding protein (RBP) into the circulation, which can then be taken up by target tissues. In adequately nourished individuals, excess dietary retinoids are stored in hepatic stellate cells in the form of retinyl esters. In times of dietary insufficiency, these stores can then be used to supply the rest of the body via retinol-RBP secreted from the hepatocytes (8).

Once at its sight of action, retinol is taken up into the cell and metabolized into RA, as summarized in Figure 2.3. Specifically, retinol in the blood is transferred into cells through membrane receptors specific to RBP called Stimulated by Retinoic Acid 6 (Stra6). Retinol can reversibly be converted to retinyl esters by LRAT and acyl-CoA:retinol acyltransferase (ARAT) and back to retinol by retinyl ester hydrolase. In cells, retinol is oxidized to retinaldehyde by retinol dehydrogenase (RDH) and can be reduced back to retinol by retinal reductase. Retinaldehyde can be converted to 11-cis-retinylaldehyde in eyes which is essential for vision or it can be further oxidized to RA by Retinaldehyde dehydrogenase (8). Cellular RA levels are controlled by Cytochrome P26 (Cyp26) enzymes which oxidize retinoids for elimination. Once it has been generated from retinol, RA can act as a ligand for the nuclear transcription factor RARs, signalling through which can control the expression level of >500 genes (10). The large number of RA target genes can be explained by the presence of three RAR isoforms (RARα, β and γ), which function as homo- or hetero-dimers- partnering with different RARs or other nuclear transcription factors.

2.2.3 Vitamin A in Pregnancy

Even before development of the fetus begins, vitamin A plays a role in both male and female reproduction including spermatogenesis and germ cell development (81). During gestation RA, has an important role in cell differentiation, proliferation and apoptosis (6,7). In mammals, the developing fetus relies on the maternal circulating retinoids for its supply. It is transferred through the placenta which is able to express RARs and retinoic X receptors. In pregnancy, the fetus receives its retinoids from retinyl esters within chylomicrons or chylomicron remnants, or as retinol bound to RBP being taken up by the placenta and yolk sac (79). Figure 2.4 shows a simplified model of uptake of vitamin A by the placenta and delivery to the fetus. In rodent models, it has been shown that both placental and fetal retinoid levels increase when female mice are on a vitamin A excess diet. This positive correlation shows that maternal diet has a direct effect on infants. The exact mechanism by which retinol is transferred to the fetus from the placenta remains elusive (79).

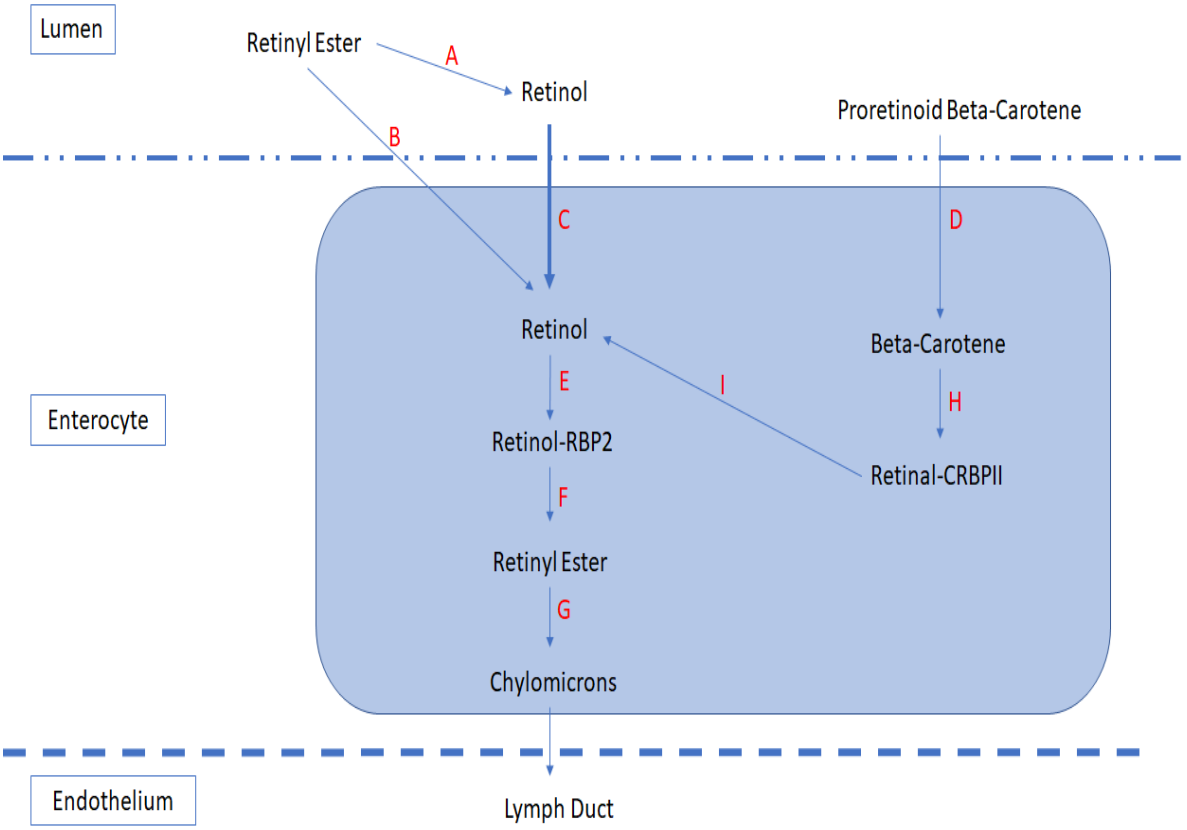

Figure 2.2- Intestinal uptake of Dietary retinoids A) Dietary retinyl ester is hydrolyzed to retinol by pancreatic enzymes, pancreatic triglyceride lipase (PTL) and pancreatic lipase-related protein 2 (PLRP2). B) Dietary retinyl ester can also be hydrolyzed at the brush border by a membrane-bound REH. C) Retinol is absorbed by the enterocyte. D) Dietary proretinoid carotenoids are taken up by enterocytes by scavenger receptors. E) Once retinol is in the enterocyte it is bound to RBP2. F) Retinol-RBP2 complex is re-esterified by lecithin retinol acyltransferase (LRAT)/diacylglycerol O-acyltransferase 1 (DGAT1). G) Retinyl esters are packaged into nascent chylomicrons and secreted into the lymphatic system. H) β-Carotene is cleaved to retinaldehyde by BCO1 and is then bound to RBP2. I) Retinaldehyde bound to RBP2 is acted upon by retinal reductase, giving rise to retinol-RBP2 complex. This β-carotene derived retinol is then processed similarly to retinyl-ester derived retinol (steps E-G). Adapted from "Vitamin A Absorption, Storage and Mobilization" Blaner *et al* 2016 (8).

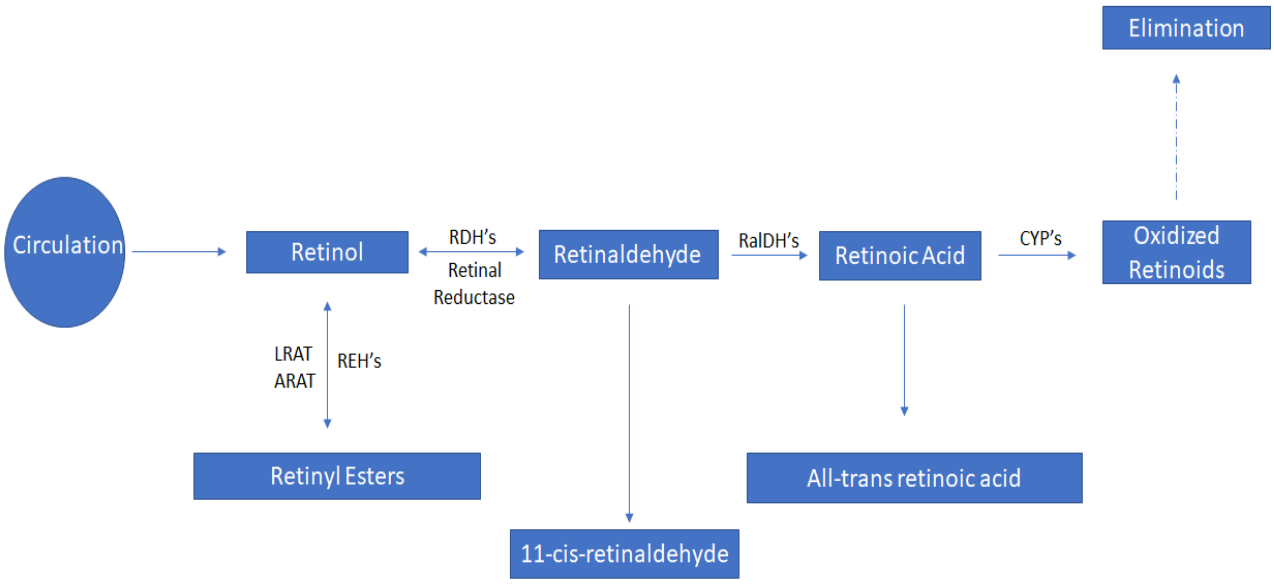

Figure 2.3 - Schematic of Retinoid metabolism in the cell. Preformed retinoids are taken up by cells from the circulation. Retinol is metabolized to Retinyl esters by LRAT/ARAT and back to retinol via REH's. RDH's convert retinol to retinaldehyde while retinal reductase can reduce it back retinol. Retinaldehyde is used as 11-cis-retinaldehyde or converted to RA by RALDHs. RA is transformed to metabolically active compounds, all-trans retinoic acid. RA levels are controlled CYP's that oxidize retinoids for elimination. Adapted from "Vitamin A Absorption, Storage and Mobilization" Blaner *et al.* 2016 (8).

2.2.4 Vitamin A in development

In the fetus retinol is converted to RA and is used for the development of many organs. Throughout development there is tight control of the amount of RA at any given time in tissues to ensure there is enough for cellular processes to occur but not too much to be teratogenic (80). Using an animal model to highlight the importance of vitamin A in development, Wilson *et al.* documented that offspring of rats on a vitamin A deficient diet had multiple abnormalities including ocular anomalies, cardiac malformations, pulmonary agenesis, diaphragmatic hernias and underdevelopment of the renal pelvis (11). Further to that, vitamin A has been proposed to have a role in the development of the pancreas, nervous system and limbs (81). For the purposes of this thesis, the emphasis will be placed on the development of the diaphragm and lungs.

2.2.5 Retinoic acid signaling and lung development

RA is essential for many milestones during development, including organogenesis of the lungs (81). In 1953, Wilson *et al* commented that a small number of offspring from rats on a Vitamin A deficient diet showed malformations of the respiratory system, including lung agenesis (11). With the many studies on the role of Vitamin A during development it was found that RA binds to nuclear receptors and can work to activate or repress transcription of a large number of downstream genes involved in lung morphogenesis (80, 82). RA concentration in lung is regulated by the retinoid synthesis and degradation (83). Histologically, lung development can be divided into five stages being the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages with the embryonic stage starting approximately in the 4th week of gestation (84). Table 2.1 outlines the developmental stages in lung formation and the known role of RA signaling in these processes.

Studies done where the vitamin A metabolic pathway is disrupted show how important vitamin A and it's metabolites are. In *Raldh2*$^{-/-}$ mice it becomes evident that RA is needed to maintain differentiation of lung progenitor cells and to initiate bud morphogenesis (85). Adding to the role of RA metabolism in lung development, Malpel *et al.* describes that RA downregulation is important to allow for gene expression in branching morphogenesis. Furthermore, they identified the three phases during which the expression of the components of RA metabolism vary during lung development. During the first phase, RA signaling is high and there is no degradation. In phases two and three, the fine counterbalance between RA synthesis and degradation is seen in

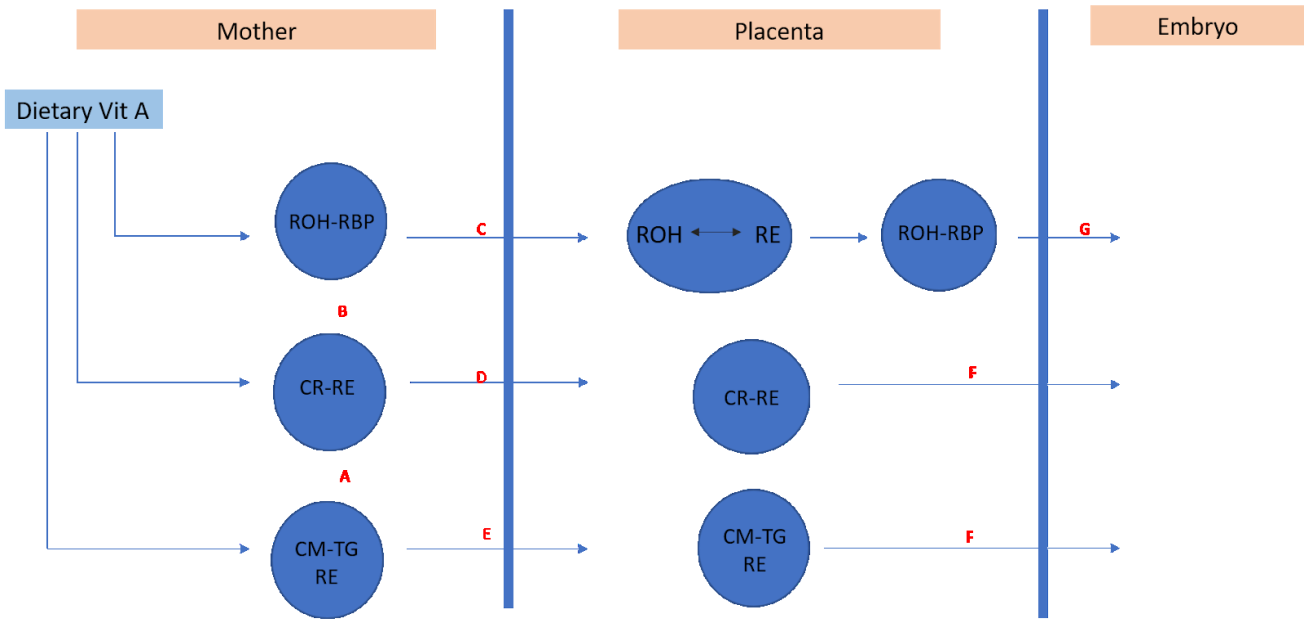

Figure 2.4. Simplified model of vitamin A placental transfer. The maternal circulation includes several sources of vitamin A for placental delivery to the embryo, this includes retinyl ester in chylomicrons and chylomicron remnants (A), and retinol-RBP secreted from the liver (B). They are absorbed in their different forms as C) Placental uptake of retinol. D) Chylomicron remnant uptake via LPL receptors. E) Whole nascent chylomicron uptake, which is either LPL dependent or independent. Once in the placenta it can be transferred to the embryo in two ways: F) Transfer of retinyl esters to fetus independent of LPL and RBP. G) Transfer of retinol to fetus dependent on LPL/RBP. Adapted from Wassef and Quadro 2011 (79)

different parts of the developing lung (83). To further support the importance of vitamin A and its metabolites in lung development, Coste *et al.* screened twenty-five critical RA signaling genes during different stages of the developing lung (30).

Similar to Malpel *et al.*, it was discovered that the timing of expression of the RA signaling genes were dependent on the stage of lung morphogenesis. When considering the long-term effects of Vitamin A deficiency during development, Checkley *et al.* found that vitamin A supplementation pre-, peri- and post-natally had a positive effect on lung function in offspring 9-13 years later when compared to children born to other women who were chronically undernourished (86). Taken together, it is clear that RA has an important role in the developing lungs, the significance of this with respect to CDH is discussed below.

2.3 Vitamin A and Congenital Diaphragmatic Hernia

2.3.1 History of the Retinoid Hypothesis

In Congenital Diaphragmatic Hernia (CDH) the integrity of the diaphragm is compromised, leading to sometimes fatal postnatal complications. While there has been some research on the etiology of CDH, there are still many unanswered questions. The etiology of CDH is both diverse and complex. To date, etiologies based on teratogens, diet and genetics have been investigated, but by far the most widely discussed, is the Retinoid Hypothesis. The Retinoid Hypothesis was first described in 2003 by Greer *et al*. (17). They noted that while no direct link has been made between vitamin A and the etiology of CDH, there is a growing body of evidence from human and animal studies that justify the hypothesis.

As outlined in the original proposal, the Retinoid Hypothesis is based on several studies conducted investigating the role of Vitamin A in CDH (17). One experiment as early as 1941 showed that pregnant rats fed a diet deficient of vitamin A had pups with CDH. Furthermore, the incidence of herniation decreased when supraphysiological levels of vitamin A were supplemented during gestation. (11). In 1981, the teratogen, Nitrofen, was first identified as causing CDH in offspring, similar to that in humans, when given to pregnant rats (19). Nitrofen and other teratogens such as Bisdiamine, SB-210661, and 4-biphenyl carboxylic acid were subsequently found to affect the synthesis of RA, by specifically inhibiting RALDH2 (20,13). These findings linked one of the most widely used animal models of CDH to altered retinoid signaling. To further support the

Table 2.1 - Developmental stages in human lung formation and role of retinoic acid signaling. (87,84,81,83)

Stage	Gestational Age	Description	Role of Retinoic Acdi (RA)
Embryonic	4-7 weeks	Branching of primitive lung bud from primitive gut to form two lung buds	Maintain differentiation of lung progenitor cells to initiate bud morphogenesis
Pseudoglandular	7-17 weeks	Branching morphogenesis-forming of pre-acinar airways	Downregulation of RA signaling to allow for gene expression
Canalicular	16-25 weeks	Formation of air-blood barrier and initiation of surfactant secretion	Downregulation of RA signaling to allow for genes expression
Saccular	24-38 weeks	Formation of primitive terminal airspaces and surfactant secretion	Downregulation of RA signalling to allow for gene expression
Alveolar	36 weeks-after birth	Pulmonary angiogenesis and secondary septation	Regulation of septal eruption and alveologenesis

retinoid hypothesis, it has been shown that when a supraphysiological dose of vitamin A is given to rats exposed to Nitrofen, the incidence of CDH is decreased (18). Underpinning the retinoid hypothesis is studies showing that offspring of mice lacking retinoid receptor expression were born with diaphragmatic defects similar to that seen in the Nitrofen model (88). When investigating the mechanism underlying the animal model of CDH, it was shown that Nitrofen suppressed retinoic acid response element (RARE) and the suppression could be dampened with supplementation of RA (89).

2.3.2 Retinoic acid signaling and abnormal diaphragm development in Animal Models

Several hypotheses have been proposed to explain the etiology of CDH including altered maternal vitamin A status, genetic disturbances and environmental factors. Dietary studies in both animal models and humans have led to a deeper understanding of the link between vitamin A and CDH. In addition to dietary studies, as stated previously, researchers use teratogenic, surgical and genetic models to further their knowledge (34). Since Greer *et al.* outlined the basis for the Retinoid Hypothesis in 2003, there have been many studies done on animal models that further support this hypothesis.

In one experiment, Clugston *et al.* used a transgenic mouse model expressing β-galactosidase under the control of a Retinoic Acid Response Element. (RARE-lacZ). In this model, RA signaling patterns can be directly observed through beta-galactosidase activity. They exposed these animals to four CDH-inducing compounds (Nitrofen, SB-210661, Bisdiamine and BPCA) known to inhibit RALDH-2 and showed that they all suppress RARE-lacZ activation 24 hours after exposure. From this, they suggested that the reduced RARE-lacZ activation in the teratogen model is caused by a decrease in RA production (90). Added to this support of the retinoid hypothesis, stemming from the knowledge that the PPF is essential in the formation of the diaphragm, the researchers showed that RALDH2 is responsible for the synthesis of RA in the PPF (90). Furthermore, it was shown that when pregnant mice are exposed to a pan-antagonist of RARs, a significant number of offspring develop CDH. When supplemental RA was given in conjunction with these teratogens, the incidence of CDH in offspring decreased dramatically. It can be concluded from their studies that retinoid signaling is crucial in the embryogenesis of the diaphragm as it has been shown that retinoid signaling is affected with the use of these various teratogens. Similarly, Cipillone *et al.* documented the occurrence of CDH in offspring of mice

given a triple RA competitive antagonist, BMS-189453. While there was only a 1/279 incidence reported, it is still important to note as RA is often used in the clinical setting in acne treatment (91,92).

To further investigate the retinoid hypothesis Babuik *et al.* administered RA and vitamin A to Nitrofen treated mice. In the group of mice that were given a supraphysiological dose of vitamin A after being treated with Nitrofen, the incidence of CDH decreased from 54% to 32%. In the group treated with RA, the incidence was significantly reduced to 15% from 54%, and when RA was given continuously for 3 days following teratogen exposure, the incidence decreased below 10%. This supported the theory that Nitrofen is affecting the production of RA and the importance of RA in diaphragm development (93).

2.3.3 Retinoid acid signaling and CDH in Humans

While most of the support for the Retinoid Hypothesis from clinical cases come from genetic studies, there are a few lines of evidence that exist from non-genetic clinical cases. One such study is when human vitamin A levels were investigated in correlation with CDH, Major *et al.* reported that plasma retinol and RBP levels in CDH newborns were 50% less than control values (14). This finding was later validated by Beurskens *et al.* investigating the same parameters but with a larger sample size. Similarly, in the study mentioned before, Beurskens noted that CDH is strongly associated with low levels of retinol and RBP in newborns (94). This finding was also independent of maternal vitamin A status. While the two previous studies mentioned noted correlation of vitamin A independent of maternal status, another human study looking at the maternal vitamin status showed that lower maternal vitamin A status was associated with a higher risk of offspring developing CDH (15). In another interesting study, fetal skin fibroblasts were used to investigate the retinoid hypothesis. Based on the fact that skin is retinoid responsive, Goumy *et al.*, measured the levels of various enzymes and receptors involved in the retinoid pathway in patients with and without CDH. In the fetal skin fibroblasts of patients with CDH, RALDH2, CYP26B1 and RARs were not present (16).

When looking at the genetic studies, in the original proposal of the Retinoid Hypothesis, Greer *et al.* had little information on the genetic contribution toward CDH but noted that once continued advances were made on characterization of the genome more examination could be done on the genes and their relationship with the retinoid-signaling pathway (17). Subsequent analysis

performed on candidate genes shows that RA signaling is a continuing theme in both animal models and human non-isolated cases (30). As mentioned above, the majority of new evidence linking the Retinoid Hypothesis with CDH in humans comes from genetic models. For example, Dalmer *et al.* found that 54 out of 218 genes associated with CDH have a direct or indirect role in RA metabolism and signaling (35,67). Some of these genes include *Crabp1, Crabp2, Lrat, Lrp2, Rara, Rarb, Rbp11, Rbp2, Rbp5, Stra6 and Wt1*. Furthermore, Goumy *et al.* also identified several chromosomal hot spots involved in CDH that are linked with vitamin A metabolism (34). Interestingly, the RA signaling pathway-associated genes identified in cases of CDH are mostly related to Bochdalek type hernias, this supports the Retinoid Hypothesis as explaining this most common type of CDH (67).

As mentioned above, *WT1* mutations have been linked with human cases of CDH, and *Wt1*-null mice have CDH (68,72). With the knowledge that offspring of mice lacking *Wt1* present with CDH, Carmona *et al.* went further to explore the role of *Wt1* in the developing diaphragm (95). By conditionally knocking out *Wt1* in the PPF, offspring developed CDH. Supporting the retinoid hypothesis, when these animals were treated with RA, it significantly reduced the size of the diaphragmatic defect. Matthew-Wood syndrome is another genetic syndrome where CDH is seen. It involves a gene that has been identified in retinoid signaling. This gene is *Stra6* and has been identified as a membrane protein essential in the assimilation of vitamin A into cells (97). A mutation in *Stra6* has been documented in many cases where CDH is present along with other congenital abnormalities such as bilateral anophthalmia and mild facial dysmorphism (96). Understanding the genetic contributions and its correlation with the retinoid pathway to CDH is important as it can be used to target therapeutic management and aid in counseling families.

2.3.4 Relevance of Retinoid Hypothesis to lung development in Congenital Diaphragmatic Hernia

With regards to CDH, patients suffer from significant pulmonary sequelae. Researchers have investigated different aspects of lung injury in human CDH cases and animal models. As previously stated, in cases of CDH, disruption in lung development is thought to not only be an outcome of mechanical obstruction but from an insult similar to the one causing the diaphragmatic hernia (50). When human CDH lungs were investigated, it was found that two RA signaling genes, CRBP2 and CYP26B1, were completely transcriptionally extinct. Interestingly these two genes

are known to be controlled by RA levels, which lead researchers to believe that intracellular fetal lung RA levels are lower in CDH cases (30). In addition to that, it was found that ALDH1A2 was upregulated in the injured lungs, which can be interpreted as either a disruption in the retinoid pathway or an effect of lung injury (30).

The Nitrofen model has been an excellent model to study the significance of vitamin A metabolism in not only the development of the diaphragm but also of the lung. In a comprehensive overview of the effect of Nitrofen on the lung, Montalva and Zani concluded that not only did half the litter exposed to Nitrofen develop CDH, but all fetuses showed some part of the signaling pathways critical to lung development were affected (98). When investigating possible treatments for improved lung function in patients with CDH, the Nitrofen model is also used. One study demonstrated that when RA is given prenatally to animals exposed to Nitrofen, it positively affects lung function by decreasing pulmonary artery resistance and improves PO2 levels. Overall, it improved lung maturation (99). One study went one step further and evaluated the effect of Nitrofen on erythropoietin and its correlation to pulmonary hypertension and hyperplasia. The researchers speculated that erythropoietin played a part in fetal lung development and suggested that due to the decrease in erythropoietin in the liver and kidney caused by Nitrofen in rats, alveolar and vascular remodelling could possibly be affected (100).

Further supporting the importance of RA in lung development utilizing a surgical model, Gallot *et al.* investigated the impact of RA supplementation on the lungs of rabbits. They found that RA improved normal type II/I pneumocyte ratio back to levels seen in control animals (105). Also utilizing the surgical model, Lewis *et al.* investigated the effect of late antenatal vitamin A (retinyl palmitate) supplementation on lung growth in the lambs. They concluded that supplementation improved lung compliance, acid-base balance and ventilation in the young lambs (101). Further experimentation and understanding of the interplay between CDH and lung injury is very important in the overall management of CDH patients. In the future, knowing the exact role of retinoid signaling in the development of CDH and lung injury can be a guide to therapeutic measures.

2.4 Summary

As discussed, CDH is a complicated birth defect with significant mortality and morbidity rates. It is evident that vitamin A metabolism plays an integral role not only in overall development

but especially in the diaphragm and the lungs. While the link between vitamin A and CDH has been investigated by many researchers, there are still many unknowns. The goal of this thesis is to further investigate the Retinoid Hypothesis and hopefully add a contribution to understanding the pathophysiology of CDH.

Chapter 3: General Methods

The following sections contain methodological details that are common to the research described in Chapters 4 and 5. Methodological details that are specific to the research described within these chapters can be found in that chapter's specific methods section.

3.1 Animals

All experiments were conducted in accordance with the guidelines established by The Canadian Council on Animal Care and approved by the University of Alberta Animal Research Ethics Committee (Animal use protocol #00001948). A breeding colony of wild type BALB/c mice (Jackson Laboratory, Bar Harbor, Maine, USA) were housed in the conventional animal facility in Heritage Medical Research Center basement at the University of Alberta. In this facility mice were kept in a 12L:12D light cycle (12 hours with lights on and 12 hours with lights off) at a temperature of 21 °C (± 2 degrees Celsius). Humidity was kept at 40-70% and cages were individually ventilated with HEPA filtered air. Animals used for breeding purposes were fed an enhanced breeder chow, while experimental animals were fed a regular chow diet unless otherwise specified. Table 3.1 details the dietary macronutrient composition and vitamin A content of these two diets.

3.2 Timed-pregnant mice

Female BALB/C mice at 110 days old were transferred to male BALB/C cages between 1500 and 1800 hrs and left overnight. The female genital region was examined between 0800 and 0900 hrs the following day for presence of a viscous vaginal plug as a sign of copulation. If a vaginal plug was present, midday of that day was considered Embryonic Day (E) 0.5. Animals were identified by cage number and ear punching. Mice were subsequently weighed on Day 7.5 and 8.5 between 0800 and 0900 hrs to confirm pregnancy. Mice with a weight gain of 1.5 g or more were considered pregnant and used for further experimentation as described below.

Table 3.1 - Macronutrient composition and vitamin A content of Breeder and Regular chow*

	Breeder	Regular
Fat	21.6%	13.2%
Protein	23.1%	24.6%
Carbohydrates	55.1%	62.1%
Vitamin A	15 IU/g	15 IU/g

*Table shows the percent of total calories obtained from the different macronutrients; Vitamin A content is shown as the number of international units (IU) per gram of chow.

3.3 Teratogen Administration

In order to induce the formation of CDH, pregnant mice were treated with a combination of the herbicide Nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether, China National Chemical Construction Jiangsu Company, Nanjing, China), and Bisdiamine ([dichloroacetyl]- 1,8-octamethylenediamine, MP Biomedicals LLC, Solon, Ohio, USA). Nitrofen-administration is a well-established model to induce CDH in rats although it is relatively ineffective in mice, requiring its combination with another CDH-inducing teratogen, Bisdiamine (102,103). For example, in 2002, Babiuk *et al.* conducted a study using the addition of Bisdiamine to Nitrofen to increase their incidence of CDH in the offspring of pregnant mice (103). Nitrofen and Bisdiamine were dissolved in olive oil by vortexing for one minute and sonicating for twenty minutes before each administration. Following mating, timed-pregnant females were administered the teratogen mixture via oral gavage using a 20G gavage needle, on day E8.5 between 1200 and 1300 hrs.

3.4 Tissue Collection

On day E18.5 mice were euthanized by Isoflurane inhalation (5% Isoflurane delivered in 1L/M of O_2). Once animals reached surgical plane anesthesia, they underwent cervical dislocation. Next, an abdominal incision was made, and the entire uterus was removed and placed in ice-cold phosphate buffered saline (PBS), prior to further dissection. Maternal livers were collected and immediately snap frozen using liquid nitrogen and held at -80ºC until further use.

Using a stereomicroscope (Stemi 508, Zeiss, Oberkochen, Germany) the isolated uterus was dissected, and the number of fetuses and any resorptions present were recorded. Individual fetuses were then separated from the uterus one at a time. Fetal crown-rump length was measured, and gross abnormalities were recorded. For the purposes of RNA collection and retinoid quantification, fetuses were dissected under a microscope and fetal lung and liver were removed, snap frozen using liquid nitrogen, and held at -80°C until further use. For the purposes of CDH analysis, fetuses were decapitated and had their hind quarters removed, their trunk was then stored overnight at 4°C in 10% buffered formalin. The following day fetuses were dissected using a stereomicroscope (Stemi 508, Zeiss) in PBS and presence, location and severity of CDH were recorded. Photographs of the diaphragms were taken using a stereomicroscope (Stemi 508, Zeiss). The classification system we used to categorize the size of diaphragm defects is shown in Figure 3.1.

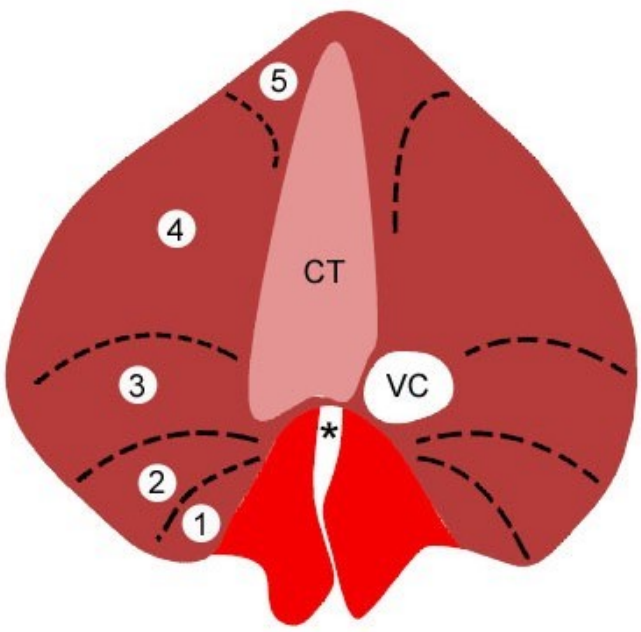

Fig 3.1 Classification of diaphragm defects by size. Schematic image showing a mouse diaphragm, with notations on how diaphragm defects of different sizes were categorized.
Area labelled 1 is considered very small, 2 is a small hernia, 3 is a medium, 4 is a large and 5 will be documented as a total absence of the hemidiaphragm. The area labelled VC is where the vena cava passes through the diaphragm. The star represents the space in-between the crural muscles where the aorta and esophagus passes through.

3.5 High Performance Liquid Chromatography

To quantify tissue vitamin A levels, specifically retinol and retinyl ester, High Performance Liquid Chromatography (HPLC) was used. The following text is a brief description of the HPLC protocol, as originally described by Kim *et al*. (104). All of the following experiments were carried out in the dark with the aid of yellow light bulbs due to the light-sensitivity of retinoids.

3.5.1 Hexane extraction and sample preparation

Retinoids were extracted from tissue samples using hexane (Fisher Chemical, Montreal, QC, Canada). Plasma samples were directly extracted into hexane, whereas liver and lung samples had to be homogenized first. To homogenize tissue, tubes were prepared with 1 ml PBS. Tissues to be analyzed were weighed and this weight was recorded. Tissues were then transferred to the tube containing PBS and homogenized with a BeadBug Microtube Homogenizer (Benchmark Scientific, Edison, NJ, USA) two times for 30 secs each on speed 20. A fraction of the tissue homogenate was then thoroughly mixed with a defined volume of internal standard, retinyl acetate (Sigma Aldrich, Oakville, Ontario, Canada). To prepare the internal standard, retinyl acetate was dissolved in ethanol to make a stock solution. The stock solution was then diluted to a concentration of ~1 ng/µl for addition to each sample. A Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA USA) was used to measure the absorbance of diluted standard solutions at 325 nm. The concentration of each standard solution was then calculated based on the optical density and the specific extinction coefficient using the Beer-Lambert equation. A standard curve was then generated using a series of dilutions with different amounts of retinyl acetate. A note of the internal standard's optical density and the volume added to each sample was made for later analysis. Ethanol was then added to bring the final ratio of ethanol to sample to 1:1. The samples were then vortexed thoroughly, and 3 ml of Hexane were added before being thoroughly vortexed again. The samples were then spun in a centrifuge (Thermo Scientific Sorvall St8 Centrifuge) for 10 minutes at maximum speed. Following centrifugation, the upper hexane layer contains the extracted retinoids. The upper phase of the centrifuged samples was removed and added to a fresh tube containing 500 µl of H_2O and vortexed. The mixture was then centrifuged again for 10 minutes at maximum speed. The upper phase from the centrifuged samples was then transferred to a clean tube and blown down using nitrogen until completely dry. Samples were

then reconstituted with 40 µl of mobile phase and added to amber vials for injection into the HPLC system.

3.5.2 Sample analysis

Samples were analyzed using an Agilent 1200 series HPLC (Santa Clara, CA, United States) running ChemStation software (Agilent), with a Zorbax Eclipse Plus C18 column (4.6 x 250 mm, 5 µm particle size; Agilent). The mobile phase was made up of Acetonitrile (70%; Fisher Scientific, Ottawa, Ontario, Canada), Methanol (15%; Fisher Scientific), and Methylene chloride (15%; Fisher Scientific). The pump flow rate was set at 1.8 mL/min, with a run time of 35 minutes per sample and an injection volume of 20 µl. Photodiode Array Detector detection wavelength was set to measure absorbance at 325 nm. The concentration of retinol and retinyl esters was based on measuring the area under the curve of chromatogram peaks, a representative example of which is shown in Figure 3.2. Sample concentration was calculated relative to the recovery of the internal standard, retinyl acetate.

3.6 RNA Extraction

Total RNA was extracted from mouse liver, lung and whole embryo samples that were harvested as previously described. Tissues were homogenized in 1 ml TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada) according to the manufacturer's protocol. Tissues were incubated at room temperature for 5 minutes, then 0.2 ml of chloroform was added, and the samples were shaken vigorously. Samples were then centrifuged in a microfuge at 2-8 °C for 10 minutes at 12,000 g (Hettich Mikro 20 microfuge, Beverly, Massachusetts, USA). The following RNA extraction steps were completed using a Qiagen RNeasy Mini Kit (Qiagen, Toronto, Ontario, Canada). The upper aqueous phase containing extracted nucleic acids was then transferred to a gDNA eliminator column and spun down. An equal volume of 70% ethanol was then added to the column flow through and mixed by pipetting. Next, 700 µl of this sample was transferred to a RNeasy mini spin column. The column was then washed with buffer and centrifuged to remove salt and other contaminants. The column was then transferred to a 1.5 ml tube and RNA was eluted using RNase-free water and centrifugation. The purity and concentration of the extracted RNA was then determined using a Nanodrop spectrophotometer (Thermo Fisher).

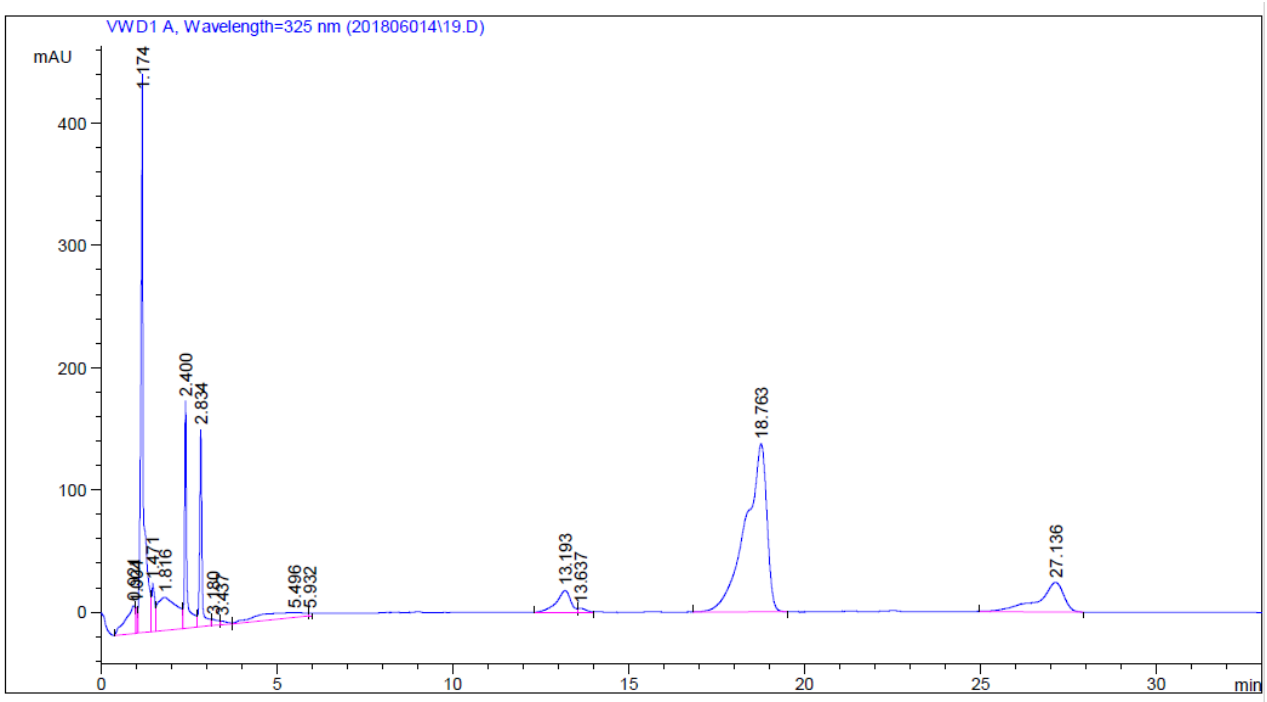

Figure 3.2-Example of HPLC profile obtained from the liver of a mouse. This figure shows a representative example of an HPLC profile obtained from mouse liver. The following peaks were used for analysis of tissue retinoid content: ~2.4 minutes, retinol; ~2.8 minutes, retinyl acetate (internal standard), ~13.2 minutes, retinyl linoleate, ~18.7 minutes, retinyl oleate/palmitate, and 27.1 minutes, retinyl stearate.

3.7 cDNA synthesis

RNA was transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA USA. The procedure was followed according to the manufacturer's protocol. In brief, the cDNA master mix contained reaction buffer, random primers, dNTPs, and reverse transcriptase. Each reaction contained 10 µl of master mix and 10 µl of RNA, with a total of 2 µg of RNA added to each reaction. The cDNA synthesis reaction was performed using a ProFlex PCR System (Applied Biosystems), and included a 2-hour incubation at 37°C, followed by a 5-min denaturation step at 85°C. Newly synthesized cDNA was diluted with nuclease-free water to be used for qPCR.

3.8 Quantitative PCR

All qPCR was performed using LightCycler 480 SYBR Green Master Mix, according to the manufacturer's protocol (Roche Life Sciences, Mannheim, Germany). In brief, the qPCR master mix contained reaction buffer, gene-specific primers, dNTPs, SYBR green and DNA polymerase. Each reaction contained 15 µl of master mix and 5 µl of cDNA. The qPCR was carried out on a Quant-Studio 3 Real Time PCR System (Applied Biosystems). The qPCR cycling conditions includes two phases. The first is 1 cycle of 2 minutes at 50°C followed by 10 minutes at 95 °C. The second includes 45 cycles of 10 seconds at 95°C followed by 10 seconds at 60°C and completed with 12 seconds at 72°C. After each cycle a melt curve ramp was performed. Table X details the primer sequences used for qPCR. Analysis of relative gene expression was performed using the ΔΔCt method. All data were analyzed relative to three references genes: 18s rRNA *(Rn18s*), beta-actin (*actb*), and β_2 microglobulin (*B2m*). Consistent results were produced for all three reference genes and only data relative to beta- actin is presented.

3.9 Statistical Analysis

All data are presented as the mean ± standard deviation (SD). Data were compiled in Microsoft Excel (Microsoft Corporation One Microsoft Way Redmond, WA, USA) and analyzed using Prism V7 (Graphpad, San Diego, CA, USA). The choice of statistical test depended on the data being analyzed. For our continuous data, when comparing two groups, a Student's t-test was used. When comparing three groups of continuous data, a one-way ANOVA was used with a

Table 3.2: Primer sequences for qPCR.

Gene	Forward Primer	Reverse Primer
18s rRNA	CCA TCC AAT CGG TAG TAG CG	GTA ACC CGT TGA ACC CCA TT
B-actin	AGCTATGAGCTGCCTGACG	TGCCACAGGATTCCATACCCAAG
β_2 microglobulin	TAC TCA CGC CAC CCA CCG	TCC CGT TCT TCA GCA TTT GGA TT
Cyp26a1	GGC ACT GTG ATT GGC AGC TTC TAA	TGC AGG GAG ATT GTC CAC AGG GTA
Cyp26b1	GCA GTA TAT GCT TAT GAC ATC TGA ATC	CCT GAC CAC TCA CCA ACA AA
Cyp26c1	CGCACCTTTGAACTGGACGGTTA	GCGGGCTACGGTACACT
Rara	CAC GCC TGAG CAA GAC ACA ATG A	GAA GGC AAA GAC CAA GTC GGT GA
Rarb	GGGCATGTCCAAAGAGTCTGTTAG	CTAGCTCCGCTGTCATCTCATAG
Rarg	ACTAAGGGAGCAGAAAGGGCTAT	TCGAGGAGTCGTCCTCAAACA
Lrat	CAG GCA TCG AAG AGA TGA CTC CG	GCT GCT GGT AAC TAA ATC CTG GTC C
Rdh 10	GCT GCT CTG CAA GCT GGA	GAC CGA GAC CTC GCC AAC
Raldh2	CCC ATT GGA GTG TGT GGA CAG AT	GCG GGT TTG ATG ACC ACG GTG TTA
Dhrs3	CCGCCTGATGTGCATCTACTATTT	TGCTGTGTAACCAGTTTGCACGA
Stra6	GTT CAG GTC TGG CAG AAA GC	CAG GAA TCC AAG ACC CAG AA
Crbp1	CTT ACT GTC CCT ACT GTG TGT CAA GCA CTA	CCT GAG ATG AAC CTC CTG AGA TGG TTT A
Crbp2	AAC ACC CTC GTG TGT GTG CAT AA	CTT GTC TCC CTC CAC CCA CTG
Crbp3	CCC GCT TGA GGC AAC TAC T	GTT TCT CAT ACA GGC TGT GTG ACA T
Wt1	GGTCTTCCGAGGCATTCAGG	TATGCACACATGAAAGGACGTTTCT
Coup-TfII	GTGTGCTTTGGAAGAGTACGTTAGG	TTGCTCTATGACTGAGGAGGAGACC
Gata 4	ACTCTTGGAACAGCCTGGTC	CCCACGTCCCAAGTCCGA
Fog 2	CCCAAGAAAGATTCTCTGCCATTGT	TGCTGTGGGTTGGCCGTA

Tukey's multiple comparison post-test. Categorical data was analyzed using Chi-Squared to trend with partitioning as the post- test.

Chapter 4: Dietary Manipulation of Vitamin A and Congenital Diaphragmatic Hernia

4.1 Introduction

During gestation, maternal vitamin A is important as it is essential for fetal development and is the only source of vitamin A for the developing embryo. There is an increased requirement for vitamin A during pregnancy and even in deficient states, it is still transferred to the fetus (120,111). According to the Retinoid Hypothesis, abnormal retinoid signaling during development leads to incomplete formation of the diaphragm (see Chapter 2). Existing data in support of this hypothesis includes the fact that when a supraphysiological dose of vitamin A was given to pregnant rats exposed to CDH causing teratogens, there was a significant decrease in the incidence of CDH compared to controls who did not receive vitamin A (18). Additional support of this hypothesis comes from human cases of CDH where it was shown that low intake of dietary vitamin A during pregnancy is associated with an increased risk of infants developing CDH (15). Furthermore, a strong association between CDH and low levels of retinol and RBP in newborns has been made (14,94). Taking these studies into account, it can be suggested that the occurrence of CDH in the context of maternal vitamin A status is two-fold. First, supraphysiological doses of vitamin A can prevent teratogen induced CDH, and second, low maternal intake may be a risk factor for CDH. While previous studies have explored maternal vitamin A status in human CDH and the rescue of CDH by vitamin A and its metabolites in teratogenic animal models, there has yet to be a study that specifically investigates maternal dietary vitamin A manipulation and its impact on teratogen induced CDH.

With this in mind, the first research question was developed, specifically asking, does maternal vitamin A status impact the susceptibility to teratogen induced CDH? We hypothesize that offspring of animals with a low vitamin A status are more susceptible to teratogen induced CDH, and those with a high vitamin A status are less susceptible to diaphragmatic injuries.

To address this research question, we wanted to determine the incidence of teratogen induced CDH in pregnant mice with different vitamin A status (Summarized in Figure 4.1). In order to achieve this, we developed the following experimental goals:

1. Establish an optimal teratogenic dose to induce CDH in mice
2. Establish a protocol to develop three groups of female mice with different vitamin A status: 1) marginal, 2) sufficient, and 3) excess.
3. Induce CDH in timed-pregnant mice with differing vitamin A status

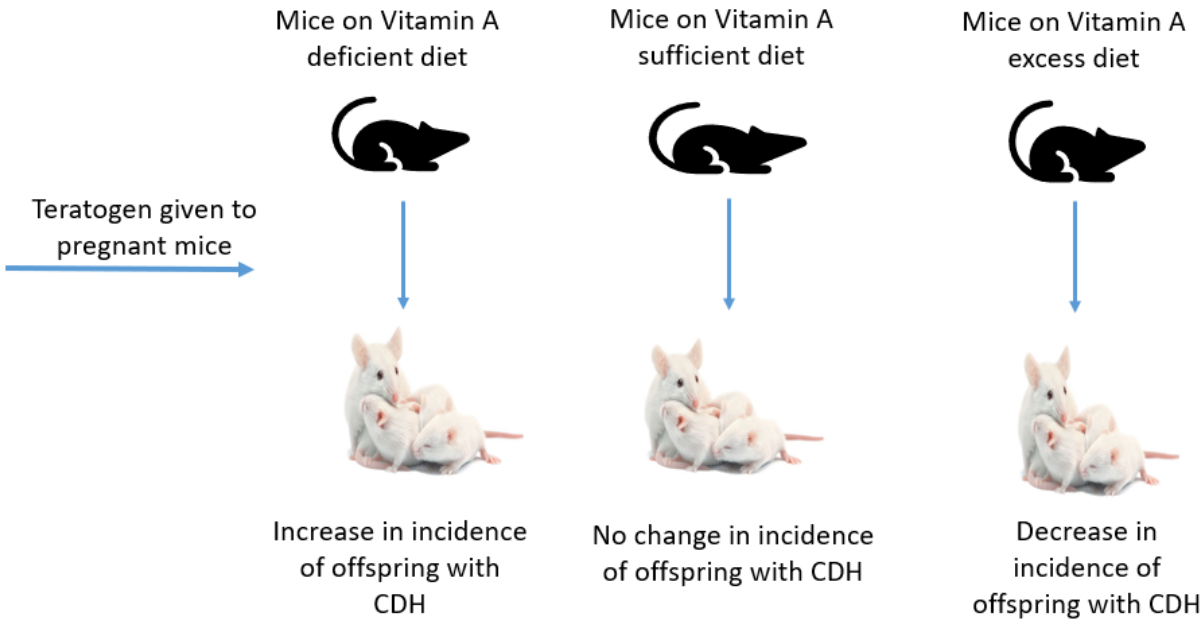

Figure 4.1 - Schematic representing the experimental design for investigating whether maternal vitamin A status impacts susceptibility of teratogen- induced CDH. Three groups of mice will be placed on diets of varying vitamin A content and then teratogen will be given to pregnant mice and incidence of CDH will be recorded. The expected results are shown for each experimental group.

To achieve these goals, first, different combinations of Nitrofen and Bisdiamine were administered to pregnant mice on various diets and the incidence of CDH, as well as resorptions, were recorded. Manipulating the combination of teratogens continued until there was a significant number of CDH without an overwhelming number of intrauterine deaths. Second, three groups of female mice with different vitamin A status: 1) marginal 2) sufficient and 3) excessive were established. To create these groups, females were fed a vitamin A deficient (VAD), sufficient (VAS) or excess (VAX) diet from weaning to 110 days of age at which time the experiment was started. Lastly, following mating, timed-pregnant females from each group were administered the optimal teratogenic combination of 0.5 mg/kg dose of Nitrofen and 0.125 mg/kg of Bisdiamine. The primary outcome measure for this experiment was the incidence of CDH in the resulting offspring. In addition, the severity and location of each diaphragm defect observed was recorded. Supplemental to this data, the hepatic vitamin A levels of the mice maintained on the three diets and their offspring were measured using HPLC.

4.2 Materials and Methods

A description of generating timed-pregnant mice, teratogen administration, tissue collection, HPLC methodology, qPCR methodology, CDH incidence tabulation and statistical analyses are provided in Chapter 3.

4.2.1 Manipulation of dietary vitamin A content

Purified rodent diets were purchased from Bio-Serv (Flemington, New Jersey, United States). Diets were custom made with three differing Vitamin A concentrations; VAD, VAS and VAX. Otherwise a standard macronutrient composition was used, with an AIN-93G vitamin and mineral mix (Table 4.1). Female mice were divided into three groups, VAD, VAS and VAX, and put on respective diets from weaning to 110 days of age (approximately 3 months). Once a week, from the day of weaning, each animal was weighed, and this value was recorded.

Table 4.1 – Macronutrient composition and vitamin A content of experimental diets

	VAD	VAS	VAX
Fat	10%	10%	10%
Protein	14.2%	14.2%	14.2%
Carbohydrates	75.4%	75.4%	75.4%
Vitamin A	0 IU/g	4 IU/g	25 IU/g

Macronutrients composition is shown as the percent of total calories obtained from the diet. Vitamin A content is shown as the number of international units (IU) per gram of chow.

4.2.2 Titration of teratogenic dose for optimal induction of CDH in mice

The effect of the teratogen Nitrofen on the development of CDH and its sequelae in animals have been widely investigated . An additional compound, Bisdiamine, has also been confirmed to cause CDH in offspring when administered to pregnant dams (25). Most experiments using these teratogens were conducted on rats with the exception of a few that utilized mice. Thus, one of the first steps in this series of experiments was to establish the optimal combination of teratogens to administer to pregnant mice. The combination of Nitrofen and Bisdiamine that mice were administered on various diets was titrated as follows:

Breeder chow:

1. 0.3 mg/kg Nitrofen and 0.3 mg/kg Bisdiamine
2. 0.4 mg/kg Nitrofen and 0.4 mg/kg Bisdiamine
3. 0.5 mg/kg Nitrofen and 0.5 mg/kg Bisdiamine
4. 0.6 mg/kg Nitrofen and 0.6 mg/kg Bisdiamine

Purified diet:

1. 0.5 mg/kg Nitrofen and 0.5 mg/kg Bisdiamine
2. 0.5 mg/kg Nitrofen and 0.25 mg/kg Bisdiamine

Regular chow:

1. 0.5 mg/kg Nitrofen and 0.125 mg/kg Bisdiamine

4.3 Results

4.3.1 Establishing an optimal teratogenic dose to induce CDH in mice

To establish the teratogenic dose needed to induce CDH in offspring without causing excess intra-uterine death, different combinations of Nitrofen and Bisdiamine were administered to pregnant mice on various diets and the incidence of CDH as well as resorptions were recorded. From the collected data, a dose of 5 mg/kg of Nitrofen and 0.125 mg/kg of Bisdiamine was chosen for ongoing experiments. This dose yielded ~50% CDH with 37% resorptions. To arrive at this dose, the amount of teratogen administered was adjusted in three phases. In phase 1, when tested on animals on a breeder chow diet, the teratogenic combinations of 0.3 mg/kg and 0.4 mg/kg of both Nitrofen and Bisdiamine yielded no offspring with CDH. When the dose was increased to 0.5 mg/kg of each teratogen, an incidence of approximately 30% CDH was recorded with only 22% resorptions. Once, this dose was deemed as adequate, phase 2 began which involved administering

this dose to mice on a purified diet. When this dose was given to mice on the purified diet, it caused almost 100% resorptions. We attributed this unexpected and substantial difference in resorption rate to the change in diet, and the switch from breeder chow to purified diets (see discussion). With the knowledge that Bisdiamine is an abortifacient in rats (121), this component was titrated down to 0.125 mg/kg with 0.5 mg/kg of Nitrofen and tested in animals fed a regular diet chow that had a similar composition to that of the purified chow, which made up phase 3 of establishing the optimal teratogenic dose to answer our primary experimental question. With this dose, an incidence of approximately 50% of CDH was recorded with 37% resorptions. This incidence and number of resorptions was decided to be adequate to test our hypothesis. CDH in half of the population was a good incidence as it allowed room to increase in offspring of mice who had a marginal vitamin A status and decrease in offspring of mice with an excess vitamin A status. Table 4.2, 4.3 and 4.4 details the various trial doses in addition to incidence of CDH and resorption rate associated with the different concentrations of teratogen and diets used.

4.3.2 Characterization of a mouse model of CDH using a teratogenic combination of Nitrofen and Bisdiamine

Having established an optimal dose of teratogens to induce CDH in mice without excess intra-uterine death, we characterized the type of diaphragm defects that this model produced. Knowing that timing of teratogen exposure leads to varied phenotypes of CDH, the side and size of teratogen induced CDH were documented in offspring of mice fed a regular chow diet and treated with the teratogenic dose of 0.5 mg/kg of Nitrofen and 0.125 mg/kg of Bisdiamine. As shown in Figure 4.2, diaphragm defects were primarily located in the posterolateral region of the diaphragm (Figure 4.2 B and E), with obvious herniation of the abdominal contents into the thoracic cavity (Figure 4.2 D), features that are characteristic of the clinical presentation of Bochdalek CDH.

4.3.3 Establishing a protocol to develop female mice with different vitamin A status

In order to achieve our primary experimental goal, we had to develop a protocol whereby the vitamin A status of female mice was manipulated. Female mice were fed either a VAD, VAS or VAX diet for three months from the time of weaning. In order to assess vitamin A status, after

Table 4.2 - Phase 1- Mice on breeder chow diet receiving an increasing concentration of Nitrofen and Bisdiamine.

Dose of Nitrofen (mg/kg)	0.3	0.4	0.5	0.6
Dose of Bisdiamine (mg/kg)	0.3	0.4	0.5	0.6
Litter (n)	1	2	7	2
Fetuses (n)	13	16	38	7
Resorptions (n)	0	3	11	13
Resorptions (%)	0	16	22	65
Diaphragmatic hernia (n)	0	0	11	5
Diaphragmatic hernia (%)	0	0	29%	71%

Table 4.3 - Phase 2 - Mice on purified diet chow receiving a steady concentration and decreasing concentration of Bisdiamine.

Dose of Nitrofen (mg/kg)	0.5	0.5
Dose of Bisdiamine (mg/kg)	0.5	0.25
Litter (n)	6	2
Fetuses (n)	2	2
Resorptions (n)	37	6
Resorptions (%)	94	87
Diaphragmatic hernia (n)	1	1
Diaphragmatic hernia (%)	50	50

Table 4.4 - Phase 3- Mice on regular chow diet receiving 0.5mg/kg of Nitrofen and 0.125mg/kg Bisdiamine.

Dose of Nitrofen (mg/kg)	0.5
Dose of Bisdiamine (mg/kg)	0.125
Litter (n)	10
Fetuses (n)	53
Resorptions (n)	32
Resorptions (%)	37
Diaphragmatic hernia (n)	26
Diaphragmatic hernia (%)	49

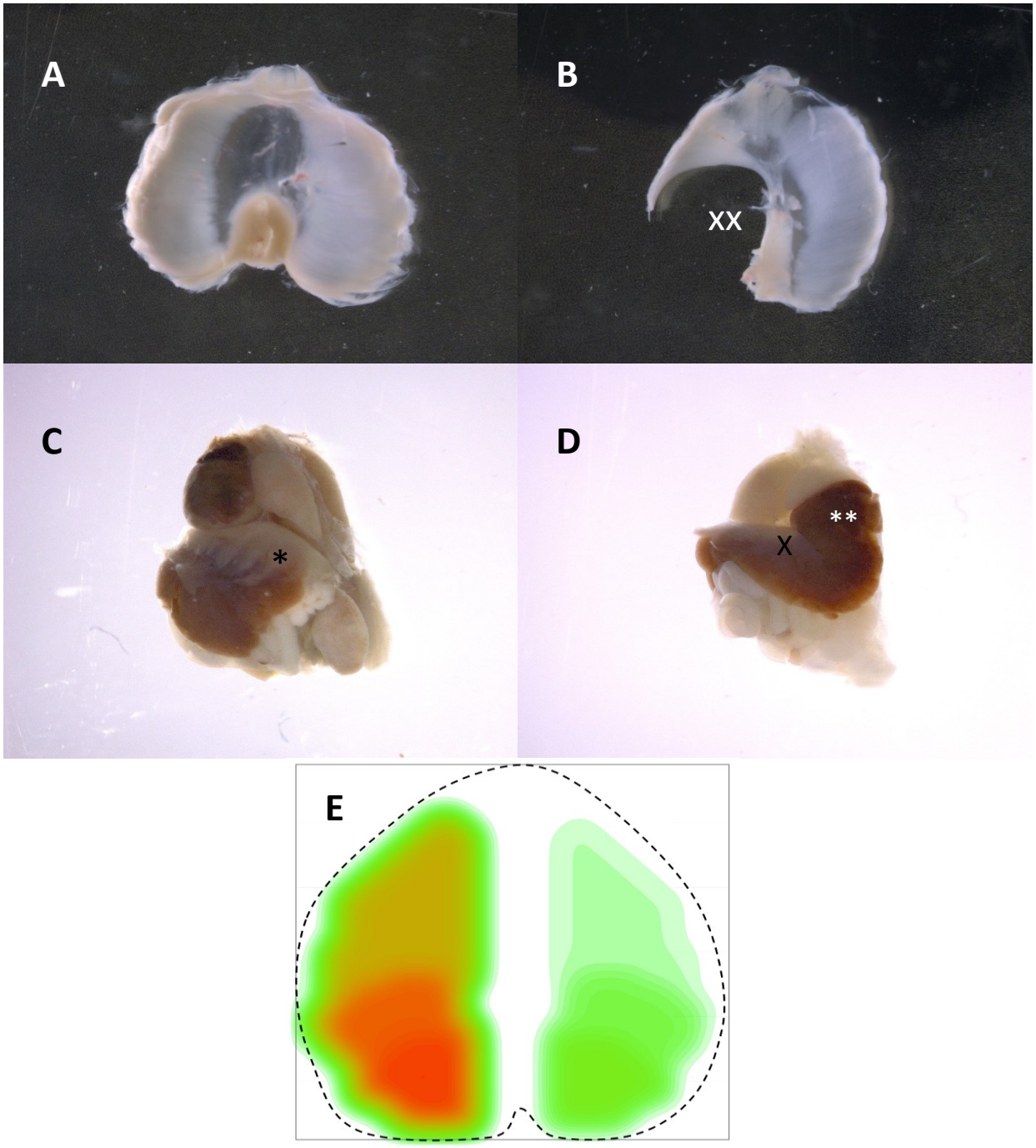

Figure 4.2- Characterization of a mouse model of CDH. A- Picture of normal mouse diaphragm. B- Picture of large left diaphragmatic hernia. XX- Diaphragm defect where abdominal contents herniate through. C- Picture of a lateral view of a normal mouse liver, lungs, heart and diaphragm. *- Normal mouse diaphragm on liver. D- Picture of a lateral view of a diaphragmatic hernia in a mouse with liver and stomach herniation and lung hypoplasia. X- Diaphragm on liver. **- Liver and stomach herniation into the chest cavity. E- Heat map showing data collected from 53 diaphragms of offspring from teratogen-treated mice showing that majority of defects are medium/large left-sided CDH. Green areas are unaffected by CDH. Intensity of red areas are the most common location for CDH.

three months on purified diets we collected liver and plasma of these mice to measure hepatic vitamin A content and the expression of vitamin A-regulated genes. As shown in Figure 4.3, plasma retinol levels between mice fed a VAD diet and those fed a VAX diet were significantly different. The mice on a VAD had lower plasma retinol levels when compared to those on a VAX diet. Similarly, as shown in Figure 4.4, hepatic retinol levels followed the same pattern, with mice fed a VAD diet having significantly lower retinol levels when compared to those fed a VAS and VAX diet. Differing from the plasma retinol, a significant change was also seen between the mice fed a VAS diet and those fed a VAX diet. Following the hepatic retinol levels, as shown in Figure 4.5 hepatic retinyl ester levels varied significantly between the three diets, with mice fed a VAD diet having the least amount of retinyl esters being detected and those fed a VAX diet having the most amongst the three diets. In summary, animals maintained on VAD, VAS and VAX diets have markedly different hepatic and circulating vitamin A levels. This is in accordance with their dietary vitamin A intake and reflects a successful change in their vitamin A status.

Having established that feeding female mice with different amounts of vitamin A changed their tissue vitamin A content, we confirmed this change in vitamin A status by looking for changes in the hepatic mRNA expression level of genes that regulate, and are regulated by, vitamin A metabolism. Figure 4.6 shows the effect of dietary vitamin A manipulation on the expression of *Cyp26a1*. Mice fed a VAD and VAS diet were both significantly lower when compared to those fed a VAX diet. Figure 4.7 shows a similar effect on the expression of *Cyp26b1*, however, mice fed a VAD diet showed a significant difference with the group fed a VAX diet. There was no difference between the mice fed a VAS diet and those fed VAD and VAX diets. Figure 4.8 shows how the dietary manipulation of vitamin A affected *Lrat* expression. Comparable to the previous genes, the mice fed a VAD diet was significantly lower to those fed VAS and VAX diets. Figure 4.9 shows the effect on *Rbp4.* No change between the different dietary groups was detected. In conclusion, mice fed diets of varied vitamin A contents, not only do their hepatic retinol and retinyl ester levels change, but also the expression of some of the genes involved in the metabolism of vitamin A are altered reflecting their altered vitamin A status.

Having established at the biochemical and gene expression level that we could successfully manipulate the vitamin A status of female mice, we wanted to confirm that this was not done at

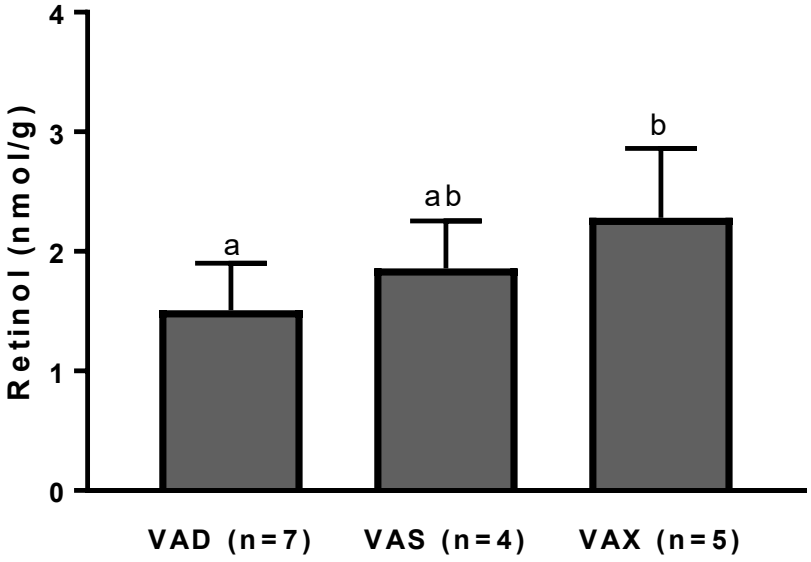

Figure 4.3 – Effect of dietary vitamin A intake on maternal plasma retinol. Graph showing the differences in plasma retinol level in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

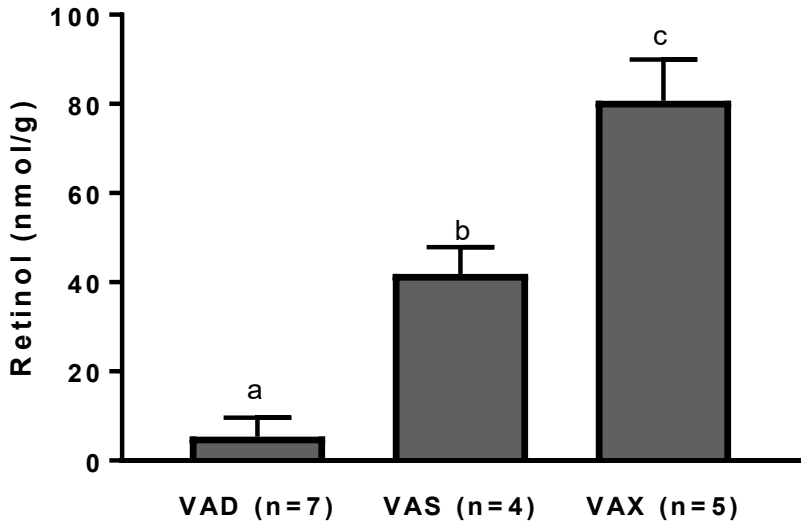

Figure 4.4 - Effect of dietary vitamin A intake on maternal hepatic retinol. Graph showing the differences in liver retinol level in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

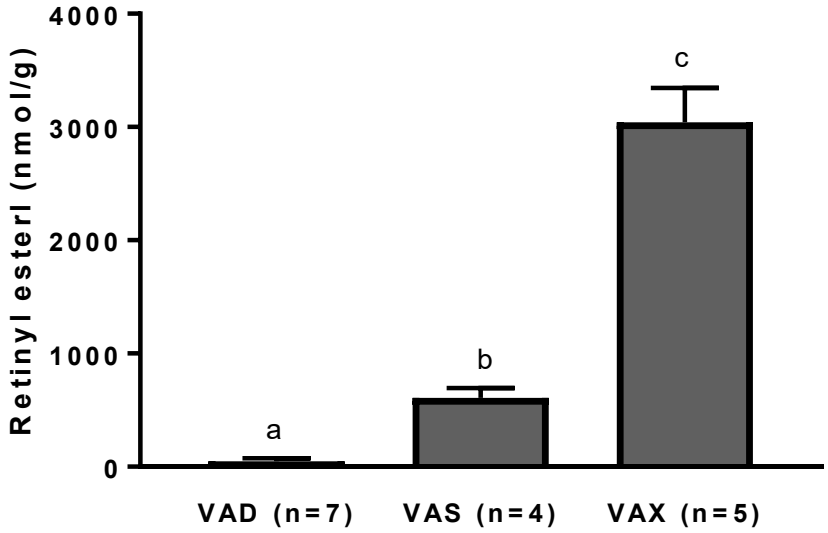

Figure 4.5- Effect of dietary vitamin A intake on maternal hepatic retinyl ester. Graph showing the differences in liver retinyl ester l level in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

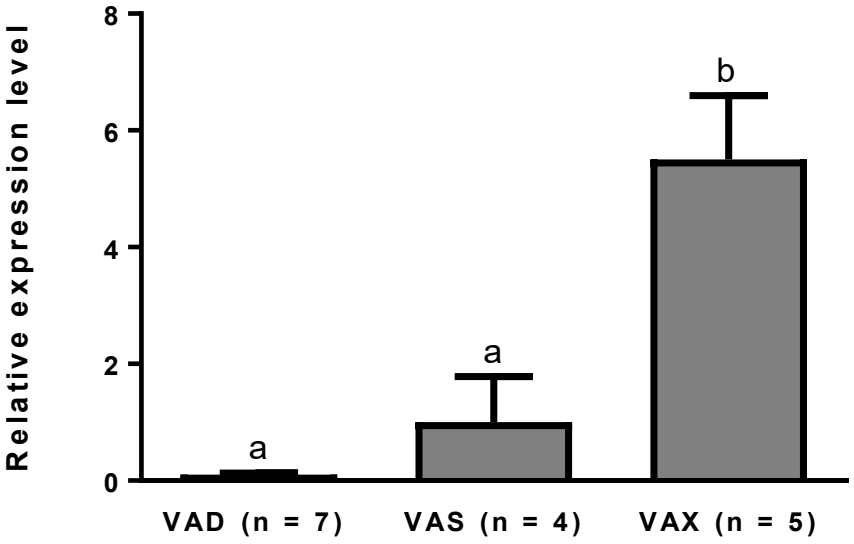

Figure 4.6- Effect of dietary vitamin A intake on *Cyp26a1* expression. Graph showing the differences in relative gene expression of *Cyp26a1* in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

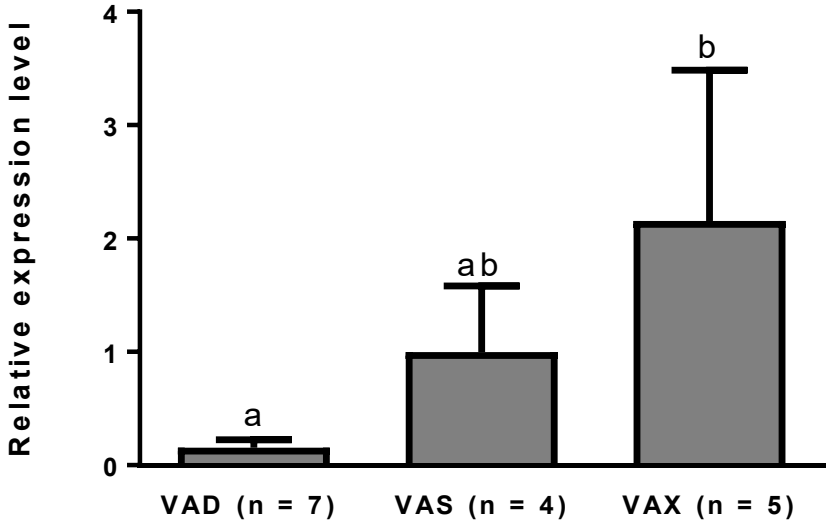

Figure 4.7- Effect of dietary vitamin A intake on *Cyp26b1* expression. - Graph showing the differences in relative gene expression of *Cyp26b1* in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

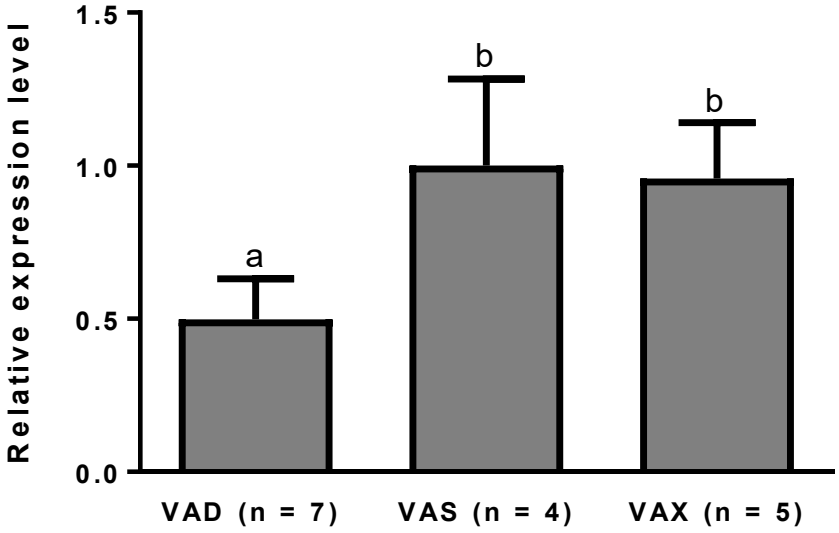

Figure 4.8- Effect of dietary vitamin A intake on *Lrat* expression. Graph showing the differences in relative gene expression of *Lrat* in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

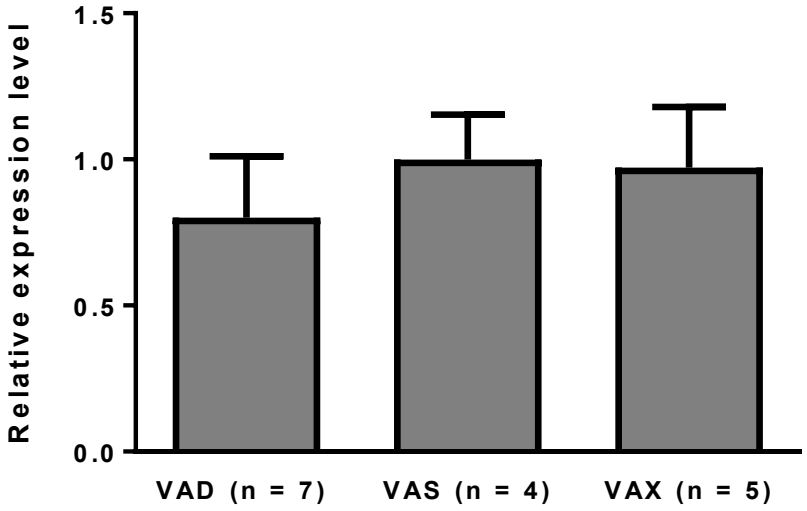

Figure 4.9- Effect of dietary vitamin A intake on *Rbp* expression. - Graph showing the differences in relative gene expression of *RBP* in groups of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

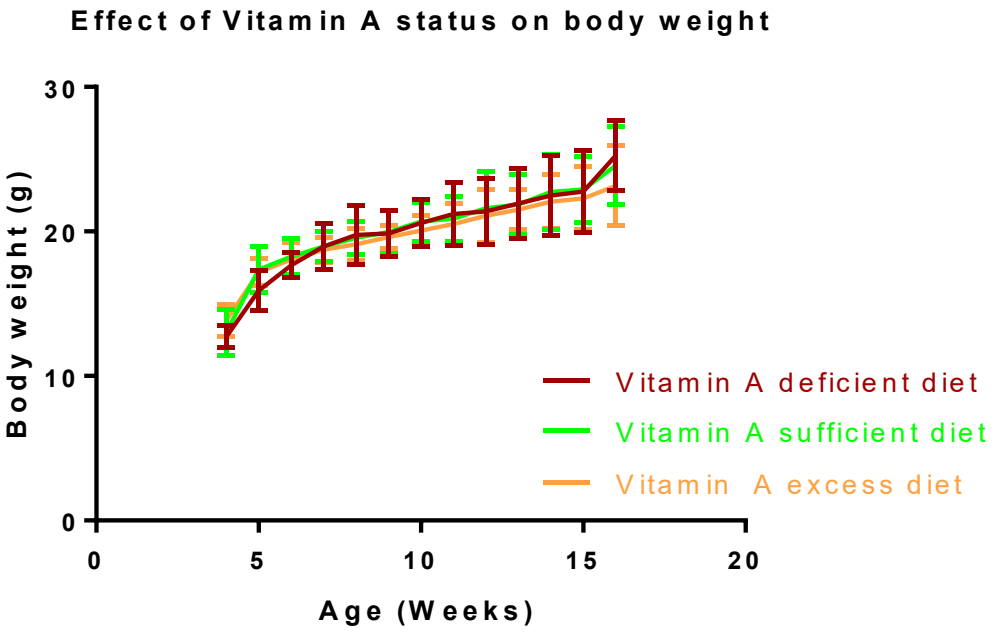

Figure 4.10– Effect of dietary intake on body weight. Graph showing progression of body weights (g) of mice fed on specific vitamin A diets from weaning to 16 weeks of life.

the expense of the growth and health status of the mice. No overt symptoms of vitamin A deficiency were observed in our mice. Similarly, data collected measuring body weights of the three groups of female mice on different diets showed no difference in mean body weight from diet to diet (Figure 4.10).

4.3.4 Effect of Maternal Diet Manipulation on fetal vitamin A status

Offspring of animals maintained on VAD, VAS, and VAX diets have markedly different hepatic vitamin A levels. This is in accordance with their dietary vitamin A intake and reflects a successful change in their vitamin A status. In order to confirm that by manipulating maternal vitamin A status we could also impact fetal vitamin A status, we measured the hepatic vitamin A content of fetal mice isolated at gestational day 18.5 from pregnant female mice fed a VAD, VAS and VAX diet. As shown in Figures 4.11 and 4.12 the retinol and retinyl ester levels in the liver of offspring of mice fed the varied vitamin A content diets differed significantly between the offspring of mice fed a VAD diet and those fed a VAX diet. No change was seen between offspring of those fed a VAS diet and those fed VAD and VAX diets.

4.3.5 Impact of maternal vitamin A status on incidence of CDH in offspring

It has previously been reported that overt vitamin A deficiency can cause CDH in the offspring of rodents (11). To ensure that the CDH seen from our teratogen treatment experiments were not secondary to overt vitamin A deficiency, offspring of mice on various vitamin A diets but were not exposed to teratogen, were dissected and their diaphragms evaluated for the presence of diaphragm defects. Table 4.5 shows the recorded incidence of diaphragmatic hernia observed in these mice. Importantly, it was noted that none of the offspring had CDH.

4.3.6 Effect of maternal vitamin A status on incidence of teratogen- induced CDH in offspring

Once we had successfully established the optimal dose of teratogen to induce CDH in our mice and established a protocol to manipulate their vitamin A status, we were able to answer our primary research question: does maternal vitamin A status impact the susceptibility to teratogen induced CDH? The three groups of mice fed VAD, VAS and VAX diets were exposed to 0.5

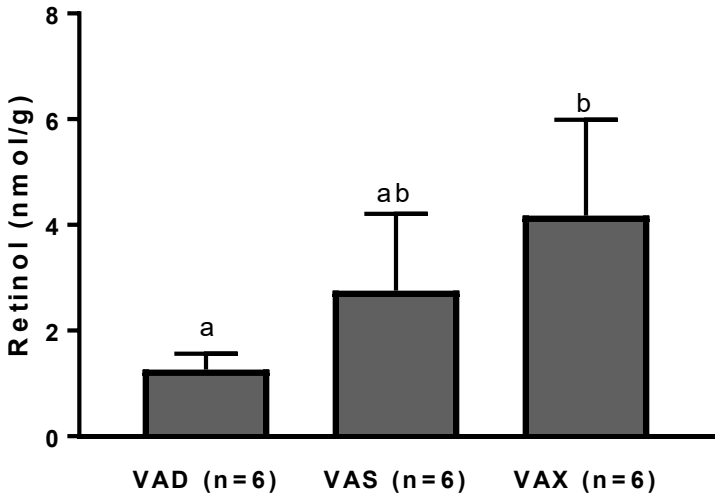

Figure 4.11- Effect of dietary vitamin A intake on fetal hepatic retinol. Graph showing the differences in plasma retinol level in offspring of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

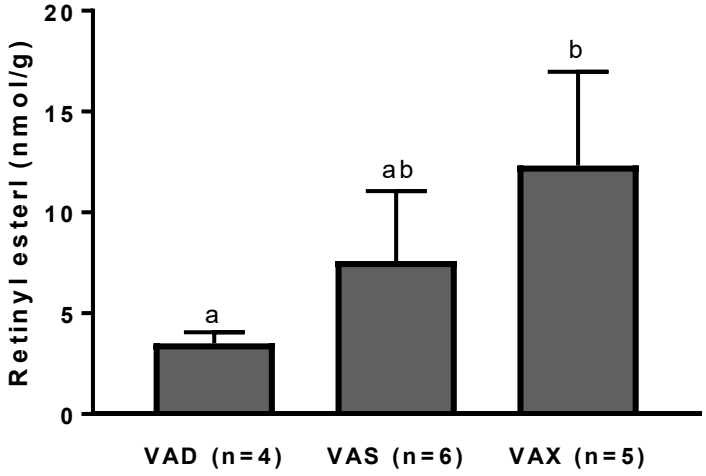

Figure 4.11- Effect of dietary vitamin A intake on fetal hepatic retinyl ester. Graph showing the differences in plasma retinol level in offspring of mice fed various vitamin A diets (VAD- Vitamin A deficient, VAS – Vitamin A sufficient, VAX- Vitamin A Excess). Data presented as mean ± SD. Analysis performed by a one-way ANOVA and Tukey's Multiple Comparison. Columns with different letters are significantly different.

Table 4.5 Table showing the incidence of CDH in offspring of mice fed specialized vitamin A diets but not exposed to teratogen.

	VAD (0 IU/g)	VAS (4 IU/g)	VAX (25 IU/g)
Average maternal age (d)	131 ± 32.4	134 ± 29.4	116 ± 18.6
Litters (n)	6	6	7
Average litter size (n)	5.6 ± 1.6	7.3 ± 1.8	6.8 ± 1.8
Fetuses (n)	34	44	47
Resorptions (n)	0	0	0
Diaphragmatic hernia (n)	0	0	0

Average data is presented as mean ± S.D.

mg/kg of Nitrofen and 0.125 mg/kg dissolved in 1 ml of olive oil. Table 4.6 shows the results of the administration of teratogen to these three experimental groups. Documentation of various parameters are also included in this table. The average maternal age was found to be 174, 155 and 145 days for mice fed VAD, VAS and VAX, respectively. The average maternal age was compared using a one-way ANOVA and was found to be significant between the mice fed a VAD diet and those fed a VAS and VAX diet. The crown rump length (CRL) of each fetus was measured and the average for each group was recorded. For the VAD group the CRL was 15.5 mm, the VAS group had an average of 15.9 mm and the VAX group had an average of 15.1 mm. When the averages were analyzed using a one-way ANOVA, there was no significant difference between groups. For each group the number of litters examined were 12, 13 and 11 for the VAD, VAS and VAX groups respectively. The average litter size for each group was also recorded. For the mice fed a VAD diet, the average litter size was 8.9. For the VAS and VAD groups, the average litter sizes were 8.5 and 8.8 respectively. A one-way ANOVA showed there was no significant difference between the litter sizes in each group. For the mice fed a VAD diet, the total number of dissectible fetuses was 39. For the mice fed VAS and VAX diets, the number of dissectible fetuses were 42 and 38 respectively. The total number of resorptions were 68, 68 and 59 for the VAD, VAS and VAX groups respectively. The percent total of resorptions were 64%, 60% and 66% for the VAD, VAS and VAX groups, respectively. The total number of resorptions was analyzed using chi-square for trend analysis and no significant difference between groups was found.

The total number of CDH observed in the VAD, VAS and VAX groups were 22, 17 and 12 respectively. The total number of CDH between groups was analyzed using a chi-square for trend analysis and a significant difference was noted between the group fed a VAD diet and those on a VAX diet. The incidence of diaphragmatic hernia represented as a percent total of dissectible fetuses was 56.4%, 40% and 31.5% for the VAD, VAS and VAX groups respectively. Figure 4.13 graphs the total number and incidence of CDH of the various groups. Tabulating the left-sided versus right-sided CDH in each group, the VAD group showed 19 left-sided CDH and 4 right-sided. The VAS group had 16 left-sided CDH and 1 right-sided. The VAX group had 11 left-sided CDH and 1 right-sided. When compared as a percentage of left versus right, the VAD, VAS and VAX groups had 86.3%, 94.1% and 91.6% respectively. A chi-square for trend analysis was used to compare the difference between left and right sided hernia between groups and no significant difference was found.

The size of diaphragmatic hernia for each group was also recorded. The animals on a VAD diet had 4 small diaphragmatic hernia and an incidence of 18.1%. The animals fed a VAS diet had 2 small defects and an incidence of 11.7%. The VAX group had 1 small CDH and an incidence of 8.3%. When looking at medium sized hernia, the VAD group had 6 hernia with an incidence of 27.2%. The VAS and VAX diet groups both had 3 medium sized diaphragmatic hernia with an incidence of 17.6% and 25% respectively. In the mice fed a VAD diet, there were 13 large CDH and an incidence of 59%. The VAS diet group showed 12 fetuses with large defects at a 70% incidence. The VAX diet group had 8 large hernia and an incidence of 66.7%. Figure 4.15 graphs the incidence of small, medium and large CDH amongst the various diet groups. Chi-square for trend analysis showed the difference between groups to be insignificant. Figure 4.16 shows representative photographs depicting examples of small, medium, large and bilateral CDH observed in mouse fetuses.

In addition to CDH, we also recorded the occurrence of two other abnormalities, cleft palate (CP) and odema (OE). Of the fetuses dissected from the VAD group, 34% had CP. In the VAS and VAX groups, there was an incidence of 27% and 19% respectively (Figure 4.14). Chi-square analysis for trend showed a significant difference between the VAD group when compared to the VAS and VAX groups. Lastly the incidence of OE was noted in fetuses from each group. The percent total incidence of OE was 28.2%, 16.6% and 25.9% for the VAD, VAS and VAX groups respectively. Chi-squared analysis for trend revealed that the differences between groups was not significant.

4.4 Discussion

The major premise of this study is centered around the Retinoid Hypothesis, stating that abnormal retinoid signaling plays a role in the formation of CDH in infants (17). Investigating this hypothesis further, we proposed that maternal vitamin A status would affect the susceptibility to teratogen induced CDH. To achieve this goal, we first had to optimize a mouse model of teratogen induced CDH. Teratogenic animal models have been used for decades to study CDH; however, experiments were mostly performed in rats. Studies utilizing teratogenic mouse models have primarily been used to investigate the impact of CDH on the lungs and not the diaphragm itself, apart from one study done by Babiuk *et al.* (102, 117,118, 112-116).

Table 4.6 Table showing the effect of maternal vitamin A status on incidence of teratogen-induced CDH in offspring

	VAD (0 IU/g)	VAS (4 IU/g)	VAX (25 IU/g)
Average maternal age (d)	174 ± 30.7	155 ± 27.5	145 ± 14.7
Average Crown Rump Length (mm)	15.5 ± 2.4	15.9 ± 1.1	15.1 ± 1.2
Litters (n)	12	13	11
Average litter size (n)	8.9 ± 1.2	8.4 ± 1.6	8.8 ± 2.4
Total fetuses (n)	39	42	38
Total resorptions (n)	68	68	59
Resorptions (% total)	64%	60%	66%
Total diaphragmatic hernia (n)	22	17	12
Diaphragmatic hernia incidence (% total)	56.4%	40.4%	31.5%
Side of diaphragm defect (L/R)	19/4	16/1	11/1
Side of diaphragm defect (% L/R)	86.3%	94.1%	91.6%
Small diaphragm defects (n)	4	2	1
Small diaphragm defects (% total)	18.1%	11.7%	8.3%
Medium diaphragm defects (n)	6	3	3
Medium diaphragm defects (% total)	27.2%	17.6%	25.0%
Large diaphragm defects (n)	13	12	8
Large diaphragm defects (% total)	59.0%	70.0%	66.7%
Cleft palate (n)	34	27	19
Cleft palate incidence (% total)	87.1%	64.2%	50.0%
Oedema (n)	11	7	7
Oedema incidence (% total)	28.2%	16.6%	25.9%

Average data is presented as mean ± S.D.

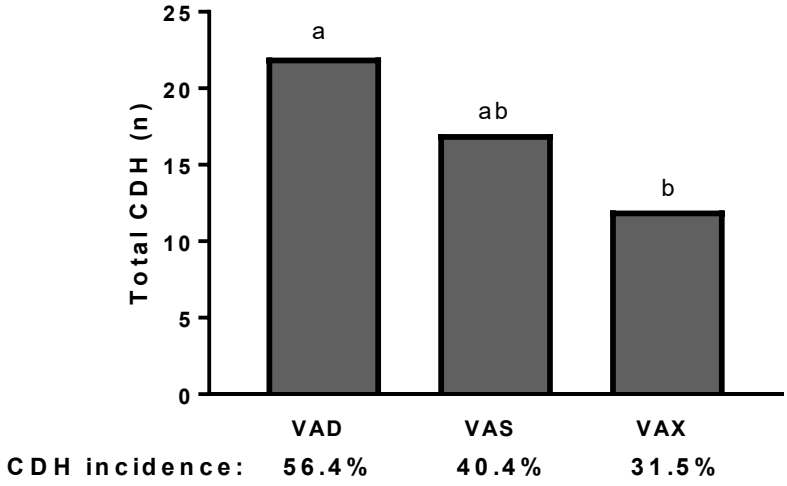

Figure 4.13- Incidence of CDH in teratogen treated mice Graph comparing the total number and incidence of CDH in mice fed varied vitamin A diets (VAD- Vitamin A deficient, VAS- Vitamin A Sufficient, VAX- Vitamin A Excess) using chi-square to trend analysis showing a significant difference (P <0.05) between animals fed a vitamin A deficient diet and those fed a vitamin A excess diet. Columns with different letters are significantly different.

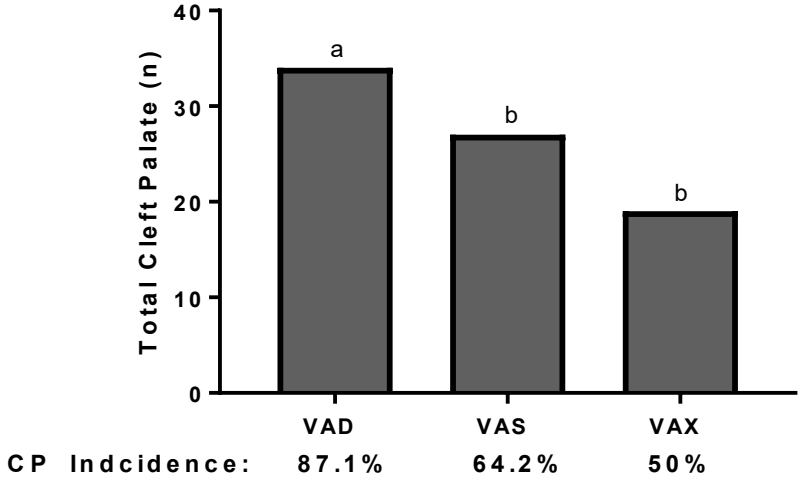

Figure 4.14- Incidence of CP in teratogen treated mice Graph comparing the total number and incidence of CP in mice fed varied vitamin A content diets (VAD- Vitamin A deficient, VAS- Vitamin A Sufficient, VAX- Vitamin A Excess) using chi-square to trend analysis showing a significant difference (P <0.05) between animals fed a vitamin A deficient diet and those fed a vitamin A sufficient excess diet. Columns with different letters are significantly different.

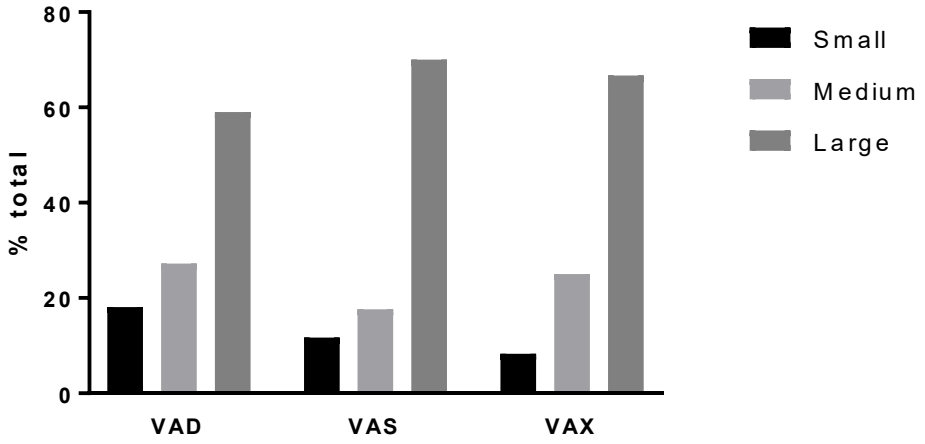

Figure 4.15- Severity of CDH in offspring of teratogen treated mice on varied diet. Graph comparing the % total incidence of small, medium and larger diaphragmatic hernia in mice fed varied vitamin A content diets diets (VAD- Vitamin A deficient, VAS- Vitamin A Sufficient, VAX- Vitamin A Excess). Using a chi-square analysis, the varying severity between diet groups was insignificant ($P > 0.05$).

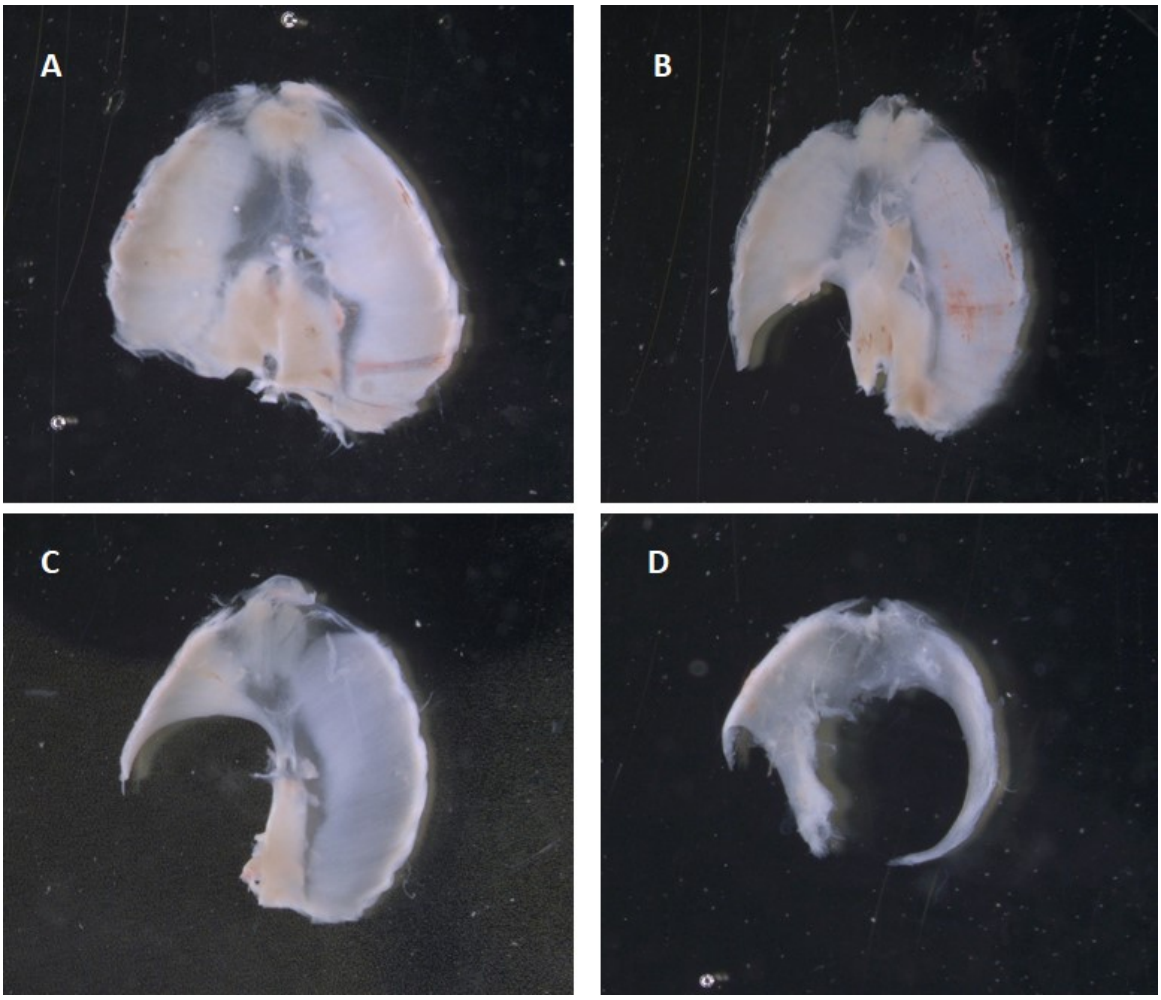

Figure 4.16 – Varying severity of CDH – Photographs of CDH of varying severities. A – Small left CDH. B- Medium left CDH. C- Large left CDH. D- Bilateral medium left and large right CDH.

With only a handful teratogen-based mouse models, establishing a teratogenic dose to induce an adequate number CDH without causing intrauterine death, was the first step in exploring this hypothesis. Babiuk *et al.* utilized a combination of 14.5 mg of Nitrofen and 14.5 mg of Bisdiamine to induce CDH in *C-met*$^{-/-}$ mice (102). In this experiment however, weight of the mouse was not taken into consideration whereas we normalized the teratogenic dose to maternal body weight. We also carried out our experiments using BALB/c mice, which are within the same species, but the strain and lack of transgenic manipulation differed, prompting a need for revision of the dose used. Even though a teratogenic rat model has been well established, inducing CDH in mice using teratogens opens doors to the use of this model in future transgenic experiments which can not be easily done in rats. As genetics has been implicated in the etiology of CDH, having the ability to alter the genetic make up of animals will aid in exploration of this etiology further.

To establish the necessary amount of teratogen, three phases of varied doses were carried out. The first phase began with animals on a breeder chow diet receiving teratogenic combinations of 0.3 mg/kg and 0.4 mg/kg of both Nitrofen and Bisdiamine. At this concentration, there was no CDH seen in the offspring of mice. When increased to 0.5 mg/kg of each teratogen, an incidence of approximately 30% CDH was recorded with only 22% resorptions. To be sure this was the best dose, it was further changed to 0.6 mg/kg of Nitrofen and Bisdiamine which led to 65% resorptions and ~72% CDH. This increase would not be feasible as not only the incidence of CDH was thought to be too high to see any differences when applied to the various diets, but the resorption rate was also too high to get a reasonable number of fetuses to evaluate. Hence, the dose of 0.5 mg/kg of Nitrofen and Bisdiamine was chosen as the possible amount for further use.

Phase two of establishing the teratogenic dose involved administering the chosen amount of 0.5 mg/kg of Nitrofen and Bisdiamine to mice fed the purified diets of varied vitamin A content. This trial yielded an unexpectedly high and unworkable number of resorptions (~90%). It was proposed that the change in diet was responsible for the increase in the number of resorptions. As noted above, the percentage of caloric intake obtained from fat is 21.6% in breeder chow, compared to 10% found in the purified diets. This led to the third phase of establishing the teratogenic dose. With the knowledge that Bisdiamine is an abortifacient in rats (121), this component was titrated down to 0.125 mg/kg with 0.5 mg/kg of Nitrofen and tested in animals fed a regular diet chow which had a similar composition to that of the purified chow. With this, an

incidence of approximately 50% of CDH was recorded with 37% resorptions. This incidence and number of resorptions was decided to be adequate to test our hypothesis. CDH in half of the population was a good incidence as it allowed room to increase in offspring of mice who had a marginal vitamin A status and decrease in offspring of mice with an excess vitamin A status.

The second goal in this series of experiments was to alter the vitamin A status of female mice through dietary manipulation. This was accomplished by separating the animals into three groups at weaning and feeding them a VAD, VAS or VAX diet. The liver is the main storage site for vitamin A in the body (8), and when the livers of these mice placed on special diets were tested it reflected their dietary changes. The livers of mice fed a VAD diet had trace amounts of retinol and retinyl esters present. The marginal vitamin A status of these animals, even after being fed a VAD diet since weaning, can be attributed to small amounts of vitamin A from the mother's milk acquired in the first few weeks of life. The livers of animals fed a diet with excess vitamin A (25 IU/g), showed an increased amount of retinol and retinyl esters when compared to that of the animals on a sufficient and deficient vitamin A diet. Interestingly, a difference was seen in the plasma levels between the mice on a deficient diet and the mice on an excess diet. Circulating plasma retinol levels are usually unaffected by changes in dietary content as retinol levels are maintained by the liver even when dietary intake is deficient. This is a method of preservation ensuring that tissues are not affected by fall in dietary vitamin A levels. Circulating levels of retinol will fall only when hepatic vitamin A levels are completely depleted (76). Overall, this data shows we successfully created three groups of mice with varying vitamin A status by manipulating dietary vitamin A intake. It is clear that the VAD diet group, even though fed a diet deficient of vitamin A, and have a lower hepatic and circulating levels of retinol, the animals have a marginal vitamin A status and are not completely vitamin A deficient. The VAX diet group has an excess of vitamin A when compared to the other two groups. We have demonstrated that consuming excess amount of dietary vitamin A (25 IU/g) causes accumulation of vitamin A in the liver. Note, this level of vitamin A is still markedly lower than the dose of 16,000 IU and 25,000 IU of vitamin A delivered to rescue CDH in previous experiments (11, 93). Thus, although we are feeding animals excess amounts of dietary vitamin A, it is several orders of magnitude lower than previous interventions that used supraphysiological doses of vitamin A.

To further characterize the alteration of vitamin A status caused by dietary modification, the expression of genes involved in retinoid signaling were measured. *Cyp26a1* and *Cyp26b1* are both responsible for oxidation of retinoic acid for elimination. As expected, they were both elevated in mice fed a vitamin A excess diet when compared to those fed a vitamin A deficient diet. *Lrat* is responsible for the esterification of retinol in the liver and extrahepatic tissues. When compared to animals fed a sufficient and excess diet of vitamin A, mice on a vitamin A deficient diet had less *Lrat* present. This change shows that the animals are responding to the change in dietary vitamin A and are making the necessary adjustments at a cellular level. Lastly, the levels of *Rbp* were measured and the changes in levels of *Rbp* between diets was insignificant. This would lead us to believe that the level of plasma retinol, while variation in deficient and excess diets were statistically significant, it is not enough to alter the amount of *Rbp*. Perhaps, if the animals fed the vitamin A deficient diet were truly vitamin A deficient, the levels of *Rbp*, would have decreased further.

During development, the fetus relies solely on the mother's supply of vitamin A. To further test the effect of dietary manipulation, the vitamin A levels of the fetuses for each diet group was measured. Evaluating the retinol and retinyl ester levels in offspring of mice fed various vitamin A diets, revealed that the level of hepatic retinol and retinyl esters mirrored those of their mothers. Offspring of mice fed a vitamin A deficient diet had little retinol and retinyl ester in their liver and was significantly lower when compared to those offspring of mice fed a vitamin A excess diet. Previous studies have suggested that during pregnancy, the nutritional requirements of the developing embryo are met before that of the mother's, and even in deficient states, vitamin A is still transferred to the fetus (111). However, the vitamin A deficiency is still seen in offspring of the mice on a VAD diet. This shows that dietary manipulation of maternal vitamin A status also affects the developing embryo and can be used to explain the impact of teratogen on mice fed varied vitamin A diets.

As vitamin A is an essential nutrient for day to day functioning, and establishing the dietary model involves altering the optimal vitamin A levels in mice, we needed to be sure that the health status of the animals was not affected. Weekly monitoring of the mice showed that all animals were bright, active and responsive with no overt changes between groups. In addition, the animals were weighed weekly from day of weaning until 16 weeks old and the progression of weight gain

did not vary from group to group. This confirmed that the health status of the animals was not overtly affected as a result of the dietary vitamin A manipulation.

One of the experiments that contributed to the formulation of the Retinoid Hypothesis showed that animals fed a VAD diet developed CDH amongst other congenital abnormalities (11). To ensure that the CDH we observed in our experiments was solely due to the teratogen exposure and not because of the dietary manipulation, we evaluated offspring of mice fed varied vitamin A diets. Of the 6 litters fed a VAD diet, none of the offspring exhibited CDH, similarly, for the 6 litters fed a VAS diet and the 7 litters fed a VAX diet. These findings underscore the fact that although the mice were fed a diet deficient of vitamin A, they have a marginal vitamin A status and are not completely deficient of vitamin A. This study is the first of its kind to measure the vitamin A status of the animals after dietary manipulation. In addition, this experiment is utilizing a mouse model while previous dietary manipulation was done in rats. These control animals successfully showed that dietary manipulation alone in mice does not cause CDH

In summary, dietary manipulation of vitamin A content can influence the vitamin A status of mice and their offspring effectively without affecting the overall health of the mouse. This is reflected in the hepatic levels of vitamin A in the animals as they mirror the vitamin A content in the diet and comparable weight progression between groups fed varied vitamin A diets. Furthermore, gene expression of retinoid metabolizing enzymes is altered reflecting the changes in the diet. Additionally, fetal vitamin A levels are impacted by dietary manipulation with hepatic retinol and retinyl ester levels in offspring showing changes similar to that of the mother, reflecting the level of vitamin A in the maternal diet. Lastly, manipulation of dietary vitamin A content alone does not induce CDH in offspring.

Having established the optimal teratogenic dose to induce CDH and developed a protocol to breed mice with differing vitamin A status, the final stage in testing the research question on whether maternal vitamin A status impacts susceptibility of teratogen induced CDH in offspring was to administer the teratogen to the three groups of mice fed the varied diets. All the female mice were plug tested after 3 months of being on their specific diets. The average maternal age differed significantly between the VAD diet group and the VAS and VAX diet groups. This can be explained by the low levels of vitamin A in the mice as Clagett-Dame and Knuston in 2011,

concluded that vitamin A deficiency in females affects reproductive capacity. Previous experiments have pointed to deficiency causing total reproductive failure due to insult before implantation occurs, fetal resorptions or fetal malformations (81). Although plug testing in all three dietary groups began at the same time it was evident that the VAD diet group took a longer time to become pregnant which can possibly mean that the animals were affected by the marginal vitamin A status before implantation occurred. As previously mentioned, in humans, increased maternal age has been associated with an increased incidence CDH in offspring (63). In addition to low vitamin A status, it can be proposed that increased incidence of CDH in this study can be attributed to advanced maternal age in the mice fed a vitamin A deficient diet. This possibility leads to future experiments that will investigate solely the effect of maternal age on incidence of teratogen induced CDH.

Before the fetuses were dissected, their CRL was measured. There was no significant difference observed in the average CRL between diet groups. This confirms that all fetuses were collected on the same day of gestation and there was no variation in fetal age between groups. Similar to CRL, the average litter size and incidence of resorptions between groups was comparable with no significant differences. Despite the increased maternal age of the VAD diet group, this shows that the marginal vitamin A status did not affect litter size or resorption rate. This supports the theory that the low vitamin A status affected the fertility before implantation.

The main aim of this research question was to compare the incidence and severity of teratogen induced CDH in offspring of mice with altered vitamin A status. We have shown that offspring of animals fed a diet deficient of vitamin A exposed to teratogen have a ~25% greater incidence of CDH when compared to offspring of animals fed a vitamin A excess diet. The incidence observed between VAD and VAS groups was insignificant, as was with the difference between the VAS and VAX groups, which can be attributed to the fact that the VAD and VAX groups have the largest difference in vitamin A status. This is in line with our proposed outcome with offspring of mice on a VAD diet being more susceptible to the actions of the teratogen. Also, excess vitamin A in the diet acts as a protective factor against the teratogen. We can also conclude that as fetal vitamin A status is also affected by maternal dietary manipulation, low fetal vitamin A status of the offspring makes it more susceptible to perturbations in RA signaling and higher vitamin A status acts as a protection against any disturbances. This reasoning is supported by the

previously mentioned studies in both humans and animals where a supraphysiological dose rescued offspring of rats exposed to teratogen and cord blood analysis of infants born with CDH had decreased levels of retinol (18,94). With regards to severity, there was no difference in size of defect between the groups with each group having a comparable number of small, medium and large hernias.

In addition to studying the incidence of CDH, we also analyzed the occurrence of CP and OE. While comparing CP incidence between dietary groups was not a primary goal of this experiment, a significant difference was observed between the offspring of animals fed a VAD diet and those fed a VAX diet. CP has previously been linked to vitamin A status and it has been shown that adequate RA can reduce the risk of CP (110). In one population study it was shown that high maternal intake of vitamin A during pregnancy had a protective association for CP (111). Our findings of CP in the teratogen treated mice are in line with previous studies as it is known that Nitrofen and Bisdiamine affect the production of RA. In addition, the increased incidence of CP in offspring of mice fed a VAD diet and decreased incidence in those fed a VAX diet, further supports the efficiency of this animal model and its ability to alter vitamin A status in mice. In our control population, no CP was observed, underscoring the fact that CP in offspring was solely as a result of teratogen exposure. Similar to CP, observance of OE was not a primary goal of this experiment and no significant difference was seen between dietary groups. In human cases of fetal thoracic anomalies, OE is attributed to obstruction of venous return to the heart increasing venous pressures (124). In our mouse model, in data not presented, all of the offspring presenting with CDH had herniation of the liver into the thoracic cavity, thus potentially causing obstruction of venous return to the heart leading to the OE seen in the offspring.

In conclusion, maternal vitamin A status impacts the susceptibility of teratogen induced CDH in offspring. We have shown that vitamin A status can be changed by manipulation of dietary vitamin A levels and concluded that a combination of 0.5 mg/kg of Nitrofen and 0.125 mg/kg of Bidsiame is needed to induce an adequate number of CDH without an overwhelming number of resorptions in the mouse model of teratogen induced CDH. We have shown that mice with a marginal vitamin A status have a ~25% higher incidence than those fed a VAX diet.

Chapter 5: Retinoic acid signaling and Congenital Diaphragmatic Hernia

5.1 Introduction

The Retinoid Hypothesis states that abnormal retinoid signaling is responsible for the formation of CDH during development (17). For many years, teratogenic animal models have been used to test this hypothesis. It has also been shown *in vitro* that CDH-inducing teratogens have an inhibitory effect on retinaldehyde dehydrogenase 2, which is needed to produce RA from retinol (13), and that teratogen-exposed embryos have decreased RA levels when compared to control embryos (20). In addition, teratogen exposure decreases markers of RA signaling (90). Even though these studies have been helpful in understanding the impact of CDH-inducing teratogens on RA homeostasis, there are still many unknowns regarding embryonic RA metabolism and downstream signaling in the developing diaphragm, and how problems in RA signalling lead to CDH. With most of the studies focusing on the specific effect on gene expression in the lung, there is a lack of information on the effect on the diaphragm. Specific effects such as gene expression during the critical period of development following teratogen exposure have yet to be explored. We proposed that exposure to CDH-inducing teratogens would change the mRNA expression levels of genes involved in embryonic RA signaling and genes that have been implicated previously in the development of CDH, providing insight into how altered RA metabolism leads to CDH. To explore this research question, timed-pregnant mice received a teratogenic combination of Nitrofen and Bisdiamine, 24 hours later offspring were harvested, and gene expression levels were analyzed. By highlighting the effect that these teratogens have on gene expression we would give a fuller understanding of the underlying mechanisms that govern the formation of CDH in infants.

5.2 Experimental Design and Methods

Timed pregnancy, teratogen administration, RNA extraction, cDNA synthesis and qPCR were carried out as described in Chapter 3.

5.2.1 Tissue Collection

On day E9.5 mice were euthanized by isoflurane inhalation (5% Isoflurane delivered in 1L/M of O_2). Once animals reached surgical plane anesthesia, they underwent cervical dislocation. Next, an abdominal incision was made and the entire uterus was removed and placed in ice-cold RNA*later* Stabilization Solution (Invitrogen, ThermoFisher Scientific), prior to further dissection.

Using a stereomicroscope (Stemi 508, Zeiss, Oberkochen, Germany) the isolated uterus was dissected and the number of fetuses and any resorptions present were recorded. Individual fetuses were then separated from the uterus one at a time and were snap frozen using liquid nitrogen, and held at -80°C until further use.

5.3. Results -Effect of teratogen exposure on gene expression

For this experiment, timed-pregnant mice were treated with teratogenic dose of 0.5 mg/kg of Nitrofen and 0.125 mg/kg of Bisdiamine and whole embryos were collected 24 hours later. The embryos were analyzed by qPCR for gene expression levels. RA associated genes were grouped into categories based on their role in vitamin A metabolism. The RA catabolizing enzymes, *Cyp26a1, Cyp26b1* and *Cyp26c1,* showed no significant difference between the control group and the group exposed to the teratogen (Figure 5.1). The retinoic acid receptors, *Rara, Rarb,* and *Rarg,* were also measured and between them *Rarb* was was the only one to show a statistically significant difference between the control group and teratogen exposed group (Figure 5.2). The group exposed to the teratogen was expressed at a lower level than those in the control group. Next, gene expression of the RA metabolizing enzymes, *Lrat, Raldh2, Rdh10* and *Dhrs3* were analyzed. It was seen that there were no significant changes between the control group and teratogen exposed groups for all of the enzymes (Figure 5.3). Next, expression of genes responsible for cellular retinoid binding and uptake were measured (Figure 5.4). This included, *Stra6, Crbp1, Crbp2* and *Crbp3. Stra6* was the only gene to exhibit a difference between the control group and the teratogen exposed group. The group exposed to teratogen had significantly lower gene expression when compared to the control group. Lastly, expression of CDH associated genes was analyzed. These genes included *Wt1, Gata4, Fog2,* and *Coup-tfII.* No significant change was noted in any of the genes (Figure 5.5).

5.4 Discussion

As previously mentioned, Nitrofen and Bisdiamine have been identified as inhibiting *Raldh2* when administered to pregnant dams (39). With this knowledge we investigated the genes involved in RA signaling to get a better picture of the effect of these teratogens. When teratogen is given, it was expected that there would be a decrease in the RA catabolizing enzymes as the cell will have less RA from decreased action of *Raldh2.* However, from our studies no change was

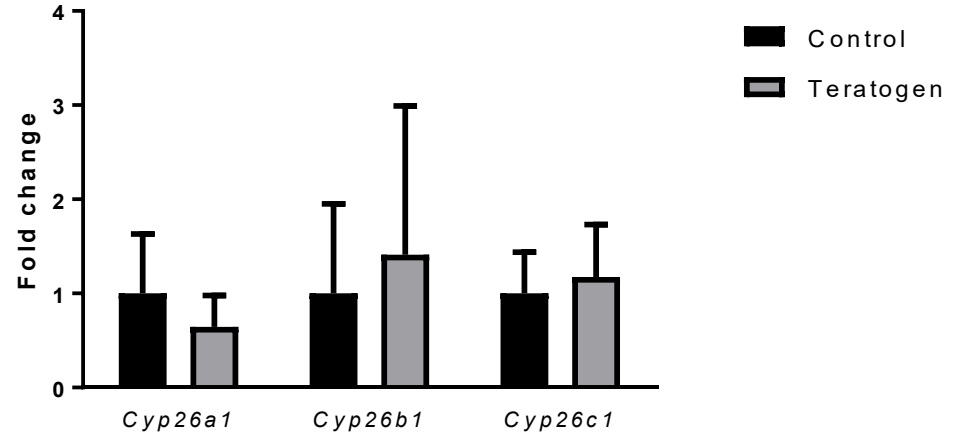

Figure 5.1- Effect of teratogen on gene expression of RA catabolizing enzymes - Effect of teratogen on gene expression of RA catabolizing enzymes, *Cyp26a1, Cyp26b1,* and *Cyp26c1.* Data presented as mean ± SD. Analysis performed by Student's t-test.

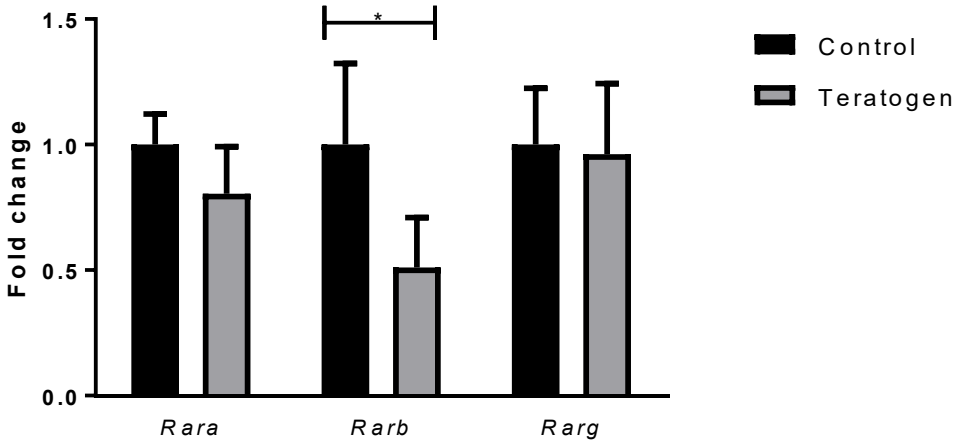

Figure 5.2- Effect of teratogen on gene expression of RA receptors- Effect of teratogen on gene expression of RA receptors, *RARa, RARb and RARg.* Data presented as mean ± SD. Analysis performed by Student's t-test. Columns with different letters are significantly different.

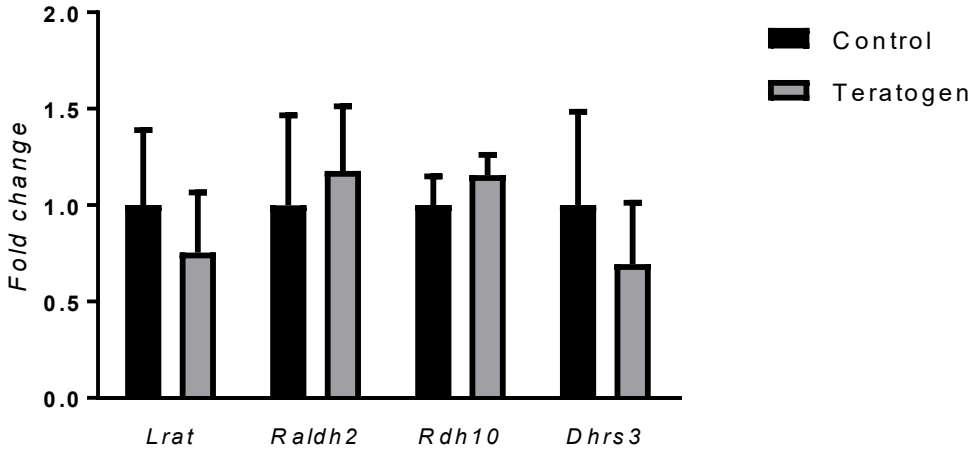

Figure 5.3- Effect of teratogen on gene expression of RA metabolizing enzymes- Effect of teratogen on gene expression of metabolizing enzymes, *Lrat, Raldh2, Rdh10 and Dhrs3.* Data presented as mean ± SD. Analysis performed by Student's t-test.

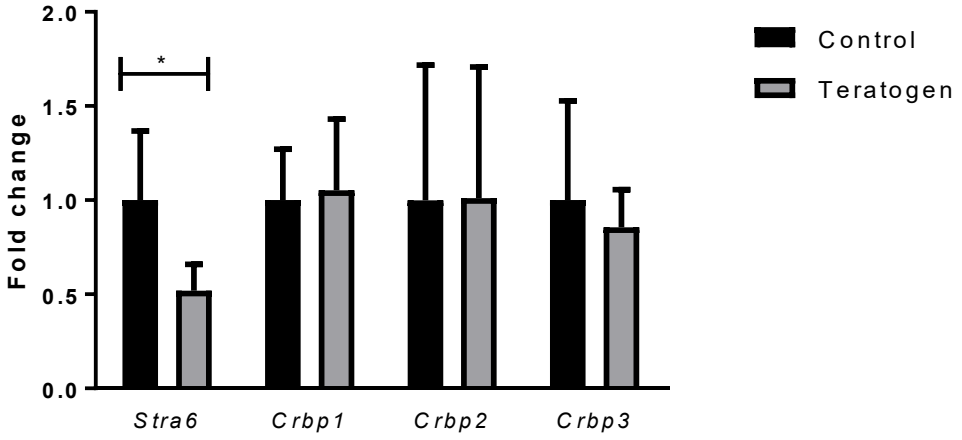

Figure 5.4- Effect of teratogen on expression of RA binding and uptake genes Effect of teratogen on expression of RA binding and uptake genes, *Stra6, Crbp1, Crbp2 and Crbp3.* Data presented as mean ± SD. Analysis performed by Student's t-test.

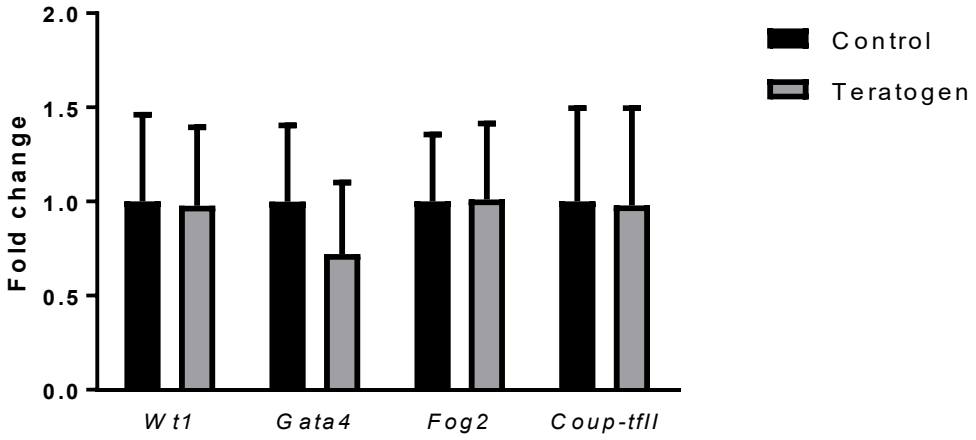

Figure 5.5- Effect of teratogen on expression of CDH associated genes- Effect of teratogen on expression of CDH Associated genes, *Wt1, Gata4, Fog2, Coup-tfII.* Data presented as mean ± SD. Analysis performed by Student's t-test.

observed in *Cyp26a1, Cyp26b1* or *Cyp26c1*. It was also expected that RAR expression will decrease as these are RA responsive genes. In this study, of the three RARs evaluated, *Rarb* is the only one that showed a significant difference. The teratogen exposed group had a ~50% decrease in expression when compared to the control group. Interestingly, in an experiment conducted by Mendelsohn *et al*, investigating the effect of knocking out the different RARs in mice, it was seen that *Rar*$\alpha^{-/-}$:*Rar*$\beta2^{-/-}$ and *Rar*$\alpha^{-/-}$:*Rar*$\beta2^{+/-}$ compound mutants were the only knockouts that exhibited CDH and in unpublished data RARβ2 was expressed in the diaphragm at a critical period in development, suggesting that it is an important player in diaphragm formation (88). Our observed change in *Rarb* is in line with this concept, as it would explain why it is the only RAR to be affected by teratogen exposure.

With regards to *Lrat*, a decrease was expected when exposed to teratogen as it is responsible for conversion of retinol to retinyl esters in the cell. As the teratogen should increase the need for retinol in the cell, a subsequent decrease in *Lrat* is expected. In our studies no change was observed after exposure to teratogen. An increase in *Raldh2, Rdh10* and *Dhrs3* was expected in the embryos exposed to teratogens as the cell is lacking RA and these three enzymes are responsible for the production of RA. Similar to *Lrat,* no change was seen in expression of any of these genes after exposure to teratogen. For the retinoid binding and uptake proteins, *Stra6, Crbp1, Crbp2* and *Crbp3,* after exposure to teratogen, a decrease was expected as these genes are RA responsive. After teratogen exposure, *Stra6* was the only gene in this group to show a difference. The teratogen exposed group had a ~50% decrease in gene expression when compared to the control group. Interestingly, *Stra6* has been previously implicated in human cases of CDH, specifically Matthew-Wood Syndrome (128). When exposed to teratogen, a decrease in CDH associated genes, *Wt1, Gata4, Fog2,* or *Coup-tfII,* was expected as it has been shown that perturbations in the expression of these genes have lead to CDH in infants (36). From our studies, no significant change was observed between teratogen exposed group and the controls.

This experiment has shown that 24 hours after teratogen exposure, offspring exhibit a change in expression of *Rarb* and *Stra6*. No other genes analyzed showed a significant change. These two genes have a strong association with CDH in both human cases and animal studies. As previously mentioned Matthew-Wood Syndrome, also known as Microphthalmic **s**yndrome 9, has been associated with mutations in *Stra6.* It is characterized by pulmonary hypoplasia/agenesis, diaphragmatic hernia/eventration, anophthalmia/microphthalmia, and cardiac defects (PDAC

syndrome). Pasutto *et al.* reported homozygous mutations in *Stra6* in infants exhibiting CDH (56). Chassaing *et al.*, noted that *Stra6* was also associated with syndromic CDH and pointed out the need for further studies looking into the association of CDH and this mutation (129). Interestingly, *Rarb* has also been associated with this specific syndrome. It has been found that both recessive and dominant mutations in *Rarb* affect function of *Rarb* in the context of the syndrome (130). Furthermore, *Rarb* was shown to be expressed in its highest level in the primordial diaphragm when compared to the mature diaphragm (109). This is in line with the previously cited work of Mendelsohn *et al.* where it was shown that knock out of *Rarb* produced CDH, leading the authors to speculate that it played a significant role in diaphragm development (88). Interestingly, when Clugston *et al*. investigated the presence of the different RAR isotypes in the developing diaphragm, RARa and RARy were the only ones found in the cells of the pleuro-peritoneal fold (90). The differences in results of these studies highlights the need for further clarification of which genes are expressed in the developing diaphragm, and by extension, genes responsible for formation of CDH.

As previously stated, Nitrofen and Bisdiamine are known to decrease RA by inhibiting the action of retinaldehyde dehydrogenase 2 (13). Decreases in *Rarb* and *Stra6* provides further evidence of decreased RA as both genes are highly RA responsive. It must be noted that while the expression of these genes was altered, other RA responsive genes studied such as *Cyp26a1, b1* and *c1,* were not affected after teratogen administration. The changes in *Rarb* and *Stra6* suggest altered RA signaling, however the lack of changes in other RA responsive genes argues against a change. This calls to attention the need for further research on the genes involved in RA metabolism and their role in the developing diaphragm. Further, it remains unknown what the downstream effects of these changes are in terms of CDH in development and morphogenesis. Alterations in these genes can have effects on a range of morphogenic processes in the developing diaphragm including cell proliferation, migration and apoptosis. Investigating these effects are imperative for understanding the mechanisms that govern the etiology of CDH.

It must be noted that there are two main limitations to this experiment. The first being that we evaluated whole embryos and not the developing diaphragm specifically. At gestational day 8.5, the diaphragm is not fully formed and the teratogens target the embryonic cells that become the diaphragm (61). The teratogen affects specific gene expression in those early diaphragmatic embryonic structures. Any effects to these genes however may be obscured by the gene expression

from other structures from the whole embryo. Second, from our previous experiment, it is documented that ~50% of offspring exposed to the teratogen exhibit CDH. If we take this into consideration, only half of the embryos tested from each group would be expected to develop CDH during development, thus masking potential changes in gene expression.

Many of the RA target genes that we expected to change were unaffected by teratogen exposure. An explanation for this result could be that 24 hours after teratogen exposure is not adequate time to observe a response in gene expression. The 24-hour time point was chosen as gestational day 8.5-9.5 has been identified as the critical period in which the primordial diaphragm is beginning to form. We propose for future experiments to extend this time to 48 hours to allow for any gene expression differences that may take longer to be manifest to be measured.

In conclusion, teratogen exposure causes a ~50% decrease in expression of two genes, *Rarb* and *Stra6*, which have well-established links to CDH in humans and mice. Thus, it seems exposure to teratogen triggers a change in the RA signaling pathway. This data helps to shed more light on the specific effects of Nitrofen and Bisdiamine on the RA signaling pathway and associated genes. Future plans involve prolonging the length of time between which the teratogen is given and gene expression is measured and increasing the number of offspring evaluated.

Chapter 6 - Conclusion and Future Directions

6.1 Introduction

The importance of vitamin A metabolism in the formation of CDH has been an ongoing area of research for over 70 years. This thesis set out to investigate the Retinoid Hypothesis, which states that abnormal retinoid signaling underlies the pathogenic mechanisms that govern the formation of CDH (17). We have successfully investigated two aspects of this hypothesis. In our first research question we explored the effect of maternal vitamin A status on the incidence of teratogen induced CDH. The second research goal took a further look into the specific effects of the teratogens Nitrofen and Bisdiamine on expression of retinoid signalling genes and CDH-associated genes. This present study has led us to further support the Retinoid Hypothesis and highlights further experiments that can be carried out to add to a fuller understanding of the etiology of CDH.

6.2 Discussion of Results

While investigating the first research question of whether maternal vitamin A status affected the susceptibility of teratogen induced CDH, we were able to achieve our three main goals. First, we established the optimal dose of teratogen to induce CDH in mice without causing too many intrauterine deaths. Second, we accomplished altering maternal vitamin A status by dietary manipulation. This was verified by measuring the retinoid levels of three groups of animals and their offspring fed VAD, VAS and VAX diets, as well as the expression of genes involved in vitamin A metabolism. Lastly, we were able to administer the teratogen to the three groups of mice on varied diets and observe the incidence and severity of CDH in offspring. We noted that the incidence of CDH is increased in animals fed a VAD diet and decreased in animals fed a VAX diet. Thus, we concluded that lower maternal vitamin A status increases susceptibility to teratogen induced CDH as the fetuses themselves have a lower vitamin A status as well, and higher maternal vitamin A levels decreases susceptibility of teratogen induced CDH as higher vitamin A levels act as a protection against teratogen-induced changes in RA signaling.

For our second research question, we utilized the teratogenic dose known to cause CDH from our first research question and measured the effect of teratogen exposure on gene expression in offspring 24 hours after teratogen administration. A 50% decrease in gene expression of *Stra6* an *Rarb* was observed in animals exposed to teratogen when compared to control animals. These two genes are known to be retinoic acid responsive and have been implicated in CDH in both

human and animal studies. Changes in their expression shows that more work needs to be done to explore the exact mechanisms by which these genes are involved in diaphragm development, including the downstream signaling that affects the morphogenesis and maturation of the diaphragm.

6.3 Limitations

Investigating the two research questions was not without limitations. As previously mentioned, maternal age in the animals fed a diet deficient of vitamin A was significantly increased when compared to those on a VAS and VAX diet. As advanced maternal age has been associated with increased risk of CDH in infants, one limitation of this study is knowing whether the increased incidence of CDH in offspring of animals fed a VAD diet can be attributed to the lack of vitamin A or to the maternal age (63). This compounding variable highlights the need for further research that separates the dietary modulation and effect of age on CDH.

In addition to maternal age, it is possible that VAD animals experienced more early embryonic deaths that were not observed during dissection, thus resorption rate and incidence of CDH could be greater than that recorded. Assessing the fetuses earlier in gestation may address this limitation and should be considered for future experiments. Another limitation was the number of experimental animals used to arrive at our conclusions. It is possible that no statistically significant change was observed in mice fed a VAS diet and those fed a VAD and VAX diet because of lack of statistical power. Similarly, no change in gene expression while investigating our second research question could be attributed in part to only one litter being used per group. Increasing the sample size in both these instances may positively alter the results obtained. As previously mentioned in Chapter 5, additional limitations existed while investigating the impact of Nitrofen and Bisdiamine on gene expression of RA related genes. These limitations included testing of the whole embryo and not the diaphragm specifically, acknowledging that approximately 50% of embryos exhibit CDH, and the need to further ascertain whether all the genes investigated are expressed in the diaphragm.

6.4 Future Directions

During our time investigating these two research questions, a third hypothesis arose. We questioned whether functional inhibition of retinoic acid signalling in the diaphragm causes CDH.

To investigate this theory, we utilized a double transgenic mouse approach, essentially knocking out *Wt1* specifically in the developing diaphragm. *Wt1* has been previously shown to be expressed in the developing diaphragm and implicated in CDH in both human and animal studies (68,95). Figure 6.1 shows the presence of *Wt1* in the early stages of diaphragm development. These double transgenic mice expressed a dominant negative retinoic acid receptor (RARdn) specifically under control of a tamoxifen-inducible Cre recombinase driven by the *Wt1* promoter (*Wt1CreERT2*). Figure 6.2 details the experimental design of this hypothesis. This approach allowed for specifically blocking retinoic acid signalling in the cells of the developing diaphragm, during the critical period of diaphragm development. To induce the expression of the *Wt1CreERT2* in timed-pregnant females, Tamoxifen was given at gestational day 8.5. Parents and offspring were genotyped to confirm the presence of both the *Rardn* and *Wt1CreERT2.* This experiment is ongoing as initial breeding of these transgenic mice has not been successful. In the future, we plan to optimize breeding of these animals and induce CDH in these double transgenic mice. In doing so, we can further support the importance of retinoid signalling in the development of CDH.

These studies above aid in working to find the mechanisms which govern the formation of CDH in infants. As vitamin A metabolism is quite dynamic involving multiple components including enzymes, receptors, activators and repressors, it would be imperative to further evaluate the complexities of the pathway and possible perturbations (82). Looking to the future, we plan to increase the number of dietary manipulated mice exposed to teratogen to expand the population of this study. Pertaining to dietary manipulation as well, we intend to investigate whether manipulating dietary vitamin A only during pregnancy has the same effect on incidence of CDH as dietary manipulation from the time of weaning. This will involve switching the diet of mice to a VAD, VAS or VAX diet immediately after plug testing instead of after weaning, exposing to teratogen and observing incidence of CDH. With regards to our gene expression work, we plan to increase our 24-hour time point to 48 hours after teratogen exposure to measure relative gene expression of offspring compared to control. In addition, we hope to investigate more genes that are involved in RA signaling such as *Dgat* and *Crabp's.* Lastly combining the transgenic mouse model and the dietary model, we will explore whether maternal vitamin A status alters susceptibility to CDH caused by functional inhibition of retinoic acid signaling. This means, once we successfully induce CDH in these double transgenic mice, we will establish VAD, VAS and VAX diet groups.

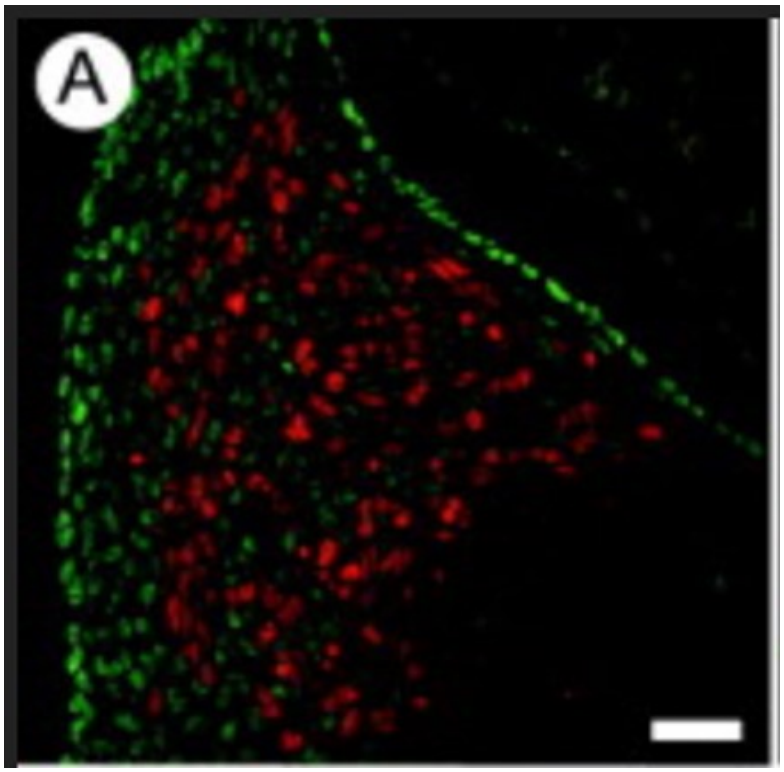

Figure 6.1 - Representation of *Wt1* in the developing diaphragm. Representative immunohistochemical staining within the developing PPF of rats at E13.5 at ×20 magnification. **A:** Labeling for wt1 (green) Adapted from "Teratogen-Induced, Dietary and Genetic Models of Congenital Diaphragmatic Hernia Share a Common Mechanism of Pathogenesis." Clugston *et al* 2006 (25)

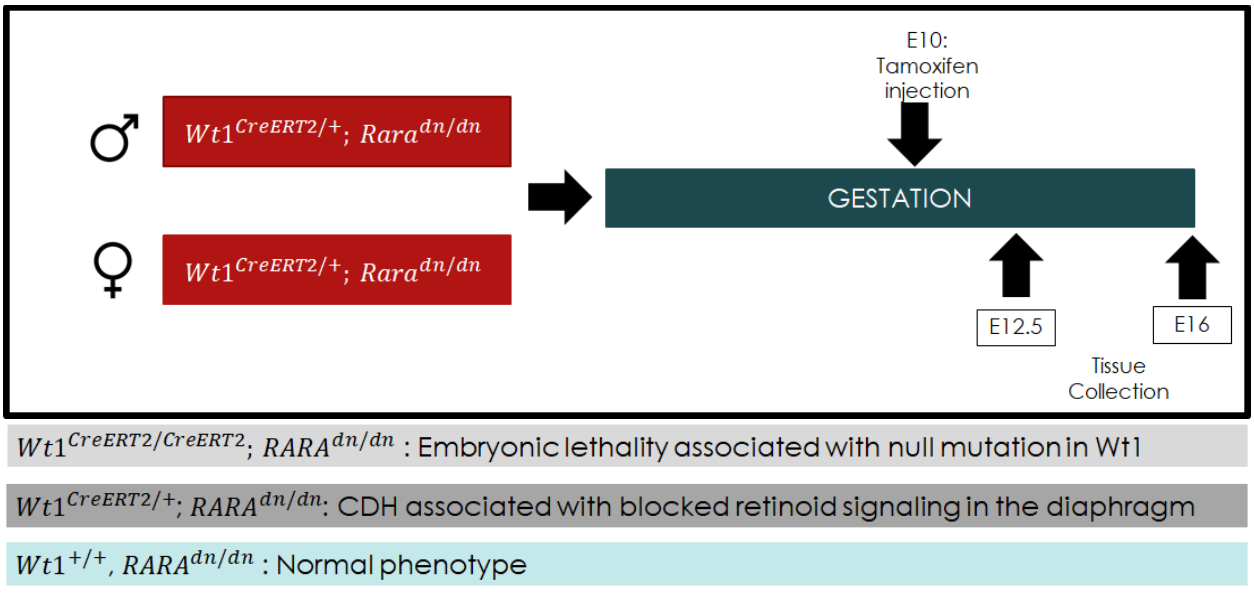

Figure 6.2 Experimental design for double transgenic mouse model.

6.5 Clinical Relevance

In Canada, the annual healthcare cost associated with CDH is estimated at $10 million (126). This figure, in addition to the high mortality and morbidity rates highlight why the need for this research is important. This thesis serves to add to the existing body of work centered around the Retinoid Hypothesis. By investigating the role of maternal vitamin A status on teratogen-induced CDH we have strengthened the theory that maternal vitamin A status impacts the formation of the defect. Recently it has been shown that in humans, mothers with lower vitamin A intake during the first trimester of pregnancy had a higher incidence of CDH (125). By combining the dietary model with the teratogenic model, we have supported the fact that abnormal retinoid signaling induces CDH, and vitamin A status can influence the susceptibility of the effects caused by the abnormal signaling. In addition, we have highlighted two genes, *Stra6* and *Rarb*, as being altered by CDH inducing teratogens. While these genes have already been implicated in the formation of CDH, our research help support their involvement and underscores need for further research into the mechanism by which altered expression of these genes contribute to CDH. It can be assumed that adequate vitamin A intake during the first trimester of pregnancy in humans could potentially lower the risk of CDH. An overall reduction in CDH can lead to significantly less burden on the healthcare system and an increase healthy live births within the population.

6.6 Overall conclusions

In conclusion, this thesis has successfully added support for the Retinoid Hypothesis in two ways. We demonstrated that: 1) marginal vitamin A status increases the risk for teratogen-induced CDH, and 2) CDH-inducing teratogens affect the retinoid signaling pathway in the developing embryo. This work suggests further studies that can be carried out to get a better understanding of the correlation between vitamin A and the formation of CDH.

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Appendix

This comment was published in Early Human Development.
Rocke, Ayanna W. and Robin D. Clugston. 2018. *Comment on "Lung and Liver Growth and Retinoic Acid Status in Human Fetuses with Congenital Diaphragmatic Hernia"*. Vol. 116.

Dear Sir,

We read with interest the recent article by Loo and colleagues titled "Lung and Liver growth and retinoic acid status in human fetuses with congenital diaphragmatic hernia" [1]. The notion that altered retinoic acid signaling contributes to the development of CDH is an intriguing hypothesis that certainly requires rigorous testing. Indeed, we commend the authors for adding data derived from human cases of CDH in support of this hypothesis, which is largely founded on data obtained from animal models. The major conclusion of the article by Loo *et al*. is that fetal retinoic acid stores influence lung and diaphragm growth in human fetuses with CDH [1]. As introduced by these authors, this conclusion rests upon the premise that hepatic stellate cells store retinoic acid, and that cellular retinol binding protein 1 (CRBP1) binds retinoic acid in these cells. Unfortunately, both these assertions are not supported by the conventional wisdom of the field. Retinoic acid is a potent transcriptional regulator, the cellular levels of which are tightly regulated – it is not stored in hepatic stellate cells, or any other tissue. Retinoic acid is synthesized from the sequential oxidation of retinol, and excess retinol can be stored in the form of retinyl ester [2]. Thus, while the authors were correct in stating that hepatic stellate cells are the primary storage site of vitamin A, it is in the form of retinyl ester not retinoic acid [3]. Moreover, CRBP1 has no affinity for retinoic acid, preferring to bind retinol [4]. The major cellular binding proteins for retinoic acid are called cellular retinoic acid binding proteins (CRABP), of which there are two major family members, CRABP1 and CRABP2 [4]. As such, the notion that CRBP1 binds retinoic acid is not supported by the literature. With these misconceptions in mind, the conclusions of the article by Loo *et al*. should be re-examined. Using CRBP1 as a marker of HSCs remains a valid approach, but caution should be used in interpreting these results in the context of fetal retinoic acid "status". The decreased number of CRBP1-positive hepatic stellate cells in the liver of fetuses with CDH (with liver herniation), is suggestive of altered vitamin A status. Similarly, the correlation between reduced lung weight and the number of CRBP1-positive hepatic stellate cells suggests the importance of fetal vitamin A status in the pulmonary outcomes of CDH, but these data should be

carefully interpreted in the context of retinoic acid availability. Further research is required to establish the relevance of altered retinoic acid signaling in human cases of CDH, the significance of which can only be realized when the data are placed within our current understanding of vitamin A metabolism and the retinoic acid signaling pathway.