# A Black Hole?

## Epidemiological and molecular biological studies on the etiology of Congenital Diaphragmatic Hernia

**L.W.J.E. Beurskens**

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**Een zwart gat?**
**Epidemiologische en moleculair biologische studies naar de etiologie van congenitale hernia diafragmatica**

**Proefschrift**

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*Ik heb nog zo veel muziek in mijn hoofd.*
*Ik heb niets gezegd, alles moet nog gezegd worden.*
Gustav Mahler

*Voor Geertje en Lucas*

# Table of Contents

# Chapter 1

General Introduction

## General Introduction and outline of this thesis

Congenital Diaphragmatic Hernia (CDH) is a life-threatening congenital anomaly. In children with CDH the diaphragm develops abnormally during the first weeks of pregnancy.[1, 2] This results in a defect in the diaphragm, compromising the natural partition between the thoracic and abdominal cavities and disturbing the formation and orientation of various organs.

The first description of a patient with CDH dates back to 1679 by Lazarus Riverius (1589-1655) as an incidental finding at autopsy of a 24-year old man.[3] Sir Charles Holt described the clinical and postmortem findings in the first paediatric patient.[3] Numerous authors have described patients with various types of diaphragm defects, including Giovanni Battista Morgagni (1682-1771) and Victor Alexander Bochdalek (1801-1883) to whom two types of diaphragm defects have been named.[4,5] Despite this long history of descriptions, the cause of CDH is largely unknown. Only in a minority of patients an identifiable genetic abnormality is present.[6]

The birth prevalence rate of CDH in the Netherlands is comparable to other countries worldwide and varies between 1 to 3 per 3000 live births. CDH is increasingly diagnosed prenatally, especially since the introduction of the standard ultrasound examination at 20 weeks of pregnancy.

Children with CDH are severely ill, mainly as a result of the associated under-development of the lungs (pulmonary hypoplasia) and increased vascular resistance (pulmonary hypertension). The pulmonary hypoplasia of variable extent and pulmonary vascular abnormalities are the main determinants of postnatal outcome. The mortality in CDH is high, but varies from 10-75% depending on case selection.[7] At 20 years of age, the survival rate is approximately 60%.[8] This number is relatively stable from the first month of life onwards, indicating that the risk of mortality is highest in the first days of life due to a failure of transition resulting in persistent hypoxia and a failure to decrease pulmonary vascular resistance and progressive damage of the lungs.

The treatment of patients with CDH focuses on stabilization of the patient after birth before the diaphragm defect is surgically closed. In 20-30% of CDH patients extra corporeal membrane oxygenation (ECMO) is needed to mainly control the pulmonary hypertension, depending on the ECMO availability and the “gut feeling” of treatment teams that ECMO may benefit individual patients. Two specialized centres in the Netherlands have ECMO facilities for newborns, i.e. Erasmus MC Rotterdam and Radboud University Medical Center Nijmegen. The availability of ECMO is one of the reasons that the care for CDH patients, both prenatally and postnatally is centered in these two university hospitals. The studies described in this thesis are conducted at Erasmus MC. Multidisciplinary teams consisting of various medical and paramedical specialists are involved in the care for the patient with CDH during pregnancy, at birth, during the stay in the hospital and after discharge. The high morbidity with severe pulmonary and gastro-intestinal problems can be a severe burden for the patient and his/her family.[9,10]

## Objectives of this thesis

In order to develop effective primary and secondary treatment options, we have to identify risk factors for CDH and understand the defective processes in lung development.
Therefore, the objectives of the studies described in this thesis are to investigate:

1) the associations between vitamin A and CDH and to identify related risk factors in humans using an epidemiological approach. These studies are described in part I.
2) normal and abnormal lung development in an experimental model for human lung growth and b) the role of the vitamin A pathway in human lung development in a variety of model systems. These studies are described in part II.

## Outline of this thesis

### Part I: Epidemiological studies

In Part I we provide a review of the current knowledge on the role of nutritional, lifestyle and genetic factors in human CDH (*Chapter 2*). The epidemiological studies described in part II are conducted within the HERNIA-study, which is an acronym for Congenital Diaphragmatic **H**ernia, **E**nvironment, **R**etinoids, **N**utrition, **I**nheritance and other **A**ssociations (figure 1.1). This case-control study was initiated to investigate the role of several risk factors related to vitamin A in the aetiology of CDH. The design is prospective with various entry and follow up moments in which three case-control studies are nested. Figure 1.2 shows the study design. In *Chapter 3* we describe the study on vitamin A and retinol-binding protein concentrations in newborn children and their mothers at delivery. The analysis of maternal dietary intake during pregnancy is described in *Chapter 4*. In *Chapter 5* we describe the study of concentrations of homocysteine, S-adenosyl methionine (SAM) and S-adenosyl homocysteine (SAH) in the newborn cord blood.

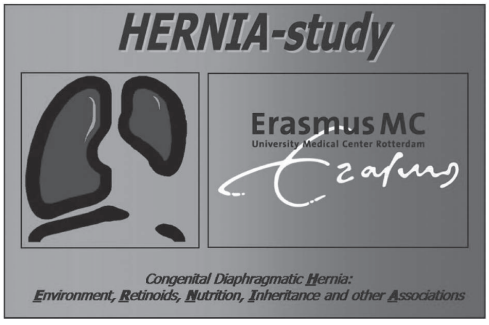


**Figure 1.1** | HERNIA study logo

**Part II: molecular biological studies**

In *Chapter 6* we provide an overview of the various animal models that have been used to study the embryology, genetics and treatment of CDH. The evidence from these animal models and human data has led to the formulation of the so-called retinoid hypothesis,[11] which states that retinoids (vitamin A derivatives) are involved in the aetiology of CDH and its associated pulmonary hypoplasia. In *Chapter 7* we describe the establishment of a human foetal lung explant model which is used to study the effect of oxygen on hypoxia-inducible factors in normal human lung tissue. In *Chapter 8* we describe our study on the expression of genes of the vitamin A pathway in human lung tissue, a rabbit model and a rat model of CDH.

In the general discussion (*Chapter 9*) the results of the studies are discussed and placed in a broader perspective. Further, we hypothesize on the possible mechanisms, treatment options and requirements of future research.

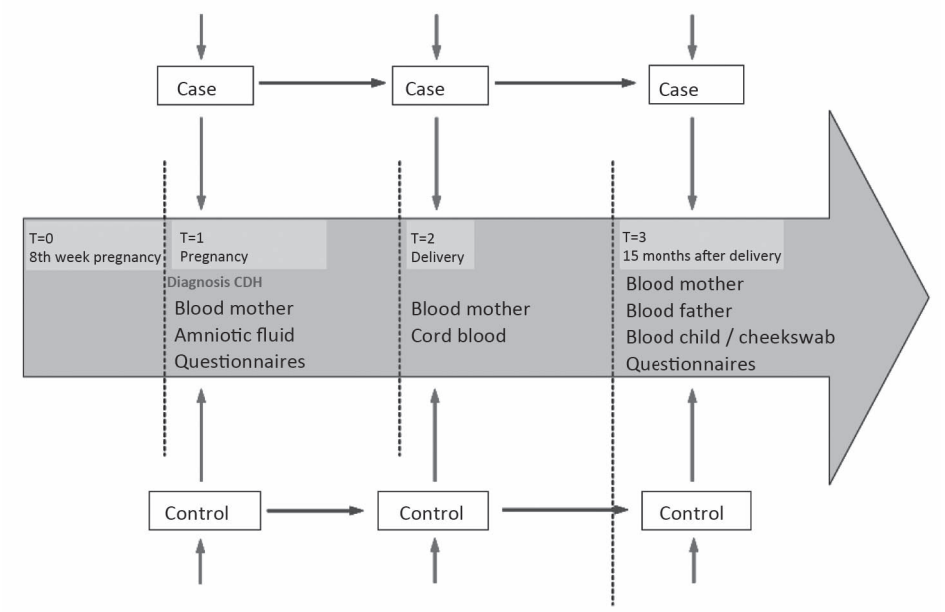


**Figure 1.2** | HERNIA study design

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Part

I

# Epidemiological studies

# Chapter 2

## The role of nutrition, lifestyle factors, and genes in the pathogenesis of congenital diaphragmatic hernia: human and animal studies

*LWJE Beurskens | D Tibboel | RPM Steegers-Theunissen*

*Nutrition Reviews* 2009; 67(12):719-730

## Abstract

Congenital Diaphragmatic Hernia (CDH) is a severe malformation with a largely unknown pathogenesis. Because an unequivocal genetic relation is diagnosed only in a minority of patients, the involvement of multiple genetic and environmental factors is suggested. Although periconceptional environmental exposures, such as maternal malnutrition and unhealthy lifestyle factors, are associated with several birth defects, they have scarcely been investigated in CDH. Nutritional and lifestyle factors can be modified and therefore may contribute to the prevention of CDH. In this review, we give an overview of the human studies in which the influences of nutrition and some related lifestyle factors during embryogenesis of the diaphragm are described. In addition, we further substantiate the findings in human by animal studies and elaborate on the nutrient-gene interactions involved. This review will contribute to the unravelling of the pathogenesis of CDH and development of preventive nutritional strategies in the future.

## Introduction

Congenital Diaphragmatic Hernia (CDH) is a malformation characterized by a defect in the diaphragm. Worldwide, every year approximately 120,000 children are born with CDH.[1-3] The intrauterine and perinatal mortality rate of CDH is around 60%, but varies between 20 and 70%.[4,5] The apparent increase in postnatal survival is probably biased by an increasing number of pregnancy terminations in patients with a poor prognosis.[6] The birth prevalence rate of CDH is comparable among countries worldwide, but ethnic differences are reported.[1,7,8] In children with a white ethnic background the frequency of CDH is higher as compared to Asian, Afro-American or Hispanic ethnicities.[9-12]

Maternal drug use has been associated with the occurrence of CDH in the child,[13-19] including anti-epileptic drugs,[20] antifungal drugs[21] and selective serotonin reuptake inhibitors.[22,23] Most reports are however sporadic and most data are from epidemiological studies on congenital anomalies including CDH, but considered as one heterogeneous group. No studies have been performed specifically on the proportion of CDH that is associated with maternal use of medication during the period in which CDH develops.

CDH is in 30% associated with another malformation.[5] In this group 50% is due to a syndrome or genetic defect. This means that the cause of CDH is unknown in 85%. The morbidity of CDH is high, with pulmonary and gastro-intestinal sequelae being most frequent.[24] The most important challenge in the immediate newborn period is the "control" of pulmonary hypertension before surgical closure of the diaphragm defect.[4] Because of the severe burden for the patients and their family, a long-term follow-up program by a multidisciplinary team is often provided.[25]

In this review we describe the embryologic background of CDH and relate this to the role of developmental and candidate genes in the interaction with nutrients and some related lifestyle factors in humans further substantiated by animal studies. The elaboration on nutrient-gene interactions will improve our understanding of the underlying mechanisms in the pathogenesis of CDH. This will potentially lead to nutritional interventions for CDH prevention in the future.

## Embryogenesis of the diaphragm

In the classical view the diaphragm is formed by the fusion of four different embryonic structures: 1) septum transversum (the primordial diaphragm), 2) pleuroperitoneal membrane, 3) oesophageal mesenchyme and 4) paraxial mesoderm of the body wall (see figure 2.1).[26,27] Muscle precursor cells migrate and differentiate within the septum transversum and eventually form the diaphragmatic muscle. These processes take place

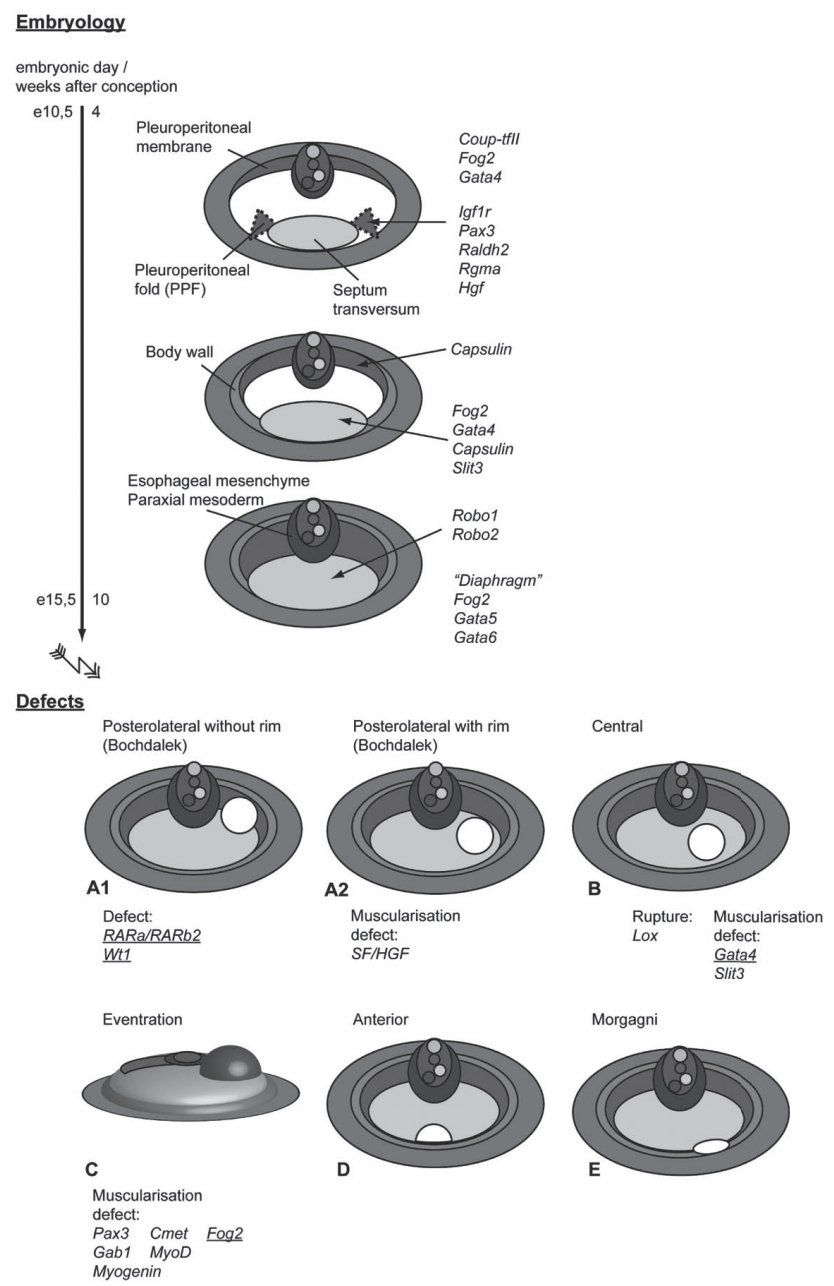


**Figure 2.1** | Schematic overview of diaphragm development. The expression of genes in the components of the diaphragm are indicated by arrows. A-E: schematic of defects that are found in CDH and animal models. Underlined: genes involved in the vitamin A pathway.

between the 4th to 10th embryonic weeks. CDH occurs if the diaphragm fails to close in this embryonic period. As a consequence of the defect the abdominal content "invades" the thoracic cavity and limits pulmonary growth. It is believed that the lungs are already hypoplastic independent of the mechanical obstruction and limitation in foetal breathing movements.[28] The defects observed in CDH are in general classified into posterolateral (Bochdalek), non-posterolateral or anterolateral (e.g. Morgagni) and central (Pentalogy of Cantrell), but not always limited to these regions. An eventration is an abnormally thin diaphragm caused by a muscularization defect and is used to describe the type of the defect rather than the location. Sometimes a thin fibrous sheet covers the intestinal organs inside the thorax. This "sac" is different from an eventration and is not considered a separate type of CDH. It illustrates the importance of the precise phenotyping of CDH and the possibility to correlate the defects with the defects reported in genetic models. This may lead to a better understanding of the deranged processes and pathways in CDH.[29]

To date, our knowledge of cell biological processes and regulation of the temporospatial expression of genes implicated in human diaphragm development is scarce. Evidence suggests that a defect in the pleuroperitoneal folds (PPF's) eventually leads to defective diaphragm formation in mice.[30-34] If this is proven true, the classical theory of the embryogenesis of the diaphragm has to be revised.

## Nutrition during the embryogenesis of the diaphragm

From gametogenesis throughout pregnancy, the conceptus is compelled to adapt at the transcriptional level to changes in its environment determined by the maternal nutritional status, metabolism and lifestyle. The maternal diet contains essential components that serve as building blocks, transcription factors and intermediates for cell signalling in the embryo and foetus. With increasing knowledge of the role of nutritional factors in developmental pathways, evidence is rising that an optimization of maternal nutrition can contribute to a reduction of the risk of congenital malformations.[35,36]

From the moment of conception the transfer of nutrients is by simple passive diffusion via the oviduct, yolk sac, extra-embryonic coelom, amniotic membranes, amniotic cavity and intervillous space.[37] The development of the placenta starts at the moment of implantation, i.e. the first week after conception. However, it is not earlier than after dissolvent of the throphoblastic plugs at the end of the first trimester that maternal blood progressively flows within the intervillous space and nutrition is provided haemotrophically.[37,38] Besides transport, the placenta also actively synthesizes nutrients, hormones and peptides that are needed for growth and differentiation of the foetus. With respect to CDH it is important to realize that at the time of defective closure of the

diaphragm the embryo is dependent on nutrient and oxygen exchange via the amniotic membranes, which at that time of pregnancy also have some metabolic functions.[39-41]

Deficiencies or an excess of nutrient supply to the embryo can be caused by maternal nutrient intake, disturbances in the absorption and transfer of nutrients to the embryo, increased nutritional needs, and genetic alterations in the mother and the child. It is likely that inadequate nutritional supplies within a certain timeframe affect the embryonic nutritional status and subsequent pathways. Derangements in these pathways may be implicated in CDH and its primary prevention by nutrition is feasible when the causes can be identified and treated preferably in the preconception period.

**Vitamin A (Retinol) and CDH**

Vitamin A is a fat-soluble vitamin which controls many developmental genes mainly by its biologically active metabolite retinoic acid (RA). Humans are not able to synthesize retinoids *de novo* and therefore obtain this vitamin by the diet. During transport from the liver to other tissues, retinol is almost solely bound to retinol binding protein (RBP). Cellular uptake of retinol is mediated by the recently discovered RBP receptor STRA6 and lecithin:retinol acyltransferase (LRAT).[41-43] Inside the cell, retinol is transformed to RA by a two-step oxidation. RA enters the nucleus and binds the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoid X receptor) which heterodimerize to the promoter region of a gene (Figure 2.2).[44,45]

The control of RA-dependent processes is achieved by the temporospatial expression of retinoid receptors and binding proteins as well as by time-dependent changes in vitamin A metabolism.[46] Vitamin A metabolism is regulated by the interplay of three enzyme families: alcohol dehydrogenases, short-chain dehydrogenases and members of the cytochrome P450 family.

From the first trimester onwards the amount of retinol supplied to the foetus is maintained at a constant level until maternal stores are almost completely depleted.[47,48] Because the foetus produces its own RBP, only free retinol passes the placental barriers.[49] The supply of retinoids to the embryo is not limited to retinol, because retinyl esters packed in lipoproteins and retinoic acid can also be taken up.[49] Moreover, the foetal membranes have an active metabolic and transport function for retinoids.[39]

The relationship between CDH and vitamin A has emerged from human studies and animal experiments. Yang et al.[50] observed an increased risk of a child with CDH in mothers using low intakes of retinol. Herewith in line is the study of Finnell et al.[51] suggesting that the elevated incidence of conotruncal heart defects in non-Hispanic whites might be related to a lower vitamin A intake in this group. Because the same differential ethnic pattern has been observed for CDH this association may also be of interest for further investigation. It is however remarkable that the birth prevalence rate of CDH is not high

in countries where vitamin A deficiency is endemic. This might be due to a failure of an adequate registration of congenital anomalies including CDH.

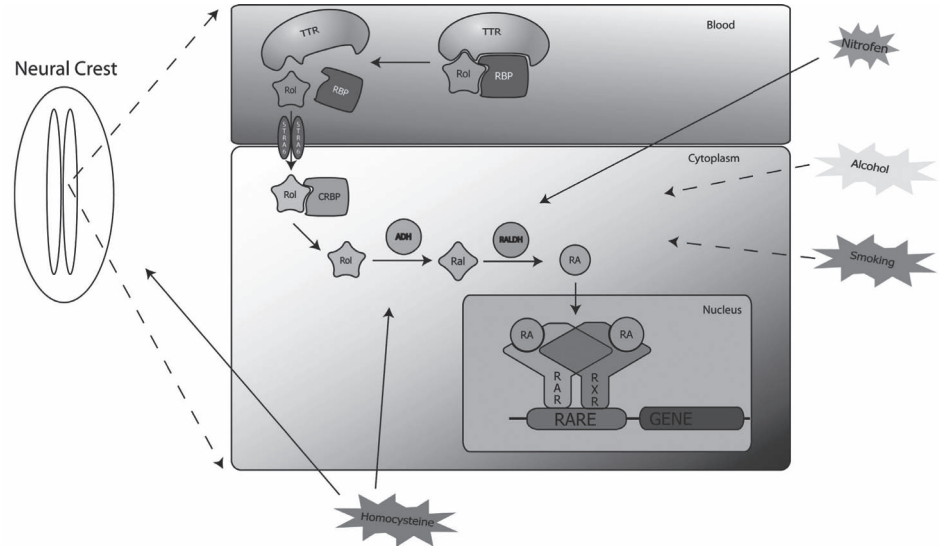


**Figure 2.2** | Schematic overview of the vitamin A pathway. Depicted are the interactions with other pathways and structures that are discussed in this review. TTR, transthyretin; Rol, retinol; RBP, retinol binding protein; STRA6, stimulated by retinoic acid 6; CRBP, cellular retinol binding protein; Ral, retinal; ADH, alcohol dehydrogenase; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element.

Nutritional experiments in rats have shown that dams fed a vitamin A deficient diet give rise to offspring with multiple congenital anomalies, including abnormalities of the diaphragm, heart, limbs, vertebral column, ocular tissues, respiratory system, cardiovascular system, and segmentation defects.[52-55] The most extensively studied animal model is based on nitrofen, a herbicide that disturbs the RA pathway. When nitrofen is administered timely to the pregnant dam, it results in CDH in a majority of the offspring, which the CDH phenotype being comparable to that observed in vitamin A deficiency models and in RAR knock-outs.[56-59] Nitrofen has been shown to block the enzyme Retinal Dehydrogenase 2 (RALDH2), resulting in RA deficiency (figure 2.2).[60] Supplementation of vitamin A or RA to the maternal diet counteracts the effect of nitrofen.[61,62] The other anti-oxidants vitamin C and E also seem to have this protective effect, although only in lung and heart tissues.[63-65]

To date, no reports are known on the effects of nitrofen in human, neither in other animals than certain rat strains with differences in susceptibility for nitrofen.

Teratogenic effects of an *excess* of dietary vitamin A have been reported in humans and animals.[66-69] The effect of high vitamin A exposure by vitamin A-containing drugs in humans, e.g. the synthetic retinoid Isotretinoin, has been described as the retinoic acid embryopathy.[70,71] This syndrome includes craniofacial, cardiovascular, thymic and central nervous system malformations and shows a remarkable similarity with those observed in hypovitaminosis A, but lacks a diaphragm defect.[71] To date only one study, by Major et al.[72], showed higher levels of retinol in mothers of children with CDH. Because their children showed lower vitamin A and Retinol Binding Protein (RBP) levels in cord blood as compared to healthy control pregnancies, it is questionable whether a high or a low vitamin A exposure in early pregnancy is teratogenic. Although the results were significant, these results should be interpreted with care because the number of patients was very low. Moreover, it is not clear whether postnatal vitamin A status of mother and child is representative of the status in the critical period early in pregnancy.

In conclusion, evidence from human studies supported by animal models, suggests that a disturbance in vitamin A metabolism, resulting in vitamin A deficiency, might be involved in the development of CDH. Prospective studies and *in vitro* experiments are needed to obtain more insight in the underlying mechanisms.

**Folate and CDH**

Folate is an essential B-vitamin present in green vegetables and meat. Folate plays an important role in several cell biological processes, such as amino acid metabolism, DNA synthesis, and methylation of DNA and RNA. The synthetic form, folic acid, is used in vitamin supplements and fortification of food because of its higher bioavailability and stability. Folate is a known modifier of the risk of birth defects in humans.[73] Periconception use of a folic acid containing supplement reduces the occurrence of neural tube defects, congenital heart defects and orofacial clefts.[74-79] These birth defects have also been related to polymorphisms in genes that encode for proteins involved in folate metabolism resulting in folate deficiency.[76,80] A comparable preventive effect on CDH has been shown by high dietary B vitamin intakes.[12,50,81] These findings are supported by studies in mice showing that maternal folate deficiency leads to an abnormal cell count and structure of the diaphragm.[82]

One explanation of the protective effect of folic acid on birth defects is based on the reduction of high homocysteine levels. It is unknown, however, whether hyperhomocysteinaemia is a primary teratogen or an epiphenomenon in the pathogenesis of these complex birth defects. It has been hypothesized that hyperhomocysteinaemia causes excessive oxidative stress and may interfere with the folate receptor.[83] Homocysteine also inhibits RA synthesis and administration of RA to homocysteine-treated chicken embryos

increased embryonic survival and diminished the number of congenital anomalies.[84] So far hyperhomocysteinaemia has not been directly linked to CDH.

## Lifestyle factors and CDH

Lifestyle factors, such as smoking and alcohol use, have been associated with several birth defects. Smoking is the strongest lifestyle factor associated with birth defects and is often accompanied by poor nutrition.[85,86] Tobacco smoke contains numerous compounds in which nicotine has been shown to disrupt vascular formation and retinoid homeostasis thereby predisposing to birth defects.[87] Although abnormal vascular formation and retinoid homeostasis might be pathogenic factors in CDH, a relationship with human CDH has not been identified. In contrast, smoking has also been associated with a lower risk of conotruncal heart defects and neural tube defects.[88]

It is well known that excessive use of alcohol by pregnant women causes foetal alcohol syndrome (FAS), in which CDH has not been reported.[89,90] In a recent paper however, Felix et al.[91] found a relationship between CDH and maternal social alcohol use in the months before conception and during the first trimester. In contrast to excessive alcohol use, social alcohol use is poorly defined but can be described as the regular use of low amounts of alcohol. There is some evidence that the metabolic pathways of alcohol and vitamin A interact. Alcohol may reduce RA status by inhibition of vitamin A metabolism and induction of RA degradation and as such potentiate the teratogenicity of vitamin A deficiency.[39] Furthermore, alcohol seems to aggravate the adverse effects of an excess of vitamin A, which has been demonstrated by the simultaneous administration of both ethanol and vitamin A in rats.[92] In addition, excessive alcohol use is associated with a poor nutritional intake, including low B vitamin intake. Whether this is critical to the preventive effect of periconception folic acid and multivitamins against CDH should be further investigated.

Obesity is a general feature of unhealthy lifestyles and is associated with malnutrition and reduced exercise. Prepregnancy obesity defined as a Body Mass Index higher than 30 is a risk factor for CDH.[93] There is some evidence that the accompanying poor glycaemic control and insulin resistance contribute to the pathogenesis of CDH.[94-96] It seems that the teratogenicity of hyperglycaemia is related to abnormal glycosylation of proteins, increase of oxidative stress, reduction of antioxidants and derangement of gene expression. Of special interest is the reduction of the levels of Pax-3 in diabetes. Pax-3 has been related to neural tube closure, but is also involved in diaphragm development (table 2.1).[95,97] The expression of *Pax-3* can be improved by vitamin A, at least in cardiac cells in the nitrofen model.[64]

**Table 2.1** | Nutrition and related genetic factors in humans and animal models with CDH

| Factor | Species | Nutritional factor | Genetic defect | Diaphragm defect and other phenotypic features | Reference |
|---|---|---|---|---|---|
| **Transcription factors** | | | | | |
| **COUP-TFII (NR2F2)** | Human | vitamin A pathway | 15q26.1-26.2 | Posterior defect | You et al. (2005)[106] Klaassens et al. (2005)[98] Slavotinek et al. (2006)[133] |
| **Coup-TFII** | Mouse | vitamin A pathway | conditional knock-out | Posterior defect | You et al. (2005)[106] |
| **GATA4** | Human | vitamin A pathway | 8p23.1 | Central *muscularization* defect | Shimokawa et al. (2005)[134] Slavotinek et al. (2006)[133] |
| **Gata4** | Mouse | vitamin A pathway | Gata 4 +/- | Central hernia (sac) | Jay et al. (2007)[107] Lopez et al. (2006)[135] |
| **FOG2 (ZFPM2)** | Human | vitamin A pathway | Mutation 8q22-23 | Muscularization defect | Temple et al. (1994)[136] Howe et al. (1996)[137] Ackerman et al. (2005)[108] |
| **Fog2 (Zfpm2)** | Mouse | vitamin A pathway | Knockout | Muscularization defect of posterior diaphragm, bilateral | Ackerman et al. (2005)[108] |
| **WT1** | Human | Possible; same mechanism as nitrofen and vitamin A deficiency models | Heterozygous loss-of-function | Diaphragm defects WAGR, Denys-Drash, Meacham and Frasier syndromes | Scott et al. (2005)[138] DeVriendt et al. (1995)[109] Cho et al. (2006)[110] |
| **Wt1** | Mouse | | Knockout | Posterolateral hernia | Clugston et al. (2006)[32] Kreidberg et al. (1993)[139] |
| **Pax3 (Splotch)** | Mouse | maternal diabetes reduces pax-3 expression in embryo. vitamin A stimulates Pax3 expression | Mutant | Amuscular diaphragm | Li et al. (1999)[69] Phelan et al. (1997)[97] Gonzalez-Reyes et al. (2006)[64] |
| **Rarα/Rarβ2** | Mouse | vitamin A pathway | Double mutant | posterior diaphragmatic hernia | Mendelsohn et al. (1994)[59] |
| **Factors in cell signaling** | | | | | |
| **STRA6** | Human | vitamin A pathway | | CDH or eventration | Pasutto et al. (2007)[112] Kawaguchi et al. (2007)[42] |
| **Factors in cell migration and mesodermal patterning** | | | | | |
| **GPC3 (Glypican 3)** | Human | Cholesterol?? | Mutation | Diaphragmatic hernia Simpson-Golabi-Behmel syndrome | Pilia et al. (1996)[115] Hughes-Benzie et al. (1996)[116] Veugelers et al. (2000)[140] Li et al. (2001)[141] |

## Genetic factors involved in the development of the diaphragm

Although genetic defects and several candidate genes have been identified in patients with CDH, most knowledge on genes and CDH is derived from animal studies.[98-102] The function of these genes can be classified into transcription factors, factors involved in cell signalling, in cell migration and mesodermal patterning, and in extracellular matrix biosynthesis (see figure 2.1 and table 2.1).[103] The following paragraphs describe only those genes related to known nutritional or lifestyle factors. For an extensive overview of other genes that may be involved in CDH, we refer to several excellent reviews in the literature.

### Transcription factors

The transcription factor COUP-TFII works together with GATA4 and FOG2 and as such is involved in retinoic acid (vitamin A) metabolism.[104] All three genes are located on the frequently altered chromosomal regions on chromosome 15 (COUP-TFII) and 8 (GATA4 and FOG2) in human CDH.[101,105] In mice, the modulation of these genes induces a muscularization defect.[106-108] Mutations in Wilms' tumour 1 (WT1) cause a spectrum of syndromes, which also includes CDH.[109,110] The WT1 gene resides in the region on chromosome 11 that is frequently altered in human CDH, and is suggested to share the same mode of pathogenesis.[32,101,105] Migratory muscle cell precursor cells in the septum transversum express the transcription factor *Pax3* which is also expressed in neural crest cells that emerge from the dorsal neural tube.[31] In the *Splotch* mouse the knockout of *Pax3* leads to an amuscular diaphragm.[69] Double mutants of the nuclear retinoic acid receptor (RARα/RAR$\beta_2$) develop CDH.[59,111] This finding suggested that a disturbance in the retinoid signalling pathway contributes to the pathogenesis of CDH.

### Factors in cell signalling

The human Matthew-Wood syndrome, which includes microphthalmia and CDH, is caused by a mutation in STRA6.[112] Recently, STRA6 has been identified as the cellular receptor for retinol.[42, 113] STRA6 has an important regulatory function in cellular vitamin A homeostasis in cooperation with lecithin:retinol acyltransferase (LRAT).[41,43]

### Cell migration and mesodermal patterning

*Glypican-3* (GPC3) is expressed selectively in mesodermal derived tissues and functions as a regulator of growth factors, apoptosis and guidance molecules, such as the *Slit-Robo* complex.[114] A role for GPC3 in the regulation of insulin-like growth factor 2 has been proposed and mutations cause Simpson-Golabi-Behmel syndrome (SGBS). Patients with SGBS have a defect in cholesterol synthase, leading to a foetal-placental overgrowth syndrome with CDH in some cases.[115, 116] So far, interactions between genes involved in cell migration and mesodermal patterning, and nutrition and lifestyle factors are not reported.

**Extracellular matrix**

The impairment of extracellular matrix (ECM) formation leads to a variety of developmental defects. In mice, a deficiency of *fibrillin-1* causes heart and lung anomalies that can be prevented by the medicine losartan, an angiotensin-II receptor antagonist or a *Tgf-β* neutralizing antibody.[117] Mutations in HCCS cause MIDAS syndrome, which is characterized by microphthalmia and CDH.[118] This combination of features has also been observed in the aforementioned Matthew-Wood syndrome.[112,119]

In summary, several genetic factors in several pathways are associated with CDH. So far some evidence is available that interactions between genetic factors and the periconception maternal vitamin A, glucose and cholesterol status may play a role in the pathogenesis of CDH.

## Conclusion and future perspectives

It is clear from human and animal studies that CDH has a heterogeneous phenotype. This suggests the involvement of multiple pathways, mechanisms and interactions. Most evidence of a nutrient-gene interaction in relation to CDH is available from human and animal studies on vitamin A. The homeostasis of the vitamin A pathway is under strict control. Disturbances in this signalling pathway during pregnancy can be harmful to the embryo due to derangements in retinoid levels, binding proteins and converting enzymes. Among species there is a different sensitivity to retinoids due to variations in placental transfer of RA isomers[120-122] or species-specific transcription factors,[123] resulting in different outcome. Phenotypic differences have been observed in animal models of CDH. In humans the differences in nutritional habits and other environmental factors may contribute to a different susceptibility and phenotypical outcome, possibly in more subtle ways than can be found by gene-expression analyses alone.[124] Some nutritional and lifestyle factors that potentially disturb retinoid homeostasis are alcohol use and smoking. However, there is only little evidence and the extrapolation of knowledge derived from animal models to the human situation is still limited.

Both vitamin A deficiency and a high vitamin A intake are suggested to be teratogenic. Therefore, the vitamin A recommendation to pregnant women is to take 800 µg retinol activity equivalents (RAE) per day, with a limit to 3000 µg RAE.[125,126] A minimal recommendation is important because of the anti-oxidant capacity of vitamins A, C and E and their demonstrated protective effect in humans[50,65] and animal models of CDH.[64] This effect may suggest that nutrient-gene interactions are implicated in CDH, but should be supported further by human studies.

The co-occurrence of CDH and facial, thyroid and cardiac anomalies, whether or not within a defined syndrome, suggests the existence of a dysmorphogenic mechanism in which the neural crest is involved.[127-129] The neural crest is an important embryonic structure from which regulatory signals are transmitted and cells migrate to form the organs. The development of the neural crest is influenced by modifiers like retinoids and folic acid.[130] Therefore, it would be very interesting to study nutrients and related genes further in relation to CDH and the neural crest.

One way to summarize the involved mechanisms in CDH is illustrated by the mesenchymal hit hypothesis.[103] This hypothesis is an extension of the dual hit hypothesis formulated by Keijzer et al.[131] which suggests that in CDH, a) similar signalling pathways are involved in the differentiation of mesenchymal cells in all of the affected organs and b) the function of these mesenchymal cells is disrupted by genetic or environmental triggers. The goal for future research is to identify and further delineate these pathways.

The investigation of the involved pathways and interactions between nutrients, lifestyles and genetic factors creates opportunities for optimizing the preconception maternal diet. This may ultimately lead to the prevention CDH or at least decrease the amount of hypoplasia or the size of the defect. The relatively low birth rate of CDH and the subtle effects of nutrition and lifestyle factors emphasizes the need of large epidemiologic studies in which international collaborations with access to birth registries are necessary, such as the recently instituted EURO-CDH consortium and the CDH-registry.[132]

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# Chapter 3

## The retinol status in newborns infants is associated with congenital diaphragmatic hernia

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## Abstract

**Objective:** Genetic analyses in humans suggest a role for retinoid-related genes in the pathogenesis of congenital diaphragmatic hernia (CDH). The goal of this study was to investigate the vitamin A status of mothers and their newborns in association with CDH.

**Methods:** We conducted a hospital based case-control study with 22 case and 34 control mothers and their newborns. In maternal and cord blood samples, retinol and retinol-binding protein (RBP) were measured with high-performance liquid chromatography and an enzyme-linked immunosorbent assay, respectively. Univariate and multivariable logistic-regression analyses were performed to determine crude and adjusted risk estimates.

**Results**: Case newborns had significantly lower levels of retinol (0.60 vs. 0.76 µmol/l, p=0.003) and RBP (5.42 vs. 7.11 mg/l, p=0.02) than did control newborns. The multivariable logistic-regression analysis showed lower levels of retinol and RBP in association with CDH risk, i.e. the odds ratio (OR) for retinol levels of <15$^{th}$ percentile (<0.61 µmol/l) was 11.11 (95% confidence interval [CI] 2.54 to 48.66; p=0.001) and that for RBP levels of <15$^{th}$ percentile (<4.54 mg/l) was 4.00 (95% CI 1.00 to 15.99; p=0.05). Retinol and RBP levels were not different between case and control mothers.

**Conclusions:** CDH is strongly associated with low retinol and RBP levels in newborns, independent of maternal retinol status. This is an important finding supporting the idea that human CDH is linked with abnormal retinoid homeostasis.

## Introduction

Congenital diaphragmatic hernia is a serious developmental disorder that is characterized by a defect of the diaphragm, pulmonary hypoplasia and pulmonary hypertension, with significant mortality and morbidity rates.[1,2] The worldwide birth prevalence rate of CDH is approximately 3 per 10,000 births and rates are comparable among countries throughout the world,[3] although children with white ethnic backgrounds might be affected more frequently.[4,5] Prenatal diagnosis, postnatal therapy and the lack of individualized treatment algorithms make CDH a major challenge in obstetrics, prenatal medicine, neonatology and paediatric surgery.[6,7]

The pathogenesis of CDH is largely unclear. Data from rodent models of CDH and analyses of human postmortem diaphragm tissue point at the initial defect occurring at very early stages of primordial diaphragm formation, before week 5 of gestation.[8] Because the variation in phenotypes is large and a genetic diagnosis is lacking for most the patients, the pathogenesis of CDH is likely to be multifactorial. Subtle derangements in genes combined with maternal nutrition, lifestyle factors and environmental exposures may be responsible for defective diaphragm formation.[9] A significant body of evidence from animal models supports a role of the vitamin A pathway or transcriptional mechanisms influenced by retinoids (vitamin A derivatives), including associations between CDH and maternal dietary deficiency of vitamin A,[10] teratogenic disturbances of retinoid signaling,[11,12] genetic abnormalities of retinoid signaling,[13,14] the reduction of defects after retinoid administration,[15,16] and the expression pattern of retinoic acid receptors in the primordial diaphragm.[17] In a pilot study, lower levels of retinol and retinol-binding protein (RBP) were found in 7 newborns with CDH, compared with 7 healthy control subjects. Case mothers had higher levels of retinol and RBP.[18] In addition, genetic studies in humans have shown that frequently deleted or duplicated regions in CDH contain genes that are related to the vitamin A pathway (ie, COUP-TFII on chromosome 15q26).[19]

Collectively, these data provided the rationale for our case-control study to investigate the vitamin A status in mothers and their newborns in association with CDH.

## Materials and Methods

### Study population

The Congenital Diaphragmatic Hernia, Environment, Retinoids, Nutrition, Inheritance and other Associations Study (HERNIA Study) is a hospital-based, case-control study conducted from 2006 onward at Erasmus MC University Medical Center Rotterdam, a tertiary referral hospital for the western part of the Netherlands.

After the prenatal diagnosis of CDH through ultrasonography, parents were asked to participate in the study. After a case was diagnosed, control triads (father, mother and child) were selected during pregnancy from the same hospital with comparable maternal age (± 1 year), parity, ethnicity and fertilization method (risk set sampling). CDH was defined as isolated if there were no major associated anomalies, apart from pulmonary hypoplasia, patent ductus arteriosus, intestinal malrotation and abnormalities in cardiac position, which are considered secondary effects of CDH. All patients with CDH were eligible for enrolment in the study protocol. In the present study, only mother-child pairs were analyzed. Chromosomal analysis and DNA harvesting were performed as standard care for all patients. General and specific retinoid-related regions were tested, such as chromosome 15q or 1q in complex cases.

The study protocol was approved by the Central Committee of Research in Human in the Hague, the Netherlands, and by the Medical Ethical Committee of Erasmus University Rotterdam. Written informed consent was obtained from every parent and on behalf of their children.

**Sampling and analytical methods**

Nonfasting blood samples were collected from case and control mothers at 38 weeks of gestation because of the obstetrical policy of induction of labour in case mothers during this week. Cord blood samples were collected from the newborns. All blood samples were collected in lithium-heparin containing tubes (BD Vacutainer® LH 68 IU, Becton Dickinson Breda, the Netherlands), protected from light with aluminium foil wrapping and kept refrigerated (4°C) until further processing. As soon as possible after collection, samples were processed by centrifugation (1,800 x *g* for 15 minutes) and plasma was stored in amber tubes (Bioplastics, Landgraaf, the Netherlands) at -80°C. For analysis of retinol levels, the samples were extracted with hexane and, after evaporation, dissolved in methanol. Retinol levels were measured with reverse-phase high-performance liquid chromatography (Column: 150*4.8 mm, Polaris C18A, HPLC: Waters Alliance HT2795, Detector: Waters 2475, Waters Corporation, Milford, MA, USA) with excitation at 328 nm and detection of emission at 468 nm. The intraassay and interassay variabilities of retinol were 3.9% and 5.1%, respectively. Serum RBP levels were measured by using an enzyme-linked immunosorbent assay kit (Immundiagnostik, Bensheim, Germany). The intraassay and interassay variabilities of RBP were 5.0% and 9.8%, respectively.

We did not detect any significant differences in retinol and RBP levels between arterial, venous or mixed cord blood. Therefore, all results from the cord blood samples were pooled for analysis (data not shown). There was no relationship between retinol and RBP levels and the time elapsed until storage or the duration of storage at -80°C (data not shown).

**Statistical methods**

Normal distributions were checked with the Kolmogorov-Smirnov test. Nonparametric tests were used when variables were not normally distributed. Categorical variables were shown as frequencies and tested by the chi-square test. We used Pearson correlation coefficients to examine associations between continuous variables. Retinol and RBP levels were presented as means and compared with the unpaired Student's t-test. The risks for CDH were expressed as odds ratios (ORs) and 95% confidence intervals (95% CIs) in a univariate logistic-regression model, with the 15$^{th}$ percentiles as cut-off points for the levels in the control population. In a multivariable logistic-regression model we adjusted the risks for the potential confounders maternal age, birth weight and gestational age at sampling. Retinol and RBP levels were included in the regression model as continuous variables and as dichotomous variables on the basis of the cut-off values from the control population. All statistical analyses were performed by using SPSS for Windows 15.0, (SPSS, Chicago, IL, USA).

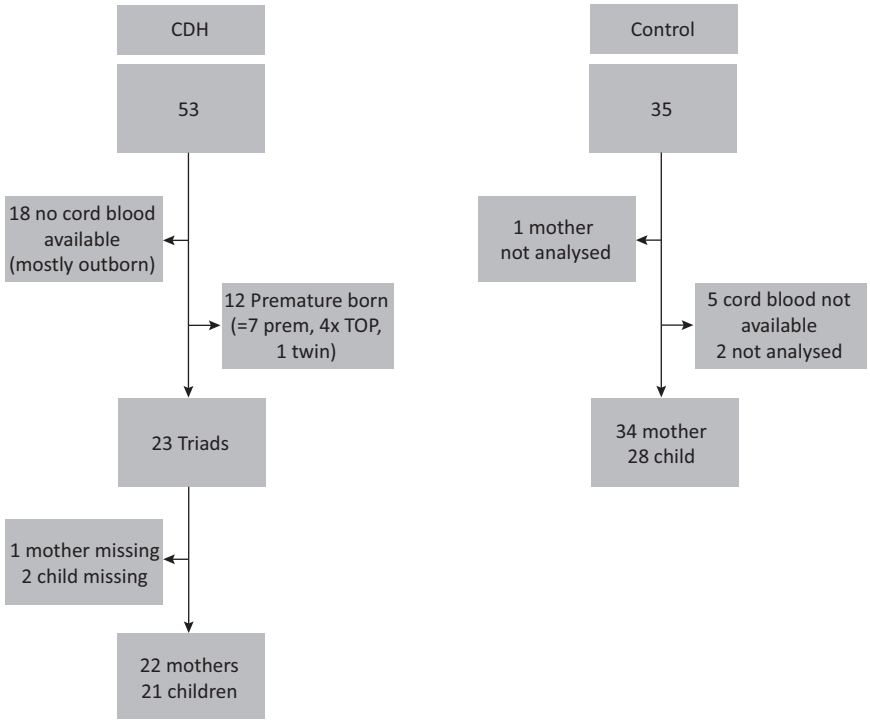


**Figure 3.1** | Flowchart of study

## Results

We included 53 case and 35 control mother-child pairs (figure 3.1). Because no control patients were available for 12 case patients born before 37 weeks of gestation, we excluded them. For 18 patients, no cord blood samples were available; 14 patients were outborn, and no cord blood was collected for 4 patients. The general characteristics of these patients are presented in table 3.1. Eventually samples were analyzed from 22 case and 34 control mothers, with 21 case and 28 control newborns.

**Table 3.1** | Clinical characteristics of the participants in the study. For the excluded cases, data on exact gestational age at sampling were available for only 7 mothers and BMI for 4 mothers. Categorical variables were tested with the chi-square test and numerical variables with the 2-sided Student t test. Birth weight was tested with the Mann-Whitney U test.

| Characteristic | CDH n=23 | Control n=35 | Excluded cases n=12 | P-value CDH vs. Control |
|---|---|---|---|---|
| **Mother** | | | | |
| Age in years, mean (SD) | 31.2 (5.2) | 33.6 (3.8) | 32.6 (5.5) | 0.04 |
| Gestational age at sampling (SD) | 38.4 (0.7) | 38.3 (0.9) | 32.7 (7.0) | 0.7 |
| Ethnicity | | | | 0.14 |
| Dutch native | 16 (70%) | 31 (89%) | 8 (67%) | |
| European other | 1 (4%) | 1 (3%) | 0 (0%) | |
| Non-European other | 6 (26%) | 3 (9%) | 4 (33%) | |
| Body Mass Index (mean, SD) | 23.0 (3.66) | 23.2 (3.66) | 26.9 (1.8) | 0.74 |
| Nulliparity (excluding this pregnancy) | 12 (52%) | 13 (37%) | 6 (50%) | 0.14 |
| Conception | | | | 0.46 |
| Spontaneous | 22 (96%) | 29 (83%) | 12 (100%) | |
| Artificial | 1 (4%) | 6 (17%) | 0 (0%) | |
| Delivery | | | | 0.15 |
| Spontaneous | 13 (57%) | 16 (46%) | 11 (92%) | |
| Instrumental | 7 (30%) | 5 (14%) | 0 (0%) | |
| Caesarean Section | 3 (13%) | 13 (37%) | 1 (8%) | |
| Unknown | 0 (0%) | 1 (3%) | 0 (0%) | |
| **Newborn** | | | | |
| Male gender | 10 (43%) | 17 (49%) | 50% | 0.7 |
| Gestational age at birth in days (SD) | 270.0 (5.2) | 278.8 (9.3) | 215.2 (45.3) | <0.001 |
| Birth weight in gram, mean (SD) | 2955 (419) | 3456 (365) | 1604 (787) | <0.001 |
| Mortality | 7 (30%) | 0 (0%) | 6 (50%) | 0.001 |
| Associated anomalies | 4 (17%) | 1[a] (3%) | 9 (75%) | 0.05 |
| Genetic defect | | – | 4 (33%) | n.a. |

[a] minor anomaly: auricular appendage; n.a. not applicable

Clinical characteristics of the case and control subjects are presented in table 3.1. Four children had multiple congenital anomalies with 1 case suspected to be Fryns syndrome and 1 clinically diagnosed as Cornelia de Lange syndrome without NIBPL mutation. CDH was associated with omphalocele, dysmorphic facial features, and a single umbilical artery in 1 case and with epicanthic folds, wide-spaced nipples, and cryptorchid testes in another. One patient in the control group showed a minor anomaly (ie auricular appendage). Karyotyping and telomeric MLPA (multiplex ligation-dependent probe amplification) for case newborns showed no specific genetic defects.

Case and control groups differed in gestational age at birth, birth weight and maternal age. There were no other significant differences between the groups. The Kolmogorov-Smirnov test indicated differences in birth weight (case: z=1.354, p=0.05; control: z=0.655, p=0.78). Therefore, birth weight was tested with the Mann-Whitney U test.

As shown in table 3.2, retinol and RBP levels were significantly lower in newborns with CDH, compared with control newborns (retinol 0.60 vs 0.76 µmol/l, p=0.003; RBP 5.42 vs 7.11 mg/l, p=0.02). In a logistic-regression model, retinol and RBP levels were significantly associated with case or control status (table 3.3). After adjustment for gestational age, birth weight and maternal age, these associations remained significant.

**Table 3.2** | Levels of Retinol and RBP. Mean levels of retinol and RBP in maternal serum and newborn cord blood.

| | CDH | | | Control | | | p-value |
|---|---|---|---|---|---|---|---|
| | n | Level, mean | SD | n | Level, mean | SD | |
| **Mother** | | | | | | | |
| Retinol (µmol/l) | 22 | 1.21 | 0.33 | 34 | 1.24 | 0.31 | 0.74 |
| RBP (mg/l) | 22 | 11.07 | 4.11 | 34 | 11.85 | 3.90 | 0.48 |
| **Newborn** | | | | | | | |
| Retinol (µmol/l) | 21 | 0.60 | 0.19 | 28 | 0.76 | 0.18 | 0.003 |
| RBP (mg/l) | 20[a] | 5.42 | 2.04 | 28 | 7.11 | 2.62 | 0.02 |

[a] One RBP sample was missing

**Table 3.3** | Associations between biomarkers of vitamin A in newborns and CDH risk.

| | β | Exp(β) (95% CI) | p | n | | Crude OR (95% CI) | p | Corrected OR (95% CI)[a] | p |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CDH | Control | | | | |
| Retinol per 1 µmol/l | -7.136 | 0.001 (0.000 -0.223) | 0.013 | | | | | | |
| RBP per 1 mg/l | -0.465 | 0.628 (0.140 -0.953) | 0.029 | | | | | | |
| Retinol levels of <15$^{th}$ percentile (<0.61 µmol/l) | | | | 12 | 3 | 11.11 (2.54 -48.66) | 0.001 | 14.11 (1.95 -102.00) | 0.009 |
| RBP levels of <15$^{th}$ percentile (<4.54 mg/l) | | | | 8 | 4 | 4.00 (1.00 -15.99) | 0.05 | 3.65 (0.72 -18.59) | 0.12 |

[a] Corrected for maternal age, birth weight and gestational age.

**Table 3.4** | Maternal and newborn levels of retinol and RBP at birth.

| Study | Gestational age | Remark | Cord Retinol level | | Cord RBP level | | Maternal Retinol level | | Maternal RBP level | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | N | Mean | N | Mean | N | Mean | N | Mean |
| Inder et al. 1998[44] | | VLBW | 22 | 157 µg/l (0.54 µmol/l) | | | 42 | 357 µg/l (1.25 µmol/l) | | |
| Brandt et al. 1978[45] | Term | | 51 | 22.4 µg/dl (0.78 µmol/l) | | | | | | |
| | Preterm (32 w) | | 42 | 14.9 µg/dl (0.52 µmol/l) | | | | | | |
| Shenai et al. 1981[46] | | | 32 | 239 µg/l (0.83 µmol/l) | 32 | 36 mg/l | | | | |
| Bhatia et al. 1983[47] | | | | | 53 | 36.9 mg/l | | | | |
| Yeum et al., 1998[48] | Term | | 10 | 0.79 µmol/l | | | 10 | 1.67 µmol/l | | |
| Sapin et al. 2000[22] | Term | Venous | 27 | 0.74 µmol/l | 27 | 1.07 µmol/l (22.4 mg/l) | 27 | 1.27 µmol/l | 27 | 1.49 µmol/l (31.29 mg/l) |

| Study | Gestational age | Remark | Cord Retinol level | | Cord RBP level | | Maternal Retinol level | | Maternal RBP level | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | N | Mean | N | Mean | N | Mean | N | Mean |
| | | Arterial | | 0.69 µmol/l | | 1.13 µmol/l (23.7 mg/l) | | | | |
| Dallaire et al. 2003[49] | | | 112 | 20.4 µg/dl (0.71 µmol/l) | | | | | | |
| Herrera et al. 2004[50] | Term | | 21 | 0.61 µmol/l | | | 13 | 1.02 µmol/l | | |
| Söderlund et al. 2005[51] | | | 10 | 1.0 µmol/l | | | 10 | 1.7 µmol/l | | |
| Galinier et al. 2005[52] | Preterm | | 244 | 0.55 µmol/l | 244 | 15 mg/l | | | | |
| | Term | | 259 | 0.73 µmol/l | 259 | 20 mg/l | | | | |
| Masters et al. 2007[29] | Term | | 251 | 20.86 µg/dl (0.72 µmol/l) | | | | 37.97 µg/dl (1.32 µmol/l) | | |
| Wang et al. 2009[21] | Term | | | 0.69 µmol/l | | | | 1.13 µmol/l | | |
| Major et al. 1998[18] | Term | Control | 7 | 293 µg/l (1.02 µmol/l) | 5 | 27 mg/l | 7 | 288 µg/l (1.01 µmol/l) | 7 | 28 mg/l |
| | | CDH | 7 | 179 µg/l (0.62 µmol/l) | 5 | 11 mg/l | 5 | 434 µg/l (1.51 µmol/l) | 5 | 51 mg/l |
| Current study | Term | Control | 28 | 0.76 µmol/l | 28 | 7.11 mg/l | 34 | 1.24 µmol/l | 34 | 11.85 mg/l |
| | | CDH | 22 | 0.60 µmol/l | 21 | 5.45 mg/l | 22 | 1.21 µmol/l | 22 | 11.30 mg/l |

Overview of several studies on maternal and newborn levels of retinol and RBP. For comparison, the mean value has been converted to µmol/l for retinol and mg/l for RBP. VLBW: very low birth weight.

The highest risk for CDH was observed for patients with the lowest 15% of retinol levels (<0.61 µmol/l) and RBP levels (<4.54 mg/l). The crude OR for CDH with retinol levels of <15th percentile was 11.11 (95% CI: 2.54 to 48.66; p=0.001) and the OR was 14.11 (95% CI: 1.95 to 102.00; p=0.009) after correction for maternal age, birth weight and gestational age. The crude OR for RBP levels of <15th percentile was 4.00 (95% CI: 1.00 to 15.99; p=0.05), and the OR was 3.65 after correction (95% CI: 0.72 to 18.59; p=0.12). Retinol and RBP levels were slightly higher in case newborns with associated anomalies, but this difference was not significant (retinol: 0.64 vs 0.59 µmol/l, p=0.7; RBP: 5.90 vs 5.40 mg/l, p=0.7) and did not change the results from the regression analyses.

Case and control mothers had no significant differences in retinol levels (1.21 and 1.24 µmol/l, respectively; p=0.74) (table 3.2). RBP levels also were not significantly different (11.07 and 11.85 mg/l, respectively; p=0.48). Maternal serum levels of retinol and RBP were not significantly correlated with newborn cord blood levels (retinol: r=0.09, p=0.54; RBP: r=0.09, p=0.55). Restriction to pairs with both mother and newborn samples available did not change these correlations.

## Discussion

### Overall findings

This study demonstrates that newborns with CDH have significantly lower levels of retinol and RBP, compared with healthy newborns. Maternal retinol and RBP levels are comparable. Our results are consistent with a perturbation of retinoid signalling and CDH observed in rodent models and support the theory that disturbed retinol status is implicated in the pathogenesis of human CDH.

### Low retinol levels

#### *Origin in the child*

To date, low retinol levels in cord blood have not been described for newborns with other congenital anomalies but only for premature or small-for-gestational age newborns. However, the relationship between low birth weight and levels of retinol is unclear.[20-22] Low birth weight is a feature of children with congenital anomalies. In our study, birth weight was correlated with vitamin A status but this correlation was lost after adjustment for gestational age. This suggests that growth retardation is associated with the vitamin A status of the newborn.

Foetal vitamin A status might be affected by disturbances in receptors, converting enzymes, or binding proteins. Mutations in the RBP-retinol receptor STRA6 have been associated with CDH but only as part of a specific, complex, congenital anomaly

phenotype.[14] No isolated CDH cases have been described with mutations in STRA6 or other components of the vitamin A pathway. However, several genes that are frequently deleted or duplicated in CDH are related to the vitamin A pathway.[23] Retinoic acid, the metabolically active metabolite of retinol, is an important regulator of gene transcription. Therefore, it is difficult to consider vitamin A activity and gene transcription separately. Moreover, other causative or protective factors might play a role.[9] For example, in animal models, marginal vitamin A status increases the teratogenicity of penta-brominated diphenyl ether.[2] Despite risk set sampling of control subjects, some case children were born prematurely and all control children were born at term. Although the power decreased, we excluded all premature newborns with CDH, which finally strengthened the results of the study. A shortcoming of our study is that control children did not routinely undergo genetic testing, which might have resulted in an underestimation of the risk estimates.

***Origin in the mother***

In rodent models, complete removal of retinol from the maternal diet induces CDH in the offspring.[10,25] However, serum levels of retinol and RBP have not been determined in the mother rats or the offspring. The hypothesis that maternal vitamin A deficiency is a risk factor for human CDH is not supported by the comparable retinol and RBP levels in our case and control mothers and the results of an earlier study that reported significantly higher levels for case mothers.[18] A trend for maternal periconceptional low intake of retinol was associated with increased risk of children having CDH.[26] However, this finding was based on indirect measurements of the vitamin A status through retrospective questionnaires. Despite large numbers, the risk was not significantly increased.[26] Furthermore, vitamin A deficiency is a common problem throughout the developing world[27] and, although a detailed epidemiological study has not been performed, there are no reports of increased incidences of CDH among those populations. A particular explanation might be the lack of birth defect registries in most of these countries.

***Origin in the transport between mother and child***

The placenta maintains adequate foetal retinol levels even in the presence of low vitamin A reserves in the mother.[28] It has been suggested that a problem in maternal-foetal transport by the placenta causes vitamin A deficiency in the child. Some studies reported weak but significant relationships between maternal and newborn retinol levels,[20,29] while others do not.[21] This variability suggests that the foetus has its own regulation of vitamin A status that is relatively independent of maternal levels. In experiments with transgenic mice producing human RBP, no maternal RBP was transferred over the placenta and the foetus produced its own RBP.[30] From the perspective of developmental biology, however, the defect in the primordial diaphragm already exists before placental circulation

commences. When the primordial diaphragm develops, nutritional delivery to the embryo is mainly by passive diffusion, metabolic functions of the amniotic membranes, or the "uterine milk".[31-33] The regulation of foetal vitamin A homeostasis during the first weeks of development is unclear but is independent of the placenta.

***Considerations regarding measurements***

Plasma retinol concentrations are maintained at a constant level over a wide range of vitamin A intakes[34] and typically do not change until hepatic stores are almost depleted.[35] During pregnancy, maternal retinol concentrations are kept at the same level throughout all trimesters.[35,36] Therefore, retinol levels at the end of pregnancy are well correlated with levels in the first trimester and the preconceptional period. Embryonic serum levels in the first weeks of pregnancy are not known and are impossible to determine. However, the vitamin A status of the embryo is supposedly dependent on the status in the surrounding tissues and the vitamin A status of mother. The correlation between vitamin A levels in amniotic fluid and in maternal serum is unclear.[37,38] Ideally, to clarify the relationship between CDH and the retinoid pathway, vitamin A status should be investigated at the beginning of pregnancy or early in the first trimester, when the initial pathogenic processes take place. However, a prospective study is currently not feasible for a rare disease such as CDH, in the absence of clear indicators of a cohort that is predisposed to CDH.

The optimal method for determination of vitamin A status is by measurement of serum retinol and RBP levels.[39] Although the liver is the main storage site for retinol, a liver biopsy was not performed for obvious ethical reasons. Beta-carotene levels were not analyzed because serum values depend on the fasting state and the previous meal. In contrast, retinol is a more stable marker and is easily separated from its esters through HPLC. Consequently, fasting and nonfasting values should not be significantly different.[39] Standardized sampling and storage ensured the stability of retinol and RBP and thus the reliability of the levels determined. This ensured that the observed effect is not attributable to differences in sampling between the case and control groups. The validity of our measurements is further supported by the fact that the levels we found for healthy newborns and all mothers were comparable to those in other studies (table 3.4). In contrast, the levels of RBP were lower than those in other reports. This can be explained by the fact that we used an ELISA method to determine RBP levels, whereas other authors used nephelometry or radial immunodiffusion. Detection levels of these methods vary.

***Considerations regarding specificity of defects***

In about 60% of CDH cases, there are no associated anomalies. It is significant that perturbations to diaphragm, lung and cardiac tissue in the rat models occur at a time (embryonic days 10-12) when there is a severe dip in retinol levels because of acute

increases in retinol utilization.[40] Therefore, the embryo may be particularly susceptible to perturbations of retinoid levels or function during that period. It is conceivable that the 'safety margin' for retinoid-mediated regulation of primordial diaphragm, lung and heart development is relatively narrow and therefore those tissues are more susceptible to perturbations, compared with other tissues that are influenced by retinoid signalling. In our population, we found no correlation between levels of retinol and RBP and the observed versus expected lung area-to-head circumference ratios (O/E LHR), a validated measure of prenatal lung development. In both human and nitrofen-induced CDH, the spatiotemporal expression of nuclear retinoic acid receptors is normal .[41] Given the clear role of retinoids during all stages of lung development[42] and the associated lung hypoplasia in CDH, it might be interesting to study the potentially beneficial effects of vitamin A supplementation for patients with CDH, as has been suggested for bronchopulmonary dysplasia and very low birth weight infants.[43]

The fact that left- versus right-sided diaphragm defects differ in occurrence rates and timing of retinoid perturbation in rodent models may reflect relative differences in susceptibility and timing of primordial diaphragm retinoid signalling. In our study, the patient with right-sided CDH had relatively low retinol and RBP levels and the double-sided CDH higher levels, compared with left-sided CDH. Statistical testing was not useful with these small numbers. It will be of interest to analyze data from a much larger population base to determine whether there is a correlation between the degree of vitamin A compromise and the occurrence of multiple anomalies associated with CDH.

## Conclusions

The pathogenesis of CDH has been largely unexplained. This study demonstrated a significant association between low cord retinol and RBP levels and CDH in newborns, whereas vitamin A status in mothers was comparable. Therefore, our study provides no foundation for suggesting a maternal vitamin A deficiency as a risk factor for CDH, although confirmation in larger studies is needed. These data emphasize the need for better understanding of retinoid homeostasis in the embryo during the first 5 weeks of gestation. Furthermore, regulators of retinoid signalling and related genetic transcriptional factors during early embryogenesis become prime targets in ongoing genetic screening for CDH-related abnormalities. New treatment options might be considered, given the role of vitamin A in lung development and the low retinol levels in newborns with CDH.

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# Chapter 4

## Dietary vitamin intake during pregnancy and the risk of congenital diaphragmatic hernia in the offspring

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## Abstract

**Objective:** This case-control study aimed to investigate the association between maternal vitamin A intake and congenital diaphragmatic hernia (CDH) in the offspring.

**Methods:** Maternal vitamin A intake during pregnancy was investigated with a food frequency questionnaire and serum retinol and retinol-binding protein. Risk estimates were calculated and corrected for potential confounders with univariate and multivariable logistic regression models.

**Results:** Thirty-one cases and 46 controls were included. After stratification in BMI categories, normal weight case mothers showed lower energy-adjusted vitamin A intakes (685 vs. 843 µg retinol activity equivalents / day; p=0.04) and slightly lower retinol levels (1.58 vs. 1.67 mmol/l; p=0.08). Vitamin A intake < 800 µg (daily recommended intake) was associated with a significantly increased CDH risk (odds ratio 7.2; 95% confidence interval 1.5-34.4; p= 0.01).

**Conclusion:** Our findings highlight a significant association between maternal dietary vitamin A and CDH in the child.

## Introduction

Congenital Diaphragmatic Hernia (CDH) is a severe developmental anomaly with a birth prevalence rate of approximately 1 in 3000.[1] CDH originates when the diaphragm fails to close during early embryogenesis resulting in a hole in the diaphragm.[2] The mortality and morbidity of CDH is considerable, mostly because of the underlying pulmonary hypoplasia and gastro-intestinal sequelae.[3]

In the vast majority of patients the cause of CDH is unknown. Genetic defects and candidate genes have been identified in animal models, but only in a small subset of patients with CDH.[4,5] The phenotypic and genetic heterogeneity in CDH patients may be explained best by a multifactorial aetiology in which genetic factors provide a background for harmful periconceptional exposures to disrupt early diaphragm development.

Evidence is accumulating on the involvement of maternal nutritional intake in the pathogenesis of CDH.[6] The intake of B-vitamins, such as synthetic folic acid and food folate, reduces the risk of several birth defects, including CDH.[7-9] Furthermore, a low maternal intake of vitamin E is associated with an increased risk of CDH in humans.[9] This is supported by the finding in the nitrofen rat model that maternal vitamin C and E supplementation during pregnancy can rescue the associated lung and heart malformations.[10] One nutritional factor that currently receives the most attention is vitamin A, a fat soluble vitamin that functions as a signalling molecule in gene transcription. Both low and high vitamin A levels in the mother have been associated with an increased risk of birth defects. Hypovitaminosis A only, however, has been related to CDH in animal models and humans.[6] In these animal models a CDH-like phenotype has been linked to a deficient maternal intake of vitamin A,[11,12] teratogenic disturbances of retinoid homeostasis,[13,14] genetic defects in retinoid signaling,[15] and the expression of retinoid-related genes in the primordial diaphragm.[16] Furthermore, a strong argument for the involvement of vitamin A in the pathogenesis of CDH is the reduction of defects after retinoid administration.[17-19] In humans, CDH has been linked to frequently deleted or duplicated chromosomal regions that contain genes related to the vitamin A pathway.[4,20] Newborns with CDH have reduced levels of retinol and retinol binding protein (RBP) in cord blood as compared to healthy newborns.[21,22] However, in contrast to the animal models, retinol and RBP levels appear not to be decreased or even elevated in the mothers of these children at birth.[21, 22] Of interest is the reported trend for periconception low intake of retinol in association with an increased risk of CDH in the child.[9]

From this background we suggest that CDH is associated with maternal dietary intake of vitamin A during pregnancy. Because of the relatively small number of CDH patients a prospective cohort study from preconception onwards is not feasible. We therefore conducted a case-control study with risk set sampling at the moment the child with CDH

was diagnosed to investigate the association between maternal dietary intake of vitamin A and CDH risk.

## Materials and methods

### Study population

The HERNIA study (Congenital Diaphragmatic Hernia, Environment, Retinoids, Nutrition, Inheritance and other Associations) is a hospital-based case-control study conducted at Erasmus MC University Medical Center Rotterdam. The study protocol was approved by the Central Committee of Research in Human in the Hague, the Netherlands, and by the Medical Ethical Committee of Erasmus University Rotterdam. Written informed consent was obtained from every parent and on behalf of their child.

The case triads (child, mother and father) were enrolled immediately after the diagnosis of CDH by prenatal ultrasound or after birth. Controls were recruited by risk set sampling at the moment of diagnosis of a child with CDH from the same outpatient clinic. To increase homogeneity, the controls were selected with comparable maternal age, parity, ethnicity and fertilization. The main exclusion criterium for controls was a congenital anomaly in the child. Directly after inclusion both parents completed a general questionnaire on life-style factors, exposures, socio-economic status and vitamin use. Furthermore, mothers completed a food frequency questionnaire (FFQ) covering the preceding four weeks. The data are derived from the case and control inclusions between 2006 and 2009 and for the current analysis the maternal data only are evaluated.

### Data-collection

To estimate the dietary intake of energy, macronutrients and vitamin A we used a validated semi quantitative FFQ that covered the intake of the previous 4 weeks at the moment of inclusion.[23] By taking into account the covariates nausea, vomiting, and the use of a special diet during the first trimester the nutritional intake at the study moment is assumed to reflect the intake during the first trimester when CDH develops. The FFQ has been updated twice and adapted based on the data from the Dutch National Food Consumption Surveys of 1992 and 1998. The FFQ has been validated twice and has been shown reliable for the estimation of dietary intake of energy, macronutrients, folate and vitamin $B_{12}$ in women of reproductive age.[23-25] The FFQ consists of 121 items and covers 90% of the daily intake of foods or nutrients. Preparation methods, portion size, additions and frequency of foods (per day, per week, per month or not at all) are noted. The average daily nutrient intake is calculated by multiplying the frequency of consumption of food items by portion size and nutrient content per gram based on the 2006 Dutch food composition table. The vitamin A

intake is estimated in Retinol Activity Equivalents (RAEs) consisting of retinol, α-carotene, β-carotene and β-cryptoxanthin, using the conversion formula 1 RAE = 1 µg retinol = 12 µg β-carotene = 24 µg α-carotene and β-cryptoxanthin.[26] We used "RAE intake" or "vitamin A" intake.

At the moment of inclusion, non-fasting venous blood samples were collected in lithium-heparin tubes, protected from light by wrapping in aluminium foil and kept refrigerated (4°C) until further processing. As soon as possible after collection plasma was processed by centrifugation (15 min at 1800 x *g*), aliquotted into amber tubes and stored at -80°C. The level of retinol was measured with reversed phase high–performance liquid chromatography (HPLC) and RBP was measured by using an Enzyme–Linked Immuno Sorbent Assay (ELISA), as described previously.[22]

Maternal Body Mass Index (BMI) was calculated from height and weight before pregnancy using the standard formula weight (kg) / height x height (m). We defined three categories: underweight (BMI <20 kg/m$^2$), normal weight (BMI 20-25 kg/m$^2$) and overweight (BMI >25 kg/m$^2$). Gestational age was calculated from the last menstrual period or based on ultrasound examination and noted up to the date the FFQ was completed.

**Data-analysis**

We evaluated underreporting of energy intake.[27] The basal metabolic rate (BMR) was calculated with the Schofield equations.[28] The individual BMR was increased with 0.5MJ because pregnant women have a higher BMR of 0.5MJ/day on average.[29-32] The physical activity level (PAL) was calculated by the ratio of the reported energy intake (EI) EI / BMR.[27] The PAL cut-off point used for evaluation of underreporting was decreased with 10% to 1.305, because pregnant women have a 10% lower PAL compared to non-pregnant women.[29,31-33]

For comparison of the general characteristics between cases and controls, the unpaired student's t-test was used for the normal distributed continuous variables, and the chi-square test for categorical variables. The intake of various nutrients (medians) was compared between cases and controls by the Mann-Whitney U test. Odds ratio's (ORs) for the association between CDH and nutrient intakes were estimated using univariate and multivariable logistic regression models to correct for potential confounders and the use of vitamin supplements. We estimated the OR using the recommended daily intake (RDI) per nutrient as cut-off point. All analyses were performed with statistical software (SPSS 15.0, IBM, Chicago, USA).

**Table 4.1** | General characteristics of case and control mothers at the moment of CDH diagnosis and inclusion.

| Characteristic | Case (n = 31) mean ± SD / n (%) | Control (n = 46) mean ± SD / n (%) | p-value crude | p-value corrected[$] |
|---|---|---|---|---|
| At the study moment: | | | | |
| Age (years) | 32 ± 4.99 | 35 ± 4.29 | 0.004** | 0.021* |
| BMI category | | | | |
| Underweight | 1 (3.2) | 0 (0) | 0.444 | |
| Normal weight | 17 (54.8) | 26 (56.5) | | |
| Overweight | 8 (25.8) | 14 (30.4) | | |
| Unknown | 5 (16.1) | 6 (13.0) | | |
| Gestational age (weeks) | 32 ± 8.10 | 28 ± 8.76 | 0.100 | 0.100 |
| Parity | | | 0.001** | |
| 0 | 21 (67.7) | 14 (30.4) | | |
| ≥ 1 | 10 (32.3) | 32 (69.6) | | |
| Ethnicity | | | 0.553 | |
| Dutch | 27 (87.1) | 42 (91.3) | | |
| Non-Dutch | 4 (12.9) | 4 (8.7) | | |
| Education | | | 0.028* | 0.039* |
| Low | 22 (71.0) | 21 (45.7) | | |
| High | 9 (29.0) | 25 (54.3) | | |
| Basal Metabolic Rate (kJ/day) | 6776 ± 864 | 6690 ± 768 | 0.677 | |
| Physical Activity Level | 1.47 ± 0.43 | 1.54 ± 0.58 | 0.597 | |
| Periconceptional: | | | | |
| Folic acid use | | | 0.372 | |
| No | 8 (25.8) | 8 (17.4) | | |
| Yes | 23 (74.2) | 38 (82.6 | | |
| Multivitamin use | | | 0.539 | |
| No | 10 (32.3) | 18 (39.1) | | |
| Yes | 21 (67.7) | 28 (60.9) | | |
| Smoking | | | 0.154 | |
| No | 23 (74.2) | 40 (87.0) | | |
| Yes | 8 (25.8) | 6 (13.0) | | |
| | | | | |
| In the first trimester | | | | |
| Nausea | 21 (67.7) | 30 (65.2) | 0.82 | |
| Vomiting | 8 (25.8) | 11 (23.9) | 0.85 | |
| Diet | 2 (6.5) | 4 (8.6) | 0.72 | |

Values are means ± SD or numbers (%). * Significant at $p < 0.05$. ** Significant at $p < 0.001$. [$]corrected for parity

## Results

We included 31 cases and 46 controls for analyses. Table 4.1 displays the general characteristics of the case and control population. Maternal age, parity and level of education were higher in the control group compared to the case group. These covariates are correlated with nutritional intake. Moreover adjustment for parity made the differences in the other 2 covariates disappear. Because of potential overadjustment, we did not adjust the associations with parity. The gestational period of 4 weeks before the inclusion moment at which the FFQ referred was similar in cases and controls.

The intakes of energy, macro nutrients and vitamin A intake in cases and controls are presented in table 4.2. The adjusted Goldberg equation was used to evaluate underreporting of energy intake. The mean PAL for the total study population was 1.51 and comparable between cases and controls (1.47 versus 1.54; p=0.60). With the PAL being higher than the cut-off (1.305), there was no evidence for underreporting. An additional trend analysis revealed a significant trend for a decrease in PAL with an increase in BMI. The PAL for case and control mothers with normal weight was 1.55 versus 1.67, for mothers with overweight 1.25 versus 1.30, respectively (p=0.74). These values were not statistically different between cases and controls and suggested underreporting of energy intake in overweight case and control mothers.

**Table 4.2** | Maternal dietary intake and biomarkers for vitamin A intake.

| Nutrient | Case (n = 31) | | Control (n = 46) | | p-value[1] | p-value adjusted[#] |
|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | | |
| Energy (kJ/day) | 9636 | 5659-19395 | 9188 | 5836-23484 | | 0.633 |
| Total fat (En%) | 35.5 | 29.0-43.3 | 35.5 | 22.70-46.63 | | 0.876 |
| Saturated fat (En%) | 13.2 | 10.5-16.4 | 13.1 | 8.4-18.0 | | 0.950 |
| MUFA (En%) | 11.1 | 7.4-15.1 | 11.3 | 6.1-17.0 | | 0.724 |
| PUFA (En%) | 6.5 | 4.3-10.4 | 6.9 | 2.2-10.9 | | 0.526 |
| Protein (En%) | 15.2 | 11.1-17.6 | 15.3 | 8.2-20.8 | | 0.884 |
| Carbohydrates (En%) | 49.8 | 43.3-59.1 | 49.5 | 33.3-61.3 | | 0.740 |
| Retinol equivalent (µg/day) | 695 | 323-1737 | 805 | 276-2235 | 0.436 | 0.387 |
| **Biomarkers[2]** | **Mean** | **SD** | **Mean** | **SD** | | |
| Retinol (µmol/l) | 1.62 | 0.26 | 1.65 | 0.34 | 0.75 | |
| RBP (mg/l) | 10.79 | 4.07 | 10.84 | 4.89 | 0.97 | |

MUFA = Mono Unsaturated Fatty Acids. PUFA = Poly Unsaturated Fatty Acids. # Adjusted for energy intake. [1] macro nutrients are displayed in energy percentage (en%), [2] Retinol 25 cases and 42 controls. RBP 25 cases and 42 controls

**Table 4.3** | Retinal Activity Equivalent intake per BMI category.

| | RAE intake (µg/day) Median (min – max) | | | | | | RAE intake < 800 µg/day | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Case | n | Control | p-value | p-value adjusted# | Case / control | OR crude (95% CI) | p-value | OR adjusted# (95% CI) | p-value |
| Total population | 26 | | 40 | | | | 20/24 | 1.9 (0.8-4.8) | 0.176 | 2.3 (0.8-6.4) | 0.122 |
| Underweight | 1 | 698 | 0 | | - | - | | | | | |
| Normal weight | 17 | 685 (328-1737) | 26 | 843 (326-2123) | 0.053 | 0.035* | 12 / 8 | 5.4 (1.4-20.5) | 0.013* | 7.2 (1.5-34.4) | 0.013* |
| Overweight | 8 | 734 (486-1229) | 14 | 613 (276-2235) | 0.238 | 0.238 | | | | | |

[#] Adjusted for energy intake. * Significant at $p < 0.05$.

**Table 4.4** | Serum retinol and RBP levels during pregnancy in case and control mothers, stratified by BMI category. Adjustment for parity did not change significance.

| | Underweight | | Normal weight | | Overweight | |
|---|---|---|---|---|---|---|
| | Case (n=1) Mean (SD) | Control (n=0) Mean (SD) | Case (n=16) Mean (SD) | Control (n=25) Mean (SD) | Case (n=7) Mean (SD) | Control (n=14) Mean (SD) |
| Retinol (µmol/l) | 2.05 | – | 1.58 (0.26) | 1.67 (0.37) | 1.64 (0.24) | 1.64 (0.31) |
| | – | | p=0.08 | | p=0.99 | |
| RBP (mg/l) | 13.90 | – | 10.10 (4.53) | 10.79 (4.06) | 12.11 (3.13) | 11.41 (6.06) |
| | – | | p=0.31 | | p=0.78 | |

We found no differences in crude and energy adjusted nutrient intakes between cases and controls. The proportion of carotenoids within the RAE was comparable in cases and controls (0.23 vs. 0.24; p=0.20). Adjustment for parity did not change this outcome. Because of underreporting in some BMI categories, the nutrient intake was stratified into BMI categories. In case mothers with normal weight, energy-adjusted RAE intake was significantly lower (685 vs. 843 µg/day; p=0.04) (table 4.3). CDH risk was significantly increased in normal weight mothers with RAE intake below the Dutch DRI of 800 µg per day (OR 5.4; 95%CI 1.4-20.5; p=0.01; energy-adjusted OR 7.2; 95%CI 1.5-34.4; p= 0.01) (table 4.3). After adjustment for maternal age and SES, this risk remained significantly increased (OR 1.6; 95%CI 1.14 – 20.79; p=0.03). Although the overall serum levels of case and control women were comparable (table 4.2), stratification in BMI categories showed slightly lower levels of retinol in case and control mothers with normal weight, albeit not significant (table 4.4).

## Discussion

This case-control study demonstrates a significantly lower dietary vitamin A intake in case mothers with normal weight during pregnancy. In line with our previous finding in the same study population, we observed a lower serum retinol and RBP in case mothers, albeit not significantly, compared with control mothers with normal weight.[22] Vitamin A intake below the daily recommended intake (DRI; 800 µg) appeared to be strongly associated with an increased CDH risk in the offspring in the normal weight population.

Vitamin A and its metabolite retinoic acid have a crucial role in lung development,[34] and, since pulmonary function is diminished in CDH, the relevance of our data is underlined by the recent description that maternal vitamin A repletion in a vitamin A deficient population improves lung function in the offspring.[35]

Our findings are in line with the data from animal models in which a complete removal of vitamin A from the maternal diet is used to induce CDH.[11,12,36] The diet is the only source of vitamin A in humans and other mammals. While vitamin A deficiency is an endemic problem throughout the developing world,[37] there are no reports of an increased incidence of CDH amongst those populations. However, a detailed epidemiological study has not been performed and most of these countries lack registries of birth defects.

The differences in vitamin A intake and the increased risk estimate were only observed in mothers with normal weight. This can partially be explained by the observation that energy intake was underreported in the BMI category ‘overweight’, which has also been observed by others.[38-40] The underreporting was non-differential for case or control mothers. Due to underreporting the estimation of the nutrient intake is less reliable in

the “overweight” group and may disturb the inferences based on the total population. Yang et al. have described a trend for increased CDH risk in association with lower vitamin A intakes, but this was not significant. The authors did not correct for BMI and did not specifically investigate the mothers with normal weight.[9]

We found a difference in parity between case and control mothers. If parity is considered a proxy for nutrition, we should have corrected for this difference. However, the underlying mechanism for a relationship between parity and nutrition is unclear. Further, we measured nutrition and adjustment for the proxy (i.e. parity) would be overcorrection. We therefore chose not to adjust for parity. In addition to this, the risk on CDH with an intake under the DRI remained significant after adjustment for parity with a bit smaller 95% CI.

The observed difference in age and parity between cases and controls can be partially explained by the fact that older women are more likely to have given birth previously. We found a lower educational level in the case group. A low socioeconomic score (SES) may be related to an increased risk of non-chromosomal congenital anomalies,[41] but others have not identified a variation for SES in CDH.[42,43] Furthermore, a low SES is related to a poorer nutrition and in the etiological pathway it precedes nutritional intake in the association with congenital anomalies.[44,45] Still, after adjustment for these factors, the risk estimate remained significantly increased.

The biomarkers retinol and RBP were measured at the study moment reflecting the vitamin A intake of the previous 4 weeks determined with the FFQ. The serum levels of retinol and RBP per BMI category however were not significantly different between cases and controls. In an earlier report, we described that the serum levels at birth were comparable in case mothers and controls.[22] This is in contrast to one report with lower numbers showing higher levels in case mothers at birth.[21] However, in this study the levels of retinol and RBP were not stratified for BMI category. The absence of an effect of the vitamin A intake on the serum levels might be explained by the fact that only a small part of the intake is reflected in the blood retinol concentration. This is due to the tight regulation of serum retinol, a mechanism that is conserved during pregnancy.[46,47] Further, this explains why the correlation between the estimated vitamin A intake and serum level of retinol is generally found to be low or absent, whereas the level of β-carotene has a better correlation.[48,49]

The assessment of vitamin A intake using the RAE does not discriminate between the different forms of vitamin A and might neglect the other physiologic functions of carotenoids. These functions are unique and may be relevant with respect to the development of the embryo.[50] The accuracy of nutrient calculation depends on the representativeness of the food composition database. Moreover, the size, growth, processing, storage, cooking and genetic variability of vegetables and fruit may lead to differences in carotenoid levels

between food composition tables.[50] In our study the recall period of the FFQ is 4 weeks, which is an optimal time period to account for the large day-to-day variation of intake of vitamin A. The questionnaire was filled out during the whole year to account for the large seasonable variation in carotenoids.[50] Moreover, in our study, the RAE was determined largely by the same food products in both cases and controls and the retinol/carotenoid rate was comparable. Nevertheless, it would be interesting to investigate the intake of retinol and carotenoids separately in further research.

A strong point of this study is the adjustment of the Goldberg equation (underreporting of energy intake) for BMR and PAL, which was based on several reports that described an increased BMR and a decreased PAL during pregnancy.[30,33,51,52] The finding that nutritional underreporting increased together with increase of BMI, corresponds with other studies.[38-40] and improved the reliability of our data.

In conclusion, maternal intake of vitamin A under the RDI may be a risk factor for CDH in normal weight mothers. For validation of these results large cohort studies are needed.

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# Chapter 5

## Biomarkers of the methylation pathway in association with congenital diaphragmatic hernia

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Submitted

## Abstract

**Introduction:** Homocysteine is a derivative of the methylation pathway and increased concentrations have been related to the aetiology of neural crest-related congenital anomalies. The embryogenesis and phenotypic features of Congenital Diaphragmatic Hernia (CDH) patients suggest that the neural crest and the methylation pathway are involved in its aetiology. We investigated maternal and child characteristics and the correlation with some of the biomarkers of the methylation pathway in cord blood, and the relation between the biomarkers and CDH risk.

**Methods:** In 22 CDH and 28 control newborns standardized questionnaires and measurements were conducted to obtain general characteristics of the mother and child. The biomarkers homocysteine (tHcy), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were determined in newborn cord blood. Correlations between maternal and newborn factors were investigated. Univariate and multivariable logistic regression analyses were performed to assess crude and adjusted risk estimates for CDH.

**Results:** Case children had a lower weight (2962 vs. 3418 gram; $p<0.001$) and gestational age (270 vs. 277 days; $p=0.006$) at birth due to the obstetrical policy to induce labour in case mothers at 38 weeks. Control mothers were slightly older (32 vs. 35 year; $p=0.05$). Other characteristics were comparable between case and control children and mothers. The concentrations of homocysteine, SAM and SAH, and the SAM/SAH ratio were comparable in case and control cord blood (tHcy: 8.57 vs. 8.56 µmol/l, $p=0.99$; SAM: 152.7 vs. 157.3 nmol/l, $p=0.76$; SAH: 43.5 vs. 48.9, $p=0.26$; ratio: 3.8 vs. 3.5 $p=0.50$). The characteristics of children and mothers were not correlated to the levels of the biomarkers.

**Conclusion:** The biomarkers of methylation determined after delivery in cord blood are not associated with CDH risk. Maternal and child characteristics could not predict newborn biomarker concentrations of methylation.

## Introduction

Disturbances in maternal homocysteine concentrations have been linked to the risk of neural crest related birth defects.[1] Low maternal concentrations of folate and/or vitamin B12 are also associated with the risk of these birth defects. Homocysteine (tHcy), B-vitamins and S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are biomarkers of the methylation pathway. Homocysteine is formed by the hydrolysis of SAH, which in turn is the demethylated product of SAM. SAM is an important methyl group donor and plays a role in many transmethylation reactions of compounds such as DNA, RNA and proteins. The SAM/SAH ratio, or methylation index, is a reflection of the balance between SAM production and demethylation to SAH and is decreased in association with hyperhomocysteinemia.[2] Hypomethylation has been related to changed DNA methylation and gene expression and the SAM/SAH ratio or SAH would be a better measure of methylation potential than total homocysteine.[3]

The use of a folic acid-containing supplement reduces increased homocysteine concentrations and SAM/SAH ratio which explains in part the preventive effects against the occurrence of neural crest related birth defects, such as neural tube defects, congenital heart defects and orofacial clefts in the child.[4-6] The patterns of associated birth defects in experimentally induced and human Congenital Diaphragmatic Hernia (CDH) show similarities with neural crest-related birth defects.[7,8] Furthermore, a high maternal dietary intake of B-vitamins seems to reduce the risk of CDH in the child.[9,10] This is further supported by a study in mice, in which maternal folate deficiency led to an abnormal cell count and structure of the diaphragm.[7] The current study aimed to investigate the biomarkers of the methylation pathway in a nested cross sectional case-control design at delivery with CDH-affected and healthy newborns.

## Methods

Within the HERNIA-birth cohort study,[11] we analyzed venous or venous-arterial cord blood from 22 CDH and 28 healthy newborns (table 5.1) in a highly standardized manner. Cord blood samples were wrapped in aluminium foil and kept refrigerated (4°C). As soon as possible after collection, samples were further processed by centrifugation at 1,800 x *g* for 15 minutes, the plasma was separated into aliquots and stored in amber tubes (Bioplastics®) at -80°C. Homocysteine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were measured by an automated LC-MS/MS method adapted from Gellekink et al.[12] as described by van Driel et al.[13] There was no relationship between the concentrations of tHcy, SAM, SAH and the time elapsed until storage at -80°C (results not shown). Because

**Table 5.1 |** Mean values of the biomarker concentration of methylation and risk estimates using odds ratios (exp β) crude and adjusted for birth weight.

| | Mean values | | | | | Risk estimates | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Case | | Control | | | Crude | | | Adjusted | | |
| | N | mean (SD) | N | mean (SD) | p | exp β | 95%CI | p | exp β | 95%CI | p |
| tHcy (µmol/l) | 22 | 8.57 (3.05) | 28 | 8.56 (3.01) | 0.59 | 1.001 | 0.830-1.208 | 0.99 | 1.100 | 0.861-1.405 | 0.45 |
| SAM (nmol/l) | 22 | 152.68 (47.54) | 28 | 157.29 (55.93) | 0.76 | 0.998 | 0.988-1.009 | 0.75 | 0.998 | 0.984-1.012 | 0.78 |
| SAH (nmol/l) | 22 | 43.51 (15.71) | 28 | 48.93 (17.37) | 0.26 | 0.980 | 0.945-1.015 | 0.26 | 0.989 | 0.947-1.033 | 0.99 |
| SAM/SAH ratio | 22 | 3.76 (1.30) | 28 | 3.50 (1.48) | 0.50 | 1.152 | 0.763-1.738 | 0.50 | 1.060 | 0.651-1.726 | 0.82 |

the concentrations were not significantly different between venous and veno-arterial cord blood all results of the samples were pooled for statistical analysis. Self administered questionnaires on general characteristics of mother and child were completed by the mother at home and checked for completeness and consistency by the researcher.

## Results and Discussion

The maternal characteristics BMI (22.6 vs. 22.8 kg/m$^2$; p=0.86), ethnicity (68% vs. 86% Dutch; p=0.07), parity (50% vs. 40% nulliparae; p=0.45) and delivery mode (50% vs. 32% spontaneous; p=0.20) were comparable in cases versus controls. Maternal age was 32 years in cases and 35 years in controls (p=0.05). In children, gestational age (270 vs. 277 days, p=0.006) and birth weight (2962 vs. 3418 gram; p<0.001) were lower in cases versus controls. This can be explained by the obstetrical policy to deliver case mothers at 38 weeks of gestation. No case or control children were diagnosed with associated anomalies or major genetic defects.

The concentrations of tHcy, SAM, SAH and the SAM/SAH ratio were not significantly different between cases and controls (table 5.1 and figure 5.1). Crude and birth weight adjusted logistic regression analyses did not reveal increased risk estimates for tHcy, SAM, SAH or SAM/SAH ratio, both continuous as well as dichotomous with cut-off points that were defined by the control population (table 5.1).

The correlation matrix showed significant relationships between maternal age and birth weight (Pearson 0.41; p=0.004), which can be explained by the correlation between maternal age and the case/control status. Further, tHcy and SAH (Pearson 0.41; p=0.003), and tHcy and SAM/SAH ratio (Pearson -0.57; p<0.001) were significantly correlated. In a multivariable regression model no significant associations were observed between the concentrations of tHcy, SAM, SAH or SAM/SAH ratio and the determinants of the mother (age, BMI, smoking, parity, delivery mode, and ethnicity), and of the child (gestational age and birth weight). Although maternal BMI is correlated with SAM/SAH in maternal blood, this study reveals that maternal BMI is not a significant determinant of SAM/SAH in cord blood.

We did not identify a correlation between birth weight and the concentrations of the biomarkers. In the pooled group of cases and controls 3 children were born small for gestational age (SGA). Also in this small subgroup biomarker concentrations were not significantly different from the children with normal birth weight (SGA vs. normal weight: tHcy: 8.83 vs. 8.37 µmol/l, p=0.80; SAM: 156.7 vs. 157.3 nmol/l, p=0.98; SAH: 53.1 vs. 46.7, p=0.53; ratio: 2.98 vs. 3.70 p=0.39).

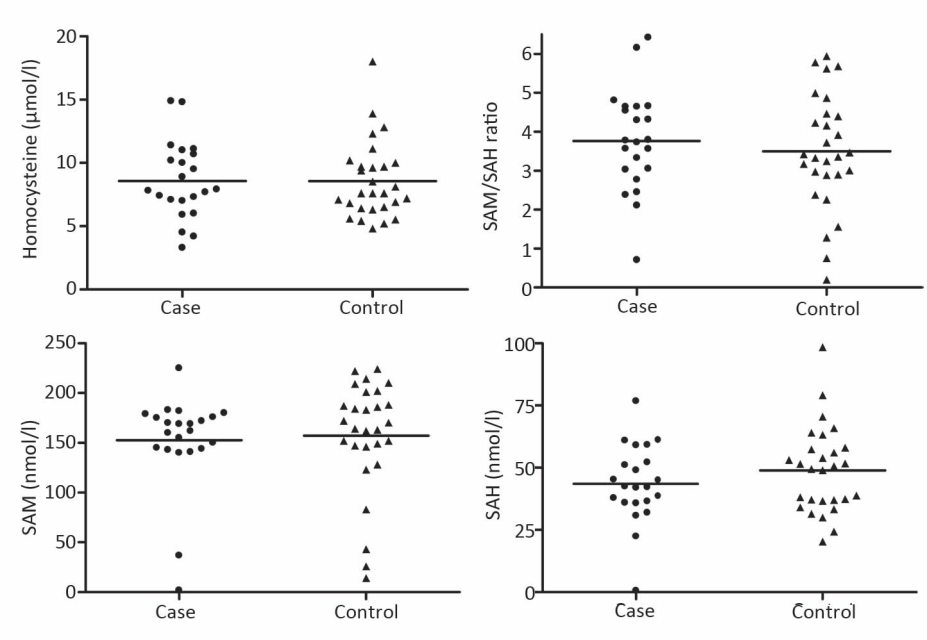


**Figure 5.1** | Concentrations of tHcy, SAM, SAH and SAM/SAH ratio in newborn cord blood. Horizontal lines represent the mean values.

Although derangements in the methylation pathway have been related to several birth defects, so far no studies reported the relationship with the biomarkers determined in cord blood. In contrast, studies on pre-eclampsia and intra-uterine growth restriction revealed that tHcy in cord blood is correlated with the concentration in maternal blood.[14,15]

The maternal use of a folic acid-containing supplement in the second and third trimester lowers maternal and newborn homocysteine levels, which may have distorted the correlations and risk estimates. Unfortunately, these data were not available in our study.

Despite the nested-cross sectional case control design gestational age was significantly different between both populations. This can completely explained by the difference in obstetrical policy between case and control pregnancies. Although we corrected for this difference in a multivariable logistic regression model, this did not lead to significant differences in biomarker concentrations. Despite the homogeneity of the CDH phenotypes, standardized cord blood sampling and analysis, the small sample size limited the estimation of associations.

## Conclusion

In conclusion, this study revealed no differences in the biomarker concentrations of methylation between CDH and control newborns. Moreover, we found no significant maternal or child determinants of the methylation status in newborns with and without CDH. Larger sample sizes are needed to gain more insight into associations between derangements in the methylation pathway and CDH risk.

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# Part II

# Molecular biological studies

# Chapter 6

## Linking animal models to human congenital diaphragmatic hernia

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## Abstract

Congenital Diaphragmatic Hernia (CDH) is a major life-threatening malformation, occurring in approximately 1 in 3000 live births. Over the years, different animal models have been used to gain insight in the aetiology of this complex congenital anomaly and to develop treatment strategies. However, to date the pathogenic mechanism is still not understood, and treatment remains difficult because of the associated pulmonary hypoplasia and pulmonary hypertension. In this review, data available from several animal models will be discussed. The retinoic acid signalling pathway (RA pathway, retinoid pathway) will be addressed as a developmental pathway that is potentially disrupted in the pathogenesis of CDH. Furthermore, genetic factors involved in diaphragm and lung development will be discussed. With this review we aim to provide a concise overview of the current most important experimental genetic data available in the field of CDH.

## Introduction

Congenital Diaphragmatic Hernia (CDH) is a major life-threatening anomaly, occurring in approximately 1 in 3000 live births. This condition not only involves a diaphragm defect but it is also associated with both pulmonary hypoplasia and pulmonary hypertension of varying severity, which usually make treatment difficult. Severe additional anomalies, such as heart defects, also increase the morbidity and mortality of patients with CDH. Mortality rates reported in the literature show a wide range of values because of the differences in patient cohorts (e.g. with or without multiple congenital anomalies), type of treatment centre and source of report, e.g. epidemiologic, genetic or surgical.[1] CDH presents as an isolated disorder in approximately 50% of the affected patients. In isolated CDH, no genetic causation has been identified, and the recurrence risk in these cases is generally suggested to be as low as 1-2%.[2,3] The remaining patients have various other congenital anomalies (non-isolated CDH). In some of these cases a defined syndrome can be diagnosed.[4,5] However, the proportion of cases with associated anomalies varies greatly, from a low of 20-50% to a high of 71%.[6-8] These proportions depend partly on the time of diagnosis of CDH, either pre- or postnatal.

Although features of patients with CDH have been described since the seventeenth century, its aetiology and pathogenesis are not well understood.[9] Presentation varies considerably among patients, but is usually marked by respiratory distress. A very small subset of patients (1%) are asymptomatic; in these patients CDH is often diagnosed coincidentally by radiographic investigations at a variable point in time, sometimes even during puberty.[10]

Part of this variation in phenotype can be explained by the location or type of the defect in the diaphragm: posterolateral (Bochdalek hernia), non-posterolateral (e.g. Morgagni and central hernias), and eventration of the diaphragm (incomplete muscularization). Distinction between these types of hernias can be difficult. Moreover, this variation makes it difficult to identify the aetiology of CDH, and could even represent different pathological mechanisms. Most likely, the aetiology of CDH of a proportion of cases is multifactorial, involving both genetic elements and environmental factors. Different strategies and models used to understand the aetiology of CDH may therefore not cover the whole spectrum of CDH in humans. Yet, experimental evidence from a variety of animal models has clarified some relevant aspects of this complex congenital anomaly. Animal models are used not only for practical reasons, but more importantly because they allow the study of early embryonic development, which is not feasible in humans.

In this review, we discuss human clinical data and experimental data from several animal models. We will show how different models lead to a potentially common pathway. Nevertheless, these experimental findings do not fully explain the pathogenesis of human CDH, whose understanding will require further research.

## Development of the lungs and diaphragm

In human development, the primordial diaphragm (septum transversum) divides the future thoracic and abdominal compartments during the period from embryonic day 22 to week 7, equivalent (e) to embryonic days 11 in the rat and to day 9.5 in the mouse.[11] Around day 26 (rat: e11.5; mouse e9.5), the lung bud arises from the foregut and bifurcates in two bronchial buds, which invade the surrounding mesenchyme. During branching morphogenesis, the interaction between the epithelium and the mesenchyme is essential for spatial formation of the lungs and diaphragm. Traditionally, it is believed that the diaphragm derives from four different embryonic structures.[11,12] The *septum transversum* originally arises as the most cranial part of the mesenchyme. During further development it descends and forms a horizontal partition that attaches ventrally and laterally to the body wall, and dorsally to the foregut mesenchyme. At the posterior wall, the *pleuroperitoneal membranes* cover the pericardioperitoneal canals between weeks 5 and 7 (rat e13.5; mouse e12). Muscle cell precursors (myoblasts) migrate and differentiate within the septum transversum. These cells will eventually form the diaphragmatic muscle and are innervated by nerve fibres originating from cervical levels 3, 4 and 5, which will form the two phrenic nerves. Finally, the *oesophageal mesenchyme* and *paraxial mesoderm of the body wall* give rise to the parts of the definitive diaphragm posterior to the oesophagus and around the body wall, respectively. In the classical view, the defect in CDH occurs in the muscular part of the diaphragm. However, observations in rats have suggested that this classical view of development may have to be revised for both animals and humans.[13-15]

## Pathogenesis of CDH

It is not clear whether the pathologic findings seen in CDH arise from a primary defect in lung development or from abnormal development of the diaphragm. Keijzer et al.,[16] using organotypic cultures in the nitrofen model, formulated the dual-hit hypothesis, proposing that two independent events (hits) cause the major features seen in diaphragmatic hernia. These (unspecified) hits disturb normal lung development (first hit) and diaphragm formation (second hit). Currently, four theories about the mechanism that causes CDH have been suggested. The first theory links malformation of the diaphragm to abnormal development of the ipsilateral lung. Iritani,[17] using the toxicological nitrofen model in rats, showed abnormalities in the ipsilateral as well as the contralateral lung even before the diaphragm starts to develop. The second theory is based on the observation that the phrenic nerve innervating the herniated side is often smaller than the contralateral nerve, hereby causing abnormal diaphragm development. However, it is now known that the

number of motor neurons is proportionate to the amount of innervated muscle tissue and is not diminished until after the period of programmed cell death. Thus, an atrophic phrenic nerve is the consequence rather than the cause of the defective development of the diaphragm.[18] The third theory proposes that fewer myotubes (muscle fibres), or a faulty distribution within the diaphragm, weakens the diaphragm and causes it to rupture. However, animal experiments have not been able to identify such an abnormality.[13] The fourth theory is based on the hypothesis that non-closure of the pleuroperitoneal canals could be caused by a defect in the pleuroperitoneal folds (PPFs), the source of diaphragm cells. Interestingly, the defect in the nitrofen model is located more medial than could be expected from non-closure of these canals. In addition, the diaphragmatic defect is already present before closure of these canals.[19] Therefore, in addition to the fourth theory, it has been proposed that the origin of the diaphragmatic defect lies in the *amuscular* mesenchymal precursor cells of the diaphragm, which are also derived from the PPFs. This is based on the observation that while migration of muscular precursors is not disturbed, a defect occurs in regions of the underlying mesenchymal substratum of the PPF. This would subsequently contribute to the defective region in CDH.[14] This latter theory is quite intriguing, since it may unite several models in the field of CDH.

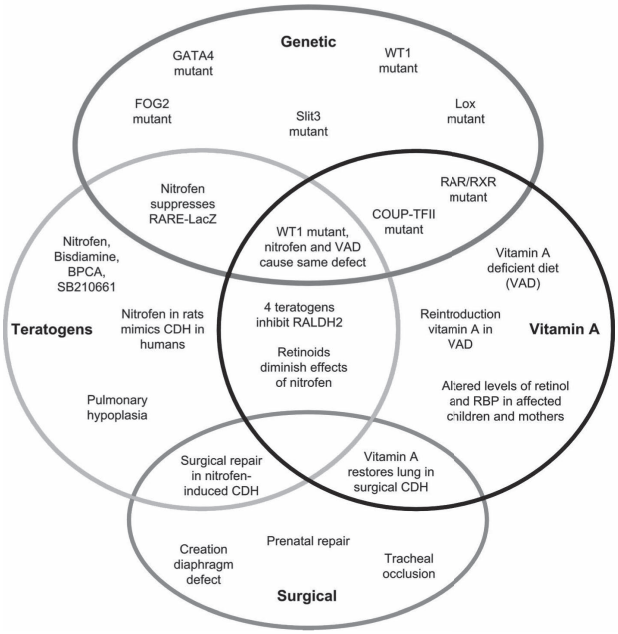


**Figure 6.1** | Overview of different types of animal models used in CDH (represented by circles). Experiments or results are referred to within their respective circles. Interesting new research is being carried out at the overlapping areas. References can be found in the text.

## Linking animal models to CDH in humans

Several animal models for CDH have been developed (figure 6.1). These animal models can be divided into three major types: surgical, genetic and teratogenic. The vitamin A model, in the figure represented by a separate circle, is in fact closely related to the teratogenic model.

The earliest models were surgical models, in which a diaphragmatic defect was surgically created, most often in foetal lambs.[20-23] They revealed that in this model, postnatal repair does not improve survival, but that prenatal repair does.[24] Several of these models have evolved over time and have been used to study therapeutic interventions such as intra-uterine repair of the defect and tracheal occlusion.[24-29] However, these procedures have not yet been able to improve survival or morbidity rates in the foetus and intra-uterine repair is not practiced anymore. Tracheal occlusion is performed only in a research setting, within a selected subset of patients that have a poor prognosis.[28,29] The results of this strategy still remain controversial.

Surgical models, however, have a major flaw from a pathogenic point of view, as they are practiced on animals that have normal lungs and diaphragm before the intervention. They therefore will be less informative about the aetiology and the mechanisms responsible for the diaphragm defect and of the disturbed growth and differentiation of the lung.

### Genetic models

Another type of model, the "genetic animal models", adds important information on these mechanisms. These data, in particular from knock-out mice, can be linked more effectively to human data, because many of the genes affected in knock-out mice reside in regions that are frequently deleted or duplicated in human CDH patients. Several knock-out mouse models for CDH have been described. In many cases, the diaphragm defect was a coincidental finding, as in the Wilms tumour 1 *(Wt1)*-deficient mouse.[30] Mice deficient in *Slit3* have a central CDH associated with cardiac and renal defects, a phenotype also seen in a small subgroup of human patients.[31] In mice, suppression of *Lox* gene expression by targeted mutagenesis produces offspring that exhibit cardiovascular instability with ruptured arterial aneurysms and rupture of the diaphragm.[32] Lox is an enzyme responsible for the cross linking of collagen and elastin. $Lox^{-/-}$ embryos also show impaired development of the proximal and distal airways, but these occur independently of the diaphragm rupture.[33] Copy number changes of some of the human loci of the LOX-like (LOXL) genes (15q24.1, 8p21.3 and 2p13.1) have been described.[34] Double null mutant mice, lacking both the alpha and beta subtype of the retinoic acid receptor (*rar*), have offspring with diaphragmatic hernia.[35,36] According to Greer et al.,[37] these retinoid receptors are expressed in the developing diaphragm.

Other gene knock-out models have been specifically developed to mimic human CDH. Ackerman et al.[38] performed a large mutant screen analysis using ENU mutagenesis and selecting for recessive mutants that had diaphragmatic defects. One of the mutants had a splice site mutation in *Fog2*, resulting in a premature stopcodon. Mice homozygous for this splice site mutation exhibit severe pulmonary hypoplasia, lobar loss, a thin non-muscularized diaphragm (eventration of the diaphragm) and cardiac abnormalities. To date, only one human patient with a nonsense mutation in FOG2 has been identified.[38] This patient was diagnosed with a left-sided eventration of the diaphragm. FOG2 was identified as an important co-factor of the gene GATA4 (GATA binding protein 4). However, mice deficient in *Gata4* die prematurely of cardiac abnormalities, but do not have lung or diaphragm defects.[39-41] Recently, Jay et al.[42] demonstrated that on a different genetic background, heterozygous *Gata4* knock-out mice indeed develop a diaphragm defect, together with lung and heart abnormalities. That defect is similar to the diaphragm defect seen in *Slit3* mutant mice with regard to its location (central), and similar to that in *Fog2* mutant mice with regard to its type (a muscularization defect with eventration of the diaphragm). You et al.,[43] developed yet another CDH model in which tissue-specific ablation of the transcription factor *Coup-tfII* (Chick Ovalbumin Upstream Promotor-Transcription Factor II) produces offspring with posterolateral CDH.

In general, translation of findings from animal models to human CDH is difficult because each model is limited in simulating human CDH. Interestingly, the human location for most of the genes used in these knock-out models, is within regions found to be deleted or duplicated in a proportion of cases of CDH. For example, in humans, COUP-TFII is located on chromosome 15q26. We recently identified the CDH critical region deleted in several human patients with non-isolated CDH. The most interesting candidate gene in this region is COUP-TFII.[44,45] So far, however, we and other groups[46] have not yet identified mutations in the coding region of this gene. Most patients with CDH and a chromosomal anomaly have additional congenital anomalies that correspond to the function of the candidate genes, e.g. cardiac defects associated with deletion of 8p23.1, the GATA4 locus. Several of the known altered chromosomal regions in human CDH patients harbour one or more genes involved in the retinoic acid pathway, e.g. RBP1 and RBP2 on chromosome 3q22 and COUP-TFII on 15q26. Figure 6.2 gives an example of these human candidate loci for which knock-out models have been described.

**Teratogen models**

Studies in rats revealed that the administration of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) to pregnant rats induces congenital anomalies in the offspring, including diaphragmatic defects, pulmonary hypoplasia and immaturity, and pulmonary vascular abnormalities, all remarkably similar to the pathologic findings in the

posterolateral or Bochdalek CDH in humans.[17,19,47-49] A major advantage of this model is that timing of the primary defect is similar to that in humans, i.e. early in embryogenesis. However, the effect of nitrofen on the development of CDH in humans has not been documented, and neither have known teratogens been identified to induce CDH in humans.

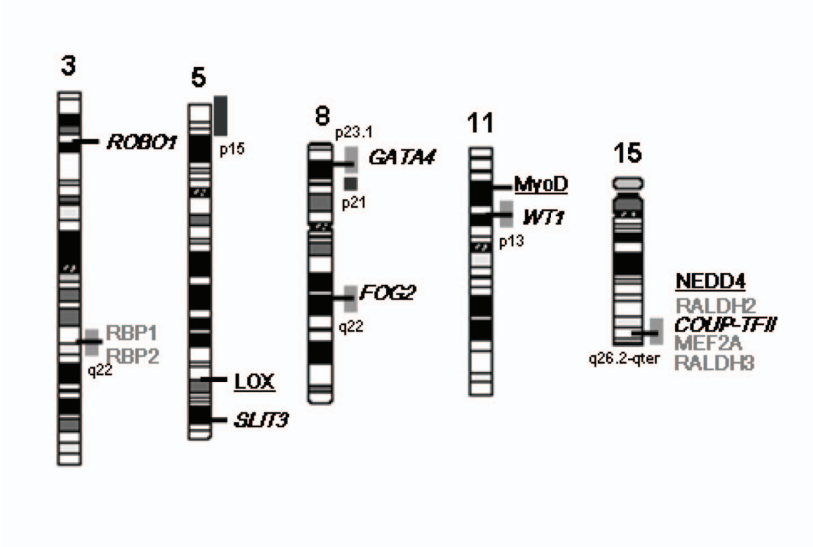


**Figure 6.2** | Most important commonly deleted or duplicated regions in human patients and their candidate genes, indicated by bars beside the chromosomes. Grey bar: deleted region. Black bar: duplicated region. Gene names in italics: muscularization defect in animal model. Gene names underlined: diaphragm defect in animal model. Gene names in grey: no animal model known, candidate gene based on cytogenetic position and/or function.

The pathogenic mechanism of nitrofen, long unknown, has recently been clarified by research into different biological pathways. Manson[50] hypothesized there was a so-called thyreomimic effect and that, because of nitrofen's stereochemical configuration, it would interfere with thyroid hormone status in a competitive way. However, we showed that nitrofen decreases the binding of triiodothyronine (T3) in a non-competitive way *in vitro*, by interacting at the nuclear receptor level.[51] Interestingly, the frequency of malformations in the nitrofen rat diminish if sufficient exogenous T4 is administered, but for herniation rates this reduction was not significant.[50,52] The current opinion is that nitrofen causes a

diaphragm defect by interfering with the retinoid signalling pathway (Figure 6.3). The first evidence of involvement of retinoids (vitamin A derivatives) in the pathogenesis of CDH came from Anderson's and Wilson's classical observations made in the middle of the past century.[53-55] They observed that 25-40% of pups born to dams fed on a vitamin A deficient diet developed a diaphragmatic hernia of the Bochdalek type, on the right side in most cases. When vitamin A was reintroduced in the diet during mid-gestation, the proportion of pups with diaphragmatic hernia diminished. Subsequently, the similarity between the above-mentioned retinoid receptor double null mutant mice[35,36] and nitrofen-treated rats raised the hypothesis that nitrofen interferes with the retinoid signalling pathway. Further evidence came from the observation that vitamin A given at the appropriate time to rats treated with nitrofen, reduces the rate of diaphragmatic hernia in the offspring by 15-30%.[56] The comparable effects of vitamin A and thyroid hormone can be explained by the fact that retinoic acid receptors, thyroid hormone receptors and steroid hormone receptors all belong to the same superfamily of ligand-inducible transcriptional regulatory factors.[57,58] Chen et al.[59] confirmed with a LacZ reporter bound to the retinoic acid response element (RARE), that nitrofen disturbs the retinoic acid pathway. Further investigation by this group showed that nitrofen works by inhibiting retinal dehydrogenase 2 (RALDH2), the major retinoic acid synthesizing enzyme (figure 6.3). Not only nitrofen, but also bisdiamine (N,N'-octamethylenebis-(dichloroacetamide)), BPCA (biphenyl carboxylic acid) and SB-210661 (a benzofuranyl urea derivative) exert their effects through RALDH2.[60] Bisdiamine, the most potent inhibitor of RALDH2, is also the most effective in inducing (left-sided) diaphragmatic defects in embryonic rats.

The finding that vitamin A counteracts the effect of nitrofen is not surprising, since an abundance of substrate (retinol) can shift the derailed enzymatic reaction (oxidation from retinal to retinoic acid by RALDH2) to a higher output of the product (RA). Addition of RA will even more strongly reduce herniation rates, as it bypasses the oxidation by RALDH2. Major et al.[61] published preliminary data showing that children with CDH had significant lower levels of retinol and retinol binding protein (RBP) than controls at birth. Maternal retinol and RBP levels at birth were significantly higher than in controls. A larger group of patients is needed to confirm these findings. Within this context a multicenter international study is underway to tackle this intriguing idea.

So far, specific strains of rodents have different susceptibilities to nitrofen's teratogenicity; for example, the Sprague-Dawley strain is more susceptible than the Wistar strain. However, there are no published epidemiological data that associate exposure to nitrofen with CDH in humans. Even in mouse models it is very difficult to induce CDH with nitrofen, which hampers the possibility of using genetically modified mice in combination with nitrofen or other teratogens to study the pathogenesis of CDH.

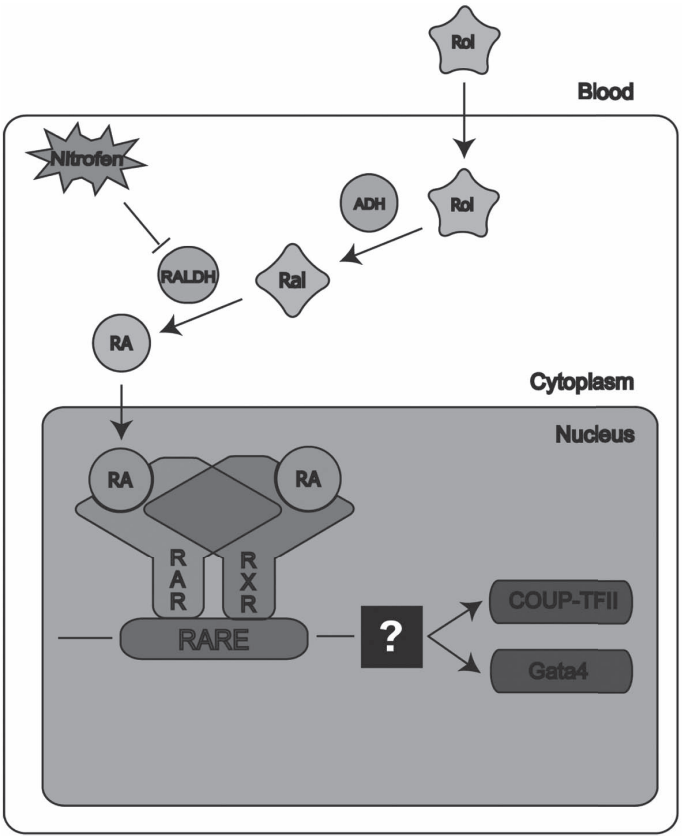


**Figure 6.3** | The Retinoic Acid signalling pathway. Retinol is transported from the blood into the cell (binding proteins not shown). Retinol is oxidized by alcohol dehydrogenases into retinal. Retinal in turn is then oxidized into retinoic acid by retinal dehydrogenases, mainly type 2 (RALDH2), which can be inhibited by nitrofen. Retinoic acid travels into the nucleus where it binds to its nuclear receptors, RAR and RXR. Heterodimerization of RAR and RXR activates the response element in the promoter region upon which transcription of genes is influenced. Some CDH candidate genes proposed to be influenced by RA are mentioned. Rol: Retinol; Ral: Retinal; RA: Retinoic Acid; RAR: Retinoic Acid Receptor; RXR Retinoic X Receptor; RARE: Retinoic Acid Response Element.

## Discussion

Although animal models have clarified some of the processes involved in the normal and the abnormal diaphragm development, they have not addressed the development of abnormal pulmonary vasculature and the ensuing pulmonary hypertension, both of which

are major clinical problems. Data on the effect of vitamin A derivatives on pulmonary vascular development are scarce. Recently we reported that the temporospatial distribution of the glucocorticoid, retinoid and thyroid hormone receptors during normal and abnormal lung development in CDH does not differ between the human CDH and normal lung, nor in nitrofen induced rodent CDH.[62]

However, the role of the retinoid signalling pathway in the pathogenesis of human CDH is worth further exploration, a challenging task because this pathway has numerous functions during pre- and postnatal development, as well as in other processes throughout the human body.[63] Retinoic acid directly influences the transcription of many genes in different pathways during development. Some of these RA-regulated genes are involved in the development of the lungs and diaphragm, including Hoxa4, TGF, N-myc, Shh (directly) and BMP4 (indirectly). Nevertheless, numerous other genes are also important in lung development, such as FGF10 and Foxa2.[64,65] The contribution of retinoids to the development of the diaphragm itself has not been studied extensively yet. Proteins associated with metabolism and binding of retinoids are expressed during diaphragm development – at least in rodents.[60] Further studies in humans are needed to confirm these data.

An important role for retinoic acid has been acknowledged in embryological lung development. From the time evagination of the lung bud takes place, RA and RALDH2 are abundantly present and essential for lung bud formation.[66-69] As the lung develops further, a proximal-distal gradient of RA is present with higher levels of RA in the proximal airway. This could be explained by lower levels of RALDH2, which is essential for the local availability of RA, and by the expression of COUP-TFII, which antagonizes retinoid signaling.[67,70] The retinoid receptors RAR and RXR are ubiquitously expressed in the developing human lung.[71,72] Furthermore, the expression patterns of several subtypes (RARα, RXRα and RXRγ) change with increasing gestational age.[72] Selective knock-out of single isoforms of RAR or RXR only leads to minor abnormalities, suggesting that different receptor subtypes have overlapping functions.[35] Consequently, if RARs or RXRs play a role in the abnormal lung development seen in CDH, more than one receptor subtype is probably compromised, or the defect must be upstream from this step in the retinoid signalling pathway.

The different animal models for CDH produce diaphragmatic defects that are all slightly different in localization. Recently, Clugston et al.[15] showed that three types of models – the teratogenic nitrofen model, the vitamin A deficient rat model (a toxicological or "dietary" model) and the *Wt1* null mutant mice model (a genetic model for CDH) – are characterized by defective development of the PPFs, thus linking different types of CDH research to a shared mechanism. Although the use of models has inherent limitations, it may nevertheless deepen our insight into the temporospatial development of the lungs and diaphragm.

Combining animal models with human data provides stronger evidence for the involvement of the retinoic acid signalling pathway in the aetiology of CDH. However, not all genes altered in these animal models, and not all chromosomal regions found in human patients, have been shown to play a role in this pathway. It follows that researchers should remain alert to possible other pathways involved in the aetiology of CDH.

## Conclusion

Evidence from different animal models suggests that a disturbance in the retinoid signalling pathway is potentially involved in the pathogenesis of CDH. This does not exclude, however, the possibility that other (unknown) pathways may play a role. To date, mutations in specific CDH candidate genes, such as those on chromosome 15q (e.g. RALDH2, RALDH3, COUP-TFII), have not yet been described. Combined data from animal models and human patients are needed to further identify genetic factors relevant to the pathogenesis of CDH. Important steps towards this aim are the establishment in the clinic of routine and prospective harvesting of patients' DNA, chromosomes, cell lines and tissues, and the integration in research of newer molecular genetics techniques, such as array Comparative Genomic Hybridization (array-CGH). Then, eventually, we will be able to unravel the pathogenesis of a disease with one name but with a significant variability in phenotypic presentation.

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# Chapter 7

## The effect of oxygen on the expression of hypoxia-inducible factors in human foetal lung explants

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## Abstract

**Introduction:** Foetal lung development requires a proper coordination between lung epithelial and vascular morphogenesis. A major determinant in lung vascular development is vascular endothelial growth factor (VEGF), which is regulated by hypoxia-inducible factors (HIFs). VEGF is expressed in the airway epithelium, while its receptors (VEGFR) are expressed in the pulmonary mesenchyme. The hypoxic environment *in utero* is beneficial for foetal organogenesis, especially vascular development. However little is known on the expression of HIFs and VEGFR-2 in the human foetal lung *in vitro*. The purpose of this study was to investigate the effects of hypoxia on foetal lung morphology and mRNA expression of VEGF, VEGFR-2, HIF-2α, and HIF-3α.

**Methods:** An explant culture technique was used to study the effects of normoxic and hypoxic conditions on human foetal lung.

**Results:** The morphology remained largely unchanged in explants cultured under hypoxic or normoxic conditions. Quantitative RT-PCR showed that the mRNA expression of VEGF-A, but not VEGFR-2 is upregulated in explants cultured at 1.5% oxygen compared with 21% oxygen. We observed a non-significant increase in HIF-2α and HIF-3α mRNA expression in explants cultured at 1.5% oxygen. These data suggest that the mRNA expression of VEGF, and possibly HIF-2α and HIF-3α, is regulated by hypoxia in the developing human lung.

**Conclusion:** This lung explant culture model appears to be a valuable model to unravel the molecular mechanisms of human lung development.

## Introduction

The molecular basis of pulmonary development has been studied extensively over the past few decades. Many morphogens, growth and transcription factors have been shown to play key roles during different stages of this process.[1-3] Accumulating evidence underlines the importance of vascular development in relation to distal airway growth and development.[4-6] It is important to realize that normal prenatal (pulmonary) development occurs in a hypoxic environment. A number of studies have shown that hypoxia *in utero* is favourable for embryological organogenesis.[5,7-9]

In mammalian systems, the cellular responses to oxygen alteration are mediated by hypoxia-inducible factors (HIFs), which are known to control more than 100 genes.[10] The HIF transcriptional complex is a heterodimer composed of one of the three oxygen-sensitive alpha subunits (HIF-1α, HIF-2α, or HIF-3α) and the constitutive HIF-β subunit, alternatively called ARNT, or aryl hydrocarbon receptor nuclear translocator.[11,12] Under hypoxic conditions, the HIF heterodimer is stable and accumulates into the nucleus where it binds to hypoxia response elements of target genes, thereby regulating their transcription. In normoxia, hydroxylation of the HIF-α subunit by prolyl hydroxylases (PHDs) mediates an interaction with the von Hippel-Lindau protein (pVHL), which subsequently leads to ubiquitination and targeting for proteasomal destruction.[11,13]

One of the most potent hypoxia-inducible growth factors is vascular endothelial growth factor-A (VEGF-A). VEGF-A signals through two high affinity tyrosine kinase receptors, VEGFR-1 (Flt-1), and VEGFR-2 (KDR in human or Flk-1 in mouse). In the lung, VEGF-A functions as a mitogen and differentiation factor for endothelial cells.[14] While VEGF-A is expressed mainly in the epithelial cells of the lung,[15] VEGFRs are expressed in the mesenchymal cells immediately underlying the epithelium and vascular structures.[16] The level of VEGF is critical for normal lung development. Overexpression of VEGF in the distal lung airway epithelium alters vascularisation and arrests airway branching,[17] whereas inhibition of VEGF results in less complex alveolar patterning and immature lung formation.[18] Additionally, by using antisense oligonucleotides against HIF-1α and VEGF, our group previously demonstrated that epithelial branching morphogenesis is abolished when pulmonary vascular development is inhibited.[5] The evidence that homozygous null VEGFR-2 mice die *in utero* as a result of a lack of mature endothelial cells and an absence of blood island formation, suggests a role of VEGFR-2 in mediating the mitogenic and chemotactic effect of VEGF-A on the endothelial cells.[19]

The variation of oxygen tension leads to a variable phenotype of lung development. *In vitro* studies using rat lung explants cultured at 3% oxygen showed an increase in epithelial branching and cellular proliferation, as compared to explants cultured at 21% oxygen.[9] Previous studies in transgenic mouse lungs showed an increase of both epithelial

and endothelial branching morphogenesis in explants cultured at 3% oxygen compared with 21% oxygen.[5]

Based on the aforementioned information, a low oxygen environment of the foetus seems critical for pulmonary angiogenesis and possibly airway branching morphogenesis. Human lung explants maintained *in vitro* have been used in a number of studies on lung development.[20-28] For instance, Acarregui et al. previously showed the effect of low oxygen level and cAMP on the expression of VEGF mRNA.[20] However, there is very little data available regarding the expression of HIF-2α, HIF-3α and VEGFR-2 in the human foetal lung *in vitro*. In this study, we used human foetal lung explants maintained in an *in vitro* culture system, to study the influence of low oxygen tension on the expression of HIF-2α, HIF-3α, VEGF-A, and VEGFR2 in the developing human lung.

## Materials and Methods

### Lung tissue and explant culture

This study has been approved by the Medical Ethical Committee of Erasmus Medical Center Rotterdam and by the board of directors of the Center for Anticonception, Sexuality and Abortion (CASA) in Leiden.

Lung tissue was obtained from normal human foetuses after surgical termination of the pregnancy at 16, 17, 21, 22 and 22 weeks of gestation and after obtaining informed consent. No medication was used to abort the pregnancy. The obtained tissue fragments were rinsed with PBS and lung tissue was identified macroscopically. The lung fragments were preserved in cooled (4°C) culture medium during transport. Within several hours, foetal lung tissue was carefully separated from the major blood vessels and airways, and dissected into 1-2 $mm^3$ pieces. Lung explants (2-3 pieces) were placed separately on Nucleopore membranes (pore size 8µm; Whatman, Den Bosch, Netherlands), and cultured as air-liquid interface cultures in serum-free Waymouth's MB752/1 medium (GIBCO, Breda, Netherlands) with 1% Penicillin-Streptomycin and 1% Insulin-Transferrin-Selenium (GIBCO). The explants were maintained either under standard culture incubator conditions of 37°C and 5% $CO_2$/ 95% air (21% oxygen; normoxia), or in an incubator with 5% $CO_2$, and 93.5% $N_2$ / 1.5% $O_2$, (hypoxia). The culture medium was changed every 24 hours and the explant tissue was harvested after 3 or 6 days of culture.

### Immunohistochemistry

Immunohistochemistry was performed using the standard avidin-biotin complex method as previously published.[29] In brief, subsequent to deparaffinization in xylene and rehydration through graded alcohol steps, slides were treated with 3% $H_2O_2$ in methanol

to block the endogenous peroxidase activity. For antigen retrieval, slides were subjected to microwave treatment in citric acid buffer, pH 6.0 (Ki-67, TTF-1) or pronase (CD-31). After the blocking step, slides were incubated for 30 minutes at room temperature with a primary antibody against Ki-67 (1:150, Dako, Heverlee, Belgium), TTF-1 (1:100, Ab-1, Neomarkers, CA, USA), or CD-31 (1:30, clone MIB-1, Dako). After rinsing with PBS, slides were incubated for 10 min with a biotinylated secondary antibody (Labvision, CA, USA), followed by incubation with peroxidase-conjugated streptavidin (Labvision). Peroxidase activity was detected by diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 0.3% $H_2O_2$ and counterstained with haematoxylin. Negative controls were performed by omission of the primary antibodies.

**RNA isolation and quantitative RT-PCR**

Uncultured lung tissue and explants cultured for 3 and 6 days were used for quantitative PCR analysis. Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, Breda, Netherlands). RNA was quantified by measuring the absorbance at 260 nm and the purity was checked by the 260/280 nm absorbance ratio. Total RNA (1µg) was added to a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 100 ng random hexamer primer, 500 mM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 10 U RNAse inhibitor and 200 U Moloney Murine Leukaemia Virus Reverse Transcriptase (all reagents were obtained from Invitrogen). The RT thermal cycle was 1 hour at 37°C followed by incubation for 15 min at 99°C (for primers see table 7.1).

**Table 7.1** | Primer sequences for quantitative PCR

| Gene | Forward Primer (5' → 3') | Reverse Primer (5' → 3') |
|---|---|---|
| HIF2-α | CCA ATC CAG CAC CCA TCC CAC | GTT GTA GAT GAC CGT CCC CTG |
| HIF3-α | ACC TGG AAG GTG CTG AAC TG | AAT CCT GTC GTC ACA GTA GG |
| VEGF-A | AGA ATC ATC ACG AAG TGG TG | TGT TGT GCT GTA GGA AGC TC |
| VEGFR-2 | CAG AGT GGC AGT GAG CAA AG | TAC ACG ACT CCA TGT TGG TC |
| POLR2A | CGG ATG AAC TGA AGC GAA TG | AGC AGA AGA AGC AGA CAC AG |

Real-time PCR was performed using an iCycler IQ Real time PCR detection system (Bio-Rad, Veenendaal, The Netherlands) and qPCR Core kit for SYBR Green I (Eurogentech, Seraing, Belgium) with the following conditions: 10 min of initial denaturation at 95°C followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 60°C for 30 s, and 75°C for 15 s. The sequences

for the gene-specific primers used in this study can be found in table 7.1. To verify the specificity of the amplified products, each PCR was followed by a melting curve analysis from 55°C to 95°C. Each sample was run as a triplicate and mRNA of each target gene was determined simultaneously in a 96-wells plate. Negative (no enzyme) and no-template (no cDNA) controls were also included. RNA Polymerase II Subunit A (POLR2A) was used as an internal reference for the relative quantitation of the PCR signals. The fold change was calculated as $2^{-\Delta\Delta Ct}$ according to Livak and Schmittgen.[30] Lung tissue from day 0 (starting material) was used as a control group (arbitrary value = 1).

### Statistical analysis

Data from the quantitative PCR are presented as mean $2^{-\Delta\Delta Ct}$ ± SEM. The differences between the experimental groups were evaluated by using one-way ANOVA with post hoc least significant difference test. A *p*-value of $< 0.05$ was considered statistically significant. All statistics were calculated using SPSS statistical package (version 11.0; SPSS Inc., Chicago, IL).

## Results

### Morphology of human foetal lung explants cultured at 21% oxygen

To evaluate the feasibility of foetal lung explant culture *in vitro* under our conditions, human foetal lung explants derived from mid-trimester abortions were maintained at 21% oxygen for 6 days. Macroscopically, the explants appeared vital until at least 6 days of culture. The microscopic appearance of mid-trimester explants after culturing for 3 and 6 days resembled the pseudoglandular stage of lung development; except that the airways were dilated and the epithelial cells appeared flattened (Figure 7.1). To evaluate cell differentiation characteristics of the explants, sections of explants cultured for 3 and 6 days were immunostained with markers for epithelial cells (TTF-1), endothelial cells (CD-31) and proliferation (Ki-67). There was no difference in the expression pattern of all markers between uncultured lungs and explants cultured for 3 or 6 days at 21% oxygen. Virtually all epithelial cells were positive for TTF-1 staining (Figure 7.2 A, D, G), while endothelial cells were positive for CD-31 (Figure 7.2 B, E, H). Ki-67 immunoreactivity was observed in both epithelial and mesenchymal cells (Figure 7.2 C, F, I). Double immunohistochemical staining with Ki-67 and CD-31 showed ongoing endothelial proliferation in the explants cultured under normoxic conditions after 6 days in culture (Figure 7.3 B).

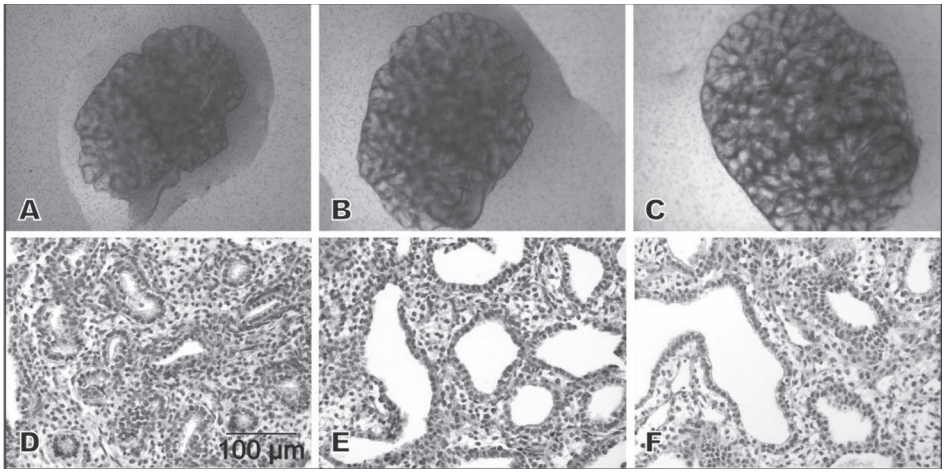


**Figure 7.1** |Representative images showing the morphology of human foetal lung explants (gestational age 16 weeks) cultured at 21% oxygen. Macroscopic pictures of uncultured foetal lung explants (A) and after culture for 3 (B) and 6 days (C). Haematoxylin & Eosin staining of uncultured lung (D) resembles the pseudoglandular stage of lung development. The airways are dilated after culture for 3 (E) and 6 days (F) at 21% oxygen. Bar, 100 µm.

### Morphologic changes associated with exposure to hypoxia

Sections of the explants were immunostained with Ki-67, TTF-1 and CD-31 after culture for 6 days at either normoxic or hypoxic conditions. Uncultured tissue from the same patient was used as a control. The airways were larger in both hypoxic and normoxic cultured lungs at day 6 as compared to control tissue. There was no difference in the localization of TTF-1 (figure 7.2 G, J) and CD-31 (Figure 7.2 H, K) between explants cultured at 21% and 1.5% oxygen. However, CD-31 immunoreactivity appeared to be stronger in hypoxia-exposed lungs as compared to explants cultured at normoxia (Figure 7.2 K vs. H). A proliferation marker, Ki-67, was expressed in both epithelial and mesenchymal cells in control tissue and explants cultured at 21% oxygen (Figure 7.2 C, F, I) but appeared to be more restricted to the epithelium in explants cultured at 1.5% oxygen (Figure 7.2 L)

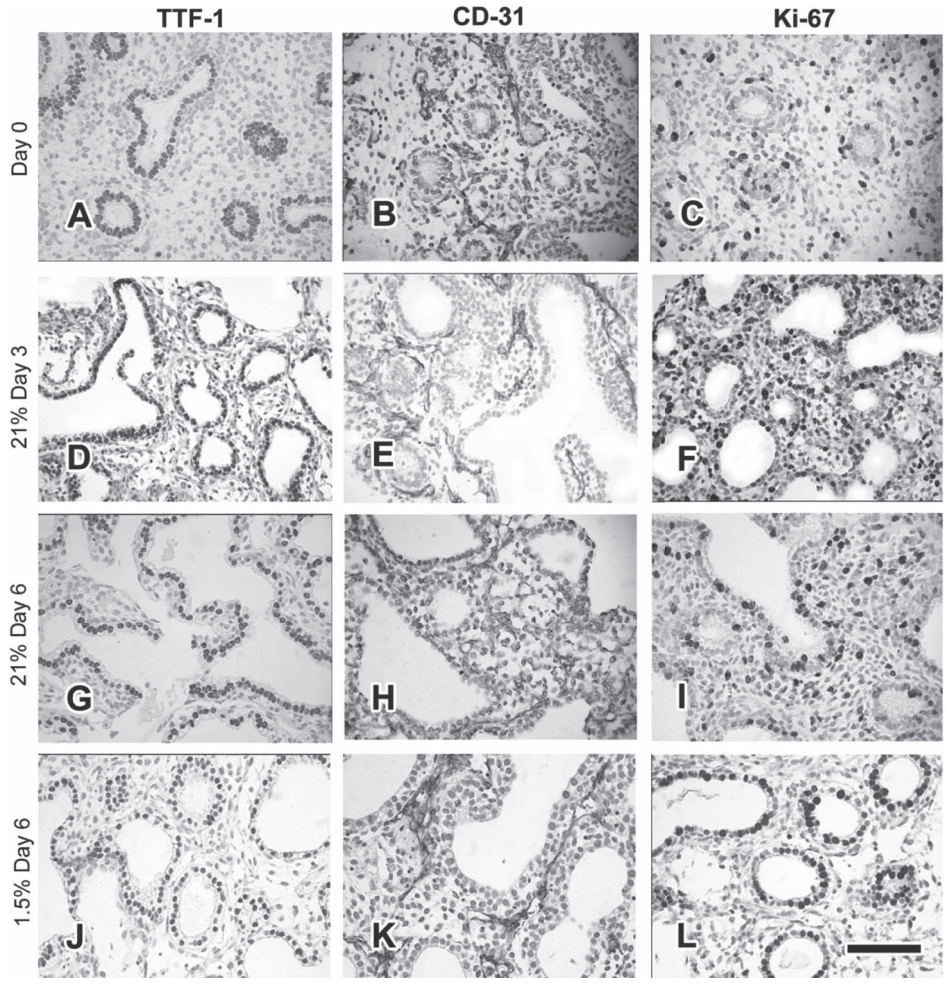


**Figure 7.2** | Immunohistochemical staining with TTF-1, CD-31, and Ki-67 of uncultured lung (A-C), explants cultured at 21% for 3 (D-F) or 6 days (G-I), and 1.5% oxygen for 6 days (J-L). TTF-1 immunoreactivity is detected in epithelial cells (A, D, G, J). Explants cultured at 1.5% oxygen (K) show stronger CD-31 staining of endothelial cells compared with control (B) and explants cultured at 21% oxygen (E, H). Ki-67 staining (C, F, I, L) shows proliferating epithelial cells and mesenchymal cells in control (C) and explants kept at 21% oxygen (F, I), while the expression is more restricted to the epithelium in explants kept at 1.5% oxygen (L). Bar, 500 µm.

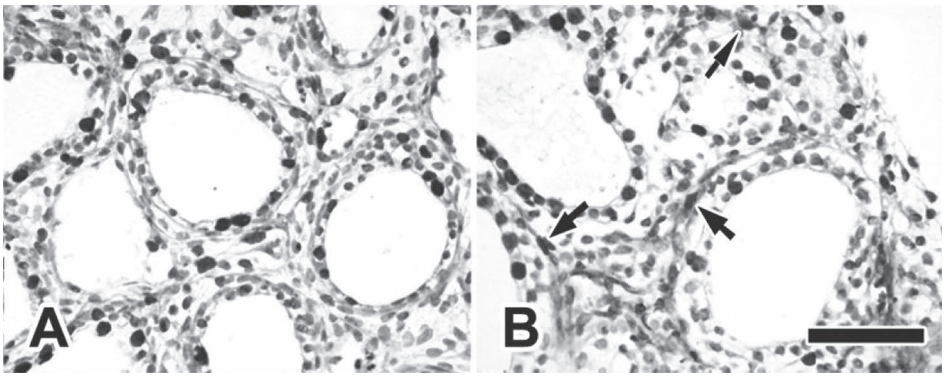


**Figure 7.3** | Double immunostaining of Ki-67 and CD-31 on explants cultured at 21% oxygen for 6 days. Ki-67 immunoreactivity is detected in epithelium and mesenchyme (A). Double staining with CD-31 (pink staining) shows proliferating endothelial cells (arrow heads; B). Bar, 250 µm.

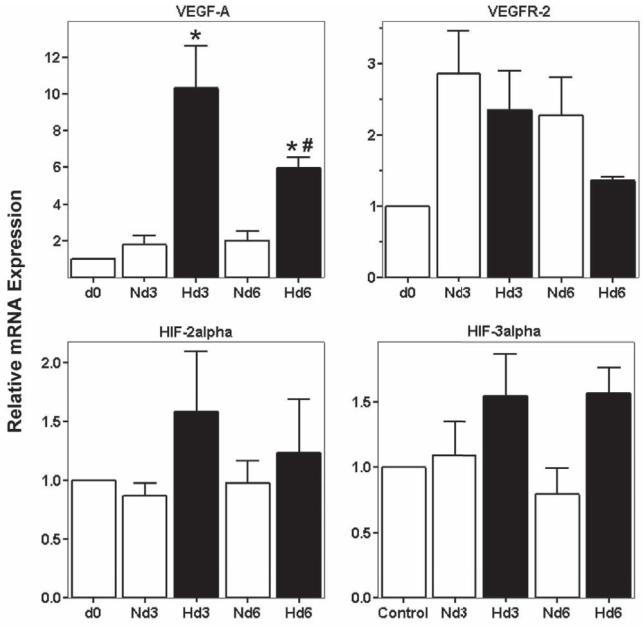


**Figure 7.4** | Relative mRNA expression of VEGF-A, VEGFR-2, HIF-2α, and HIF-3α in human foetal lung explants maintained for 3 or 6 days in 21% $O_2$ (Nd3 and Nd6, respectively) or 1.5% $O_2$ (Hd3 and Hd6, respectively) and uncultured lungs (d0). For relative quantification, the expression of POLR2A mRNA was used as reference gene. Bar represents means ± SEM (n=5). The expression VEGF-A mRNA is upregulated in explants cultured at 1.5% oxygen (Hd3 and Hd6) as compared to explants cultured at 21% oxygen (Nd3 and Nd6) and control (d0), but its expression in explants kept at hypoxic conditions is decreased when cultured for a longer period (Hd6 vs. Hd3). * $p \leq 0.01$ vs. d0, Nd3, and Nd6. # $p < 0.01$ vs. Hd3.

**Quantitative RT-PCR**

Quantitative RT-PCR showed that VEGF-A, VEGFR-2, HIF-2α, and HIF-3α mRNA were expressed in all experimental groups. Relative mRNA expression of each gene was demonstrated as mean ±SEM in figure 7.4 (n=5 per group). There was an increase in VEGF-A mRNA expression in explants cultured at 1.5% oxygen compared with explants cultured at 21% oxygen and control ($P \leq 0.01$). However, under hypoxic conditions, VEGF-A mRNA expression was downregulated in explants cultured for 6 days compared with 3 days ($P < 0.01$). There was no significant change in the expression of the other investigated genes between the explants cultured in normoxia and hypoxia, although the expression of HIF-2a appeared higher in the explants cultured at 1.5% oxygen at day 3 compared with explants cultured at 21% oxygen without reaching statistical significance.

## Discussion

The developing lung requires the formation and maintenance of a vascular network in close proximity to a layer of alveolar epithelial cells. In this study, we demonstrated that human lung explants cultured for 3 to 6 days at 1.5% oxygen maintain appropriate proliferation and differentiation of the airway epithelium as well as vascularisation. Moreover, we demonstrated that VEGF-A mRNA expression was increased in the explants cultured under hypoxic conditions. In line with the increased expression of VEGF-A, we found that both HIF-2α and HIF-3α were slightly elevated in the hypoxic cultures, although the differences with normoxic cultures were not statistically significant.

Foetal lung explants maintained *in vitro* are a well-characterized model for studying foetal lung development. The differentiation of midgestational human foetal lung explants in culture has been characterized both biochemically and morphologically in several studies,[15,20-23,25-28] as depicted in table 7.2. However, there is little information on the role of hypoxia at the cellular level in human foetal lung development. Previous studies in murine lungs have shown that hypoxia enhances the development of pulmonary vasculature and airway epithelial branching morphogenesis, as compared to ambient oxygen.[5,9]

In the present study, the airway epithelium of the explants appeared flattened within a dilated airspace after being maintained at 1.5% oxygen for 6 days. There was no obvious difference in the localization of epithelium (TTF-1) and endothelium (CD31) specific markers between human lung explants cultured at 1.5% oxygen or 21% oxygen. Immunostaining with the proliferation marker Ki-67 showed that cells in the lung explants were still dividing. A formal quantification of cell proliferation was not performed, due to the small number of samples. These findings indicate that the cultured lung tissue maintains its normal structure and growth capacity.

**Table 7.1 |** Previous studies with human fetal lung explants

| Point of interest | Culture condition | Main results | Reference |
|---|---|---|---|
| **Morphological change** | | | |
| cell differentiation | 20% $O_2$ | Spontaneous differentiation of airway epithelium into type II pneumocytes | 20 |
| apoptosis | 20% $O_2$ | ↑ number of cell undergoing apoptosis esp. in the interstitium | 25 |
| in response to hyperoxia | 95% vs. 20% $O_2$ | ↓ number of vessels and VEGF mRNA expression in hyperoxia<br>↓ cell proliferation in the interstitium but not in epithelium | 27 |
| **Surfactant protein** | | | |
| effects of $O_2$ | 70% or 95% vs. 20% | ↑ SP-A, SP-C mRNA, SP-B unchanged | 24, 30 |
| effects of retinoic acid | +/- retinoic acid | ↓ SP-A, SP-C mRNA, ↑SP-B mRNA | 22 |
| effects of glucocorticoids | +/- dexamethasone | ↑ SP-A mRNA at lower concentration but<br>↓ at concentration > $10^{-8}$M | 21 |
| **VEGF pathway** | | | |
| effects of $O_2$, cAMP | 2% vs. 20% $O_2$<br>+/- cAMP | ↑ VEGF mRNA and protein after 2-4 d in 2% $O_2$<br>cAMP ↑ VEGF mRNA in 20% but not 2% | 15 |
| effect of exogenous VEGF | +/- recombinant VEGF | VEGFR-2 expressed in distal airway epithelium<br>Exogenous VEGF ↑ epithelium volume density, tissue differentiation and<br>↑ SP-A, SP-C mRNA, SP-B unchanged | 26 |

The close anatomical relationship between airways and blood vessels in the lung suggests their putative interaction during development. The inhibition of vascularisation in mouse lungs *in vitro* results in a decrease in epithelial branching morphogenesis.[5,31] VEGF has been shown to act as a potent inducer of endothelial cell growth and hypoxia is one of its most important stimuli.[14,20] VEGF is essential for embryonic development, as it was shown that inactivation of a single VEGF allele results in embryonic lethality with impaired vessel formation.[14,32] In this study, we have shown that VEGF-A mRNA expression is significantly upregulated in lung explants cultured at 1.5% oxygen, as compared to normoxic conditions. Similar findings have been reported for VEGF expression in human,[20] mouse,[5] and rat,[9] foetal lung explants cultured at low oxygen. However, these studies have not established the relationship with expression of HIFs and VEGF. Our results confirm that low oxygen stimulates VEGF expression and probably vascular development in human foetal lung development.

The expression of VEGFR-2 is stimulated by HIF-2α,[33] but there are no reports of an oxygen-dependent upregulation of VEGFR-2 expression in human lung. Previous studies in

lung explants of a transgenic mouse model have shown that VEGFR-2 mRNA expression was significantly increased after two days in culture in hypoxic conditions. However, with extension of the culture period, this difference was no longer noticeable.[5] Our results also showed no significant difference in the expression of VEGFR-2 mRNA between 1.5% and 21% oxygen after 3 or 6 days in culture. Apparently, other factors than hypoxia are also important in the expression of VEGF receptors in human pulmonary mesenchyme. It is possible that the expression of VEGFR-2 is oxygen-dependent, but the effect of oxygen is already diminished when we studied the cultured tissue at day 3.

VEGF gene transcription is activated by hypoxia through HIF-1α and HIF-2α.[34-36] These two factors have a close sequence similarity but their modes of expression vary greatly, and HIF-1α and HIF-2α have unique targets.[10] HIF-1α is ubiquitously expressed in the developing embryo[37] and in the lung, where it is detected in the branching epithelium of the first trimester lung, but also in some mesenchymal cells.[16] HIF-1α double knockout mice die in utero at e10.5 with cardiac, vascular defects and extensive cell death.[38,39] HIF-2α is abundantly expressed in murine adult lungs under normoxic conditions,[34,40] especially in the developing mesenchyme and vasculature.[37] HIF-2α-null mice die in the embryonic stage with abnormal lung maturation and blood vessel defects,[41] and a defect in catecholamine production.[35] However, a subset of HIF-2α knockout offspring survives postnatally but suffers from respiratory distress due to surfactant deficiency.[42] Another member of the HIF family, HIF-3α, appears to be involved in negative regulation of the angiogenic response through an alternative splice variant, inhibitory PAS domain protein (IPAS).[43] IPAS can be induced by hypoxia in heart and lung resulting in a negative feedback loop for HIF-1α activity in these tissues.[44] A previous study in adult mice by Heidbreder et al.[40] reported that mRNA expression of HIF-3α, but not HIF-2α increased significantly corresponding to the duration of systemic hypoxia. In addition, they found an increase in both HIF-2α and HIF-3α protein levels.[40] In the A549 cell line, HIF-1α and HIF-3α showed a different response to hypoxia (1% $O_2$), which led to the suggestion that HIF-3α is complementary rather than redundant to HIF-1α induction.[45] Our analysis with quantitative PCR showed only a slight increase in HIF-2α and HIF-3α mRNA expression in explants cultured under hypoxic conditions at day 3 but this difference was not significant. However, it must be noted that the regulation of HIF-α factors is mainly post-transcriptional, and therefore this could very well explain the marginal differences between the hypoxic and normoxic cultures.[13] So, aside from the presence of both HIF-2α and HIF-3α, we showed that the expression of their main target, VEGF-A, is significantly upregulated upon hypoxia. This indicates that although the expression of both factors is only slightly elevated, the transcriptional activity of the HIF factors is increased under hypoxic conditions. At this point, we cannot discriminate whether HIF-2α or HIF-3α is the main inducer of VEGF.

In summary, this study has demonstrated that foetal human lung explants exposed to hypoxia (1.5% oxygen) maintain proper epithelial and mesenchymal morphogenesis. An increase in VEGF-A expression under hypoxic conditions suggests its role in regulating pulmonary vascular and airway development. Human foetal lung explants maintained in a serum-free system allowed us to focus on the local effects of oxygen tension rather than a systemic response to hypoxia. Moreover, the establishment of this model of normal human foetal lung development might also open up the possibility of studying abnormal lung development such as pulmonary hypoplasia resulting from obstructive uropathy and oligohydramnios, or in case of congenital diaphragmatic hernia. A better understanding of the abnormal processes in these conditions will enhance our knowledge of the pathogenesis of pulmonary defects. In addition, this model can be applied to investigate the effect of different ligands or medications that have an effect on lung development such as retinoic acid and steroid hormone. This may provide us with new therapeutic interventions, both pre- and postnatally.

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# Chapter 8

## Metabolic disturbances of the vitamin A pathway in congenital diaphragmatic hernia

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** equal contribution*

Submitted

## Abstract

**Introduction:** Congenital Diaphragmatic Hernia is a severe birth defect. It is a major cause of severe respiratory failure in the newborn mainly due to the associated pulmonary hypoplasia and pulmonary hypertension. Studies in animal models and humans have led to the hypothesis that the vitamin A pathway is involved in its etiology. Since the vitamin A pathway is essential for lung development, we aimed to examine the differential expression of the metabolic actors of the vitamin A pathway in two animal models of CDH and in human fetal lung tissue of CDH patients and fetal controls.

**Methods:** We investigated normal and CDH lungs in human tissue at different stages of development, in the nitrofen rat model, the surgical rabbit model and the A549 alveolar cell line. The expression of enzymes, binding proteins and receptors that are part of the vitamin A pathway was checked by quantitative and qualitative PCR experiments.

**Results:** The expression of cellular retinol-binding protein (CRBP2) and a retinoic acid-degrading enzyme (Cyp26b1) were diminished in human CDH lungs. Both genes were sensitive for retinoic acid (RA) in the A549 alveolar cell line. The RA-generating enzyme RALDH2 was significantly increased in human CDH lung, rabbit CDH lung, but not in the rat CDH lung.

**Conclusion:** Our results underline that CDH lungs differ from healthy lung with respect to the expression of key factors of the vitamin A pathway. We propose a pathogenetic mechanism to explain underline the involvement of the vitamin A pathway in CDH.

## Introduction

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly with a birth prevalence of 1 in 3,000 which accounts for about 8% of major congenital anomalies. CDH causes severe neonatal respiratory distress and is associated with ongoing mortality in high risk patients and considerable morbidity in survivors.[1,2] The etiology of CDH is poorly established; although approximately 15% of patients have an identifiable genetic correlation, the remaining 85% are idiopathic in origin.[3] The retinoid (active derivatives of vitamin A) hypothesis is one of the most common hypotheses to explain the cause of idiopathic CDH at least in animal models.[4-7]

Vitamin A (retinol) is essential for lung development and pulmonary cell differentiation and its deficiency results in alterations of lung development, morphogenesis and function.[8] To be physiologically active, retinol (Rol) has to be converted into retinoic acid (RA) by oxidative reactions that convert Rol into retinaldehyde (Ral) and Ral into RA. A large number of enzymes catalyze the oxidation of Rol to Ral (alcohol dehydrogenases [ADH], short-chain dehydrogenase-reductase [DHRS] and retinol dehydrogenase [RDH]) as well as the oxidation of Ral to RA (retinaldehyde dehydrogenases [RALDH]). RALDH2 is the main RA-synthesizing enzyme during development.[9,10] Retinoids are bound intracellular to specific binding proteins: Cellular Retinol Binding Proteins (CRBP1 and 2) and Cellular Retinoic Acid Binding Proteins (CRABP1 and 2). A balance between RA synthesis and degradation determines the intracellular RA concentration. The degradation of RA is carried out by three specific members of the cytochrome P450 family: CYP26A1, B1 and C1.[11] The two major isoforms of RA (*all-trans* and *9-cis* RA) are the ligands for the nuclear Retinoic Acid Receptors (RARα and β isoforms) and Retinoid X Receptors (RXRs) that act in heterodimeric combinations to regulate the transcription of target genes containing a RA-response element (RARE) (see Figure 8.1).

In the CDH context, the retinoid hypothesis has been based on convincing animal data on the association between the genesis of congenital diaphragmatic hernia and: i) maternal vitamin A deficiency,[12] ii) knock-out of RARα and β receptors,[13] (iii) exposure to the teratogen nitrofen, which interferes with the synthesis of RA by RALDH2,[7] (iv) reduction of defects after retinoid administration[14] and (v) the expression of retinoic acid receptors in the primordial diaphragm.[7] In human, the first link between retinoids and CDH was the finding of a 50% decrease of blood Rol and retinol binding protein (RBP) in 7 CDH newborns compared to healthy ones.[15] These findings were recently confirmed by a larger case-control study measuring the retinoid state in mothers and newborns with CDH.[16] In addition, some mutations in the human CRABP1 and STRA6 (RBP membrane receptor) genes, which interfere with the metabolic retinoid signaling pathway, were linked to CDH.[6,17] Earlier data did not reveal an altered expression of RARs and RXRs both

with regards to tissue distribution and time of appearance in human CDH lung during development.[18] Together, these data support the idea that changes in the retinoid pathway may be linked with human CDH.

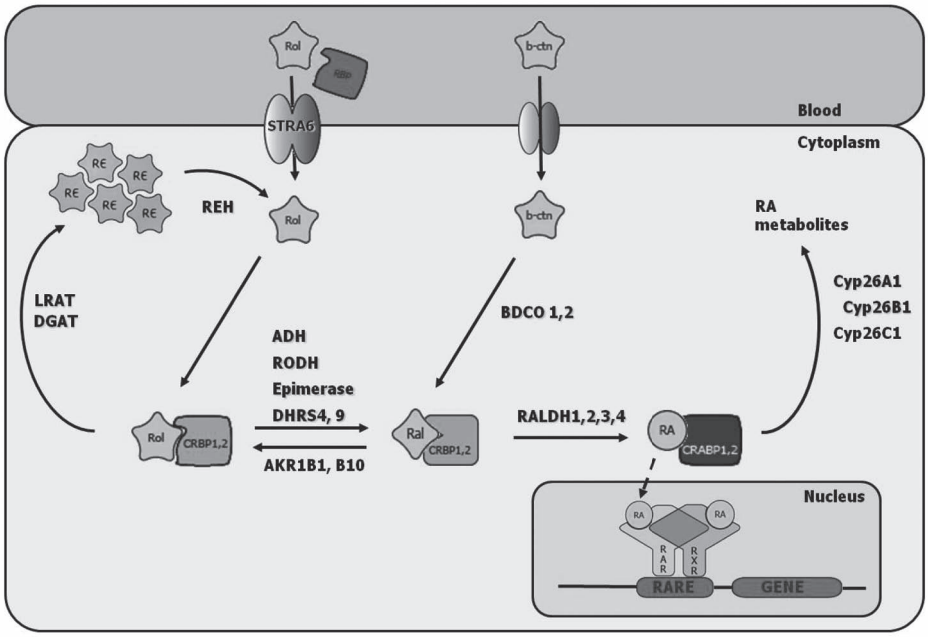


**Figure 8.1** | Schematic representation of metabolic and molecular actors of the retinoids signaling pathway. The abbreviations used in the figure can be found in the text.

Here, we evaluate the gene expression of receptors, binding proteins and enzymes involved in retinoid metabolism in normal and CDH lung development in order to evaluate the metabolic retinoid hypothesis. We propose a pathogenetic mechanism for the etiology of CDH based on human data combined with data from two animal models: a well established rabbit surgical model with intact metabolic retinoid signaling[19] and the classical teratogenic nitrofen rat model with a disturbance of retinoid signaling.[20]

## Methods

### Chemicals

*All-trans* retinol, *all-trans* RA and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich® (Saint-Quentin-Fallavier, France). For all experiments, both RA and retinol were prepared as 1000x stock solution in DMSO. Culture medium and additives (streptomycin, penicillin, dextran-coated charcoal stripped fetal calf serum [FCS]) were purchased from Invitrogen® (Cergy-Pontoise, France). The transfection reagent GeneJammer was purchased from Stratagene® Europe (Amsterdam, the Netherlands).

### Human Tissue collection

Following the approval of the Erasmus MC Medical Ethical Committee and informed consent of the pregnant women or parents, lung tissue was obtained from the archives of the Department of Pathology, Erasmus MC, Rotterdam. The selected fetal and neonatal lung tissues were obtained after elective termination of pregnancy (TOP) or at autopsy. The general characteristics of the 8 isolated CDH patients (gestational age 21-41 weeks) are described in Table 8.1. Lung tissue from 13 age-matched fetuses (gestational age 13-34 weeks) that died of reasons other than pulmonary abnormalities served as control. None of the patients included in this study were subjected to prenatal steroids or extracorporeal membrane oxygenation therapy. All tissue samples were harvested as soon as possible after death, but ultimately within 24 hours after death.

**Table 8.1 |** Clinical information of congenital diaphragmatic patients. Normal lung weight / body weight ratio < 25 weeks of gestation = 0.015, between 25 and 37 weeks of gestation = 0.012, and > 37 weeks of gestation = 0.02. TOP: termination of pregnancy, nc: not collected

| CDH lung number | Lung stage | Gestational age (weeks) | Sex | Birth weight (g) | Lung/body weight ratio | Time of death |
|---|---|---|---|---|---|---|
| 1 | canalicular | 21.4 | M | 320 | 0.02 | TOP |
| 2 | canalicular | 22 | M | nc | nc | TOP |
| 3 | canalicular | 25 | nc | 558 | 0.012 | TOP |
| 4 | saccular | 34 | M | 1250 | 0.005 | < +1 h |
| 5 | alveolar | 37.2 | M | 2500 | 0.005 | + 7 h |
| 6 | alveolar | 38 | F | 2450 | 0.011 | + 48 h |
| 7 | alveolar | 40 | M | 2880 | < 0.001 | < +24 h |
| 8 | alveolar | 41 | F | 3510 | 0,006 | +1 h |

**Animal CDH models: rabbit surgical and rat nitrofen model**

New-Zealand rabbits (Charles River®, L'Arbresle, France) and pregnant Sprague-Dawley rats (Harlan Laboratories®, Horst, the Netherlands) were respectively shipped at day 17 of gestation (term = day 31) and at day 7 of gestation (term = day 21). All animals were treated according to current guidelines on animal well-being. The Ethics Committee for Animal Experimentation of the Faculty of Medicine at Clermont-Ferrand and the Animal Welfare Committee of Erasmus MC at Rotterdam approved the experiments. The animals were housed in separate cages at normal room temperature and daylight, with free access to food and water. For rabbit spatiotemporal expression pattern studies, lung tissues were collected at day 21 (late pseudo-glandular stage), 26 (canalicular stage), 28 (saccular stage) and 31 (alveolar stage) of gestation. Surgical diaphragmatic hernia was created at day 23 of gestation (pseudo-glandular stage) and the lungs collected at day 31 of gestation, as previously described.[19] The rabbits were euthanized with 5 ml intravenous pentothal (Hospira®, Illinois, USA). Operated and immediate adjacent non-operated fetuses were harvested by caesarean section. For the teratogenic model, rats in the nitrofen group received 100 mg nitrofen dissolved in 1 ml olive oil via gavage on day 9 of gestation. The experimental approach has been published by our group before.[21] In short, rats in the control group received only olive oil on day 9. At day 21, the fetuses were delivered by caesarian section. The diaphragm of the fetuses was carefully inspected under a dissecting microscope (Olympus SZX12®, Zoeterwoude, the Netherlands) for the presence of a diaphragm defect. All the samples were snap frozen (liquid nitrogen) and stored at -80°C for immuno-histochemistry and molecular analysis.

**Alveolar epithelial cell line A549 culture and treatment**

The A549 cell line was purchased from the American Tissue Culture Collection (LGC Standards Sarl®, Molsheim, France). The cell culture was conducted using ATCC recommendations, under standard conditions (5% $CO_2$, 95% humidified air, 37°) in F-12K medium supplemented with 5% charcoal-stripped FCS (to prevent contamination by endogenous serum retinoids), 50 mg/ml of streptomycin and 50 UI/ml of penicillin. At 90% of confluence, the cells were treated with *all-trans* retinoic acid (ATRA; Sigma-Aldrich®, Saint-Quentin-Fallavier, France) in DMSO (vehicle) or with DMSO alone, during 6, 12, 24 and 48 hours. In all cases, the maximum concentration DMSO to which the cells were exposed was <0.1%. After treatment, cells were washed once with PBS and stored at -80° for RT-PCR. A total of 3 x $10^5$ A549 cells in 6-well plates were transfected using GeneJammer with 1 µg of reporter DR5-*tk*-CAT plasmid and 1 µg of cytomegalovirus (CMV)-beta-galactosidase vector serving as internal control to normalize variations in transfection efficiency. The plasmid DR5-*tk*-CAT contains one copy of the retinoic acid-responsive element DR5 (direct repeat 5) ligated to a herpes simplex thymidine kinase promoter

upstream of a chimeric chloramphenicol acetyl transferase (CAT) reporter gene.[22] The CMV-beta-galactosidase plasmid contains CMV promoter and enhancer sequences that drive a beta-galactosidase (βGAL) gene. The transfections of the corresponding vectors CAT and β-GAL, 24 h retinol or ATRA treatment, and CAT measurement by an immunoenzymatic assay (Roche Diagnostics®, Meylan, France) were performed as previously published.[23]

**RNA extraction and RT-PCR experiments**

Trizol® reagent (Invitrogen®, Cergy-Pontoise, France) was used to extract total RNA from the human, rabbit and rat lung samples and from the A549 cell line. Total RNA was quantified by measuring absorbance at 260nm. The RNA quality was studied by the RNA/protein ratio (260 nm / 280 nm) and by gel electrophoresis (2% agarose) to observe the presence of intact 28S and 18S RNA bands. Copy DNA (cDNA) was generated using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen®). Specific oligonucleotide primers were originally generated using the web program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/ primer/primer3) and PerlPrimer (http://perlprimer.sourceforge.net) based on the published full-length human, rabbit and rat mRNA sequences of each specific gene and designed to avoid genomic DNA amplification (Table 8.2 and Marceau et al., 2006[23] for human RARs and RXRs primers). All primers were first checked for their specificity to amplify defined mRNA regions, using human and rabbit tissue already reported to express these genes (positive control). PCR amplification was carried out in an DNA-Engine PTC-200 (Biorad®, Marne la Coqette, France) using 50 ng of total cDNA per reaction and according to the following program: initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 45s, annealing at 59°C for 45s, and extension at 72°C for 60s (36 cycles), terminated by a final extension of 72°C for 7 min. The PCR products were separated on a 2% agarose gel and sequenced on both strands to confirm the specificity of the reaction, with the same primers as those of the PCR, using the DNA Dye Terminator Cycle Sequencing kit and the Applied Biosystems model 377 DNA Sequencer (Applied Biosystems®, Courtaboeuf, France). Amplification of the housekeeping gene acidic ribosomal phosphoprotein P0 (36B4) was used as positive control. A negative control for amplimer contamination was set up using a complete PCR reaction mix without cDNA.

**Table 8.2**

| Gene name Abbreviation | Gene name Long | Primer sequence rat Forward | Backward |
|---|---|---|---|
| CRBP1 | Cellular Retinol Binding Protein 1 | TGAACTTCACCTGGAGATGAG | CTCGAGACCAAGGTTATCTG |
| DHRS4 | Microsomal short chain dehydrogenase 4 | GCAAATTAGAAGGCTAGGCA | TCTCGCCATTGATGTAACTG |
| RALDH1 | Retinal dehydrogenase 1 | GGTAGTGTGGGTTAACTGCT | TTCCAGACATCTTGAATCCAC |
| RALDH2 | Retinaldehydrogenase 2 | ATGGGTGAGTTTGGCTTACG | AAGGAGGCCTGGTGATAGGT |
| RALDH3 | Retinal dehydrogenase 3 | TCGAGAGTGGGAAGAAGGAA | AGAAGACGGTGGGTTTGATG |
| Cyp26a1 | Cytochrome p450 family 26 subfamily A polypeptide 1 | ACCCTTCGATTGAATCCTCC | ATCTGGTAACCGTTCAGCTC |
| Cyp26b1 | Cytochrome p450 family 26 subfamily B polypeptide 1 | AGAGCTGCAAGCTGCCTATC | CGCCCCAGTAAGTGTGTCTT |
| LRAT | Lecithin:retinol acyltransferase | CAGGCTGAGAAGTTTCAGGA | GATGCCAGGCCTGTGTAGAT |
| RPS18 | 40S ribosomal protein s18 | AAGTATAGCCAGGTTCTGGC | CCCAAAGACTCATTTCTTCTTGG |

### Real Time quantitative PCR assays

Real-time PCR was performed using Lightcycler Sybr Green technology (Roche Diagnostics®, Meylan, France) using the same couples of primers and cDNA generation as described above. Each of the 40 PCR cycles (95°C for 10s, 55°C for 10s for the rabbit primers and 59°C for human ones, 72°C for 15s) was followed by a melting curve analysis from 65°C (rabbit) or 69°C (human) to 95°C. Each sample was run in duplicate. Negative control samples and reactions mixed without cDNA templates were run in parallel. Gene expression levels (dCT) were calculated relative to the measured CT value of the housekeeping gene.

### RALDH2 immunohistochemistry protocol

Sections of normal human and rabbit lung were fixed in 4 % paraformaldehyde in PBS (pH 7.4) at 25°C for 10 minutes, rinsed three times with PBS, incubated at 25°C for 10 minutes in H2O2 (quenching of endogen peroxidases) and incubated in PBS with 3% bovine serum albumin (Sigma Aldrich®) at 25°C for 30 minutes. Cells and tissues were incubated overnight at 4°C in the presence of RALDH2 (Santa-Cruz Biotechnology®, ALDH1A2 (N-20): sc-22591) certified rabbit polyclonal primary antibody (1/200 in PBS) (Tebu®, Le Perray-en-Yvelines, France). This was followed by three PBS washes; one-hour incubation in the presence of a secondary goat HRP anti-rabbit antibody (Interchim®, Montluçon, France) at room temperature and again three washes with PBS. The samples were then examined after HOECTH nuclear staining (15 min, dilution in PBS 1/10), and after mounting in an

aqueous mounting fluid Vectashield (Vector®, Burlingame, CA) under a Zeiss Axiophot microscope. For negative controls, sections were incubated with normal rabbit IgG in place of anti-RALDH2.

**Statistical analysis**

Results are expressed as the means (± SD) of different experiments per condition. The comparison was conducted by analysis of variance (ANOVA) using Statview software (SAS Institute, City, Country) and GraphPad Prism 4 (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Statistical significance was defined as $p < 0.05$.

## Results

Expression patterns of retinoid metabolism actors during normal human lung development. The expression patterns of the genes involved in the RA pathway (figure 8.1) were analyzed by RT-PCR at 4 well-defined morphological stages of normal lung development (the pseudo-glandular, canalicular, saccular and alveolar stages) (Figure 8.2). The genes encoding for proteins involved in cellular Rol uptake (STRA6, CRBP1 and CRBP2) were expressed at all stages studied. The genes for proteins that convert Rol or β-carotene into Ral (ADH3 and ADH4, DHRS4 and DHRS9, RODH, epimerase, beta carotene 15-15' dioxygenase type 1 / BCDO1 and type 2 BCDO2) were also expressed in all four stages. A similar pattern was found for the genes of aldo keto reductase (AKR1B1 and AKRB10), enzymes able to reduce Ral into Rol; and for RALDH1 and RALDH2, two irreversible converters of Ral into RA. The mRNA expression of RALDH3 and RALDH4 was only detected during the canalicular and alveolar stages. During all stages of lung development, the cellular retinol binding proteins CRABP1 and CRABP2, as well as the RA degrading enzyme CYP26B1 were expressed. Diacylglycerol acyl transferase (DGAT) and retinyl esters hydrolase (REH), responsible for storage and hydrolysis of retinyl esters, are expressed at all stages of lung development. The mRNA for lecithin:retinol acyltransferase (LRAT), the other enzyme involved in storage of retinyl esters, was absent in all studied samples. The same expression pattern was found for all the actors of the metabolic pathway (STRA6, RA-binding proteins and enzymes) in the alveolar epithelial cell line A549 (data not shown). The functionality of the RA pathway was demonstrated in the A549 cells, since a 2.44 (± 0.28) fold induction of RA-dependent CAT gene reporter was observed after a 24 hours incubation with retinol. This indicates that the A549 cells are capable of converting the retinol into functional RA.

**Altered expression of retinoid metabolism actors in human CDH lungs.**

In order to check our hypothesis whether vitamin A metabolism is disturbed in human CDH lung we performed qualitative RT-PCR on the molecular actors established in our first screenin [Figure 8.3 and data not shown for STRA6, CYP26C1, AKR1B1 and 10]. In all four stages, the positive expression pattern was comparable between CDH and normal lung for the majority of the genes analyzed. RALDH3 and 4 showed the same alternative expression pattern in CDH and normal lungs. LRAT, CYP26A1 and CYP26C1 were not expressed in CDH lungs as well as in control lung tissues. Furthermore, a complete absence of CRBP2 and CYP26B1 expression was noted in all stages of CDH lungs. Therefore, these two genes were checked for their potential regulation by *all-trans* RA in A549 cells, which present the same expression pattern of metabolic actors as described for total lung (see figure 8.2). These cells also express RARα and RXRβ (data not shown). We found that 1 µmol/L RA induced CYP26B1 transcripts after 6 hours of treatment, and peaks at 24 hours of treatment (Figure 8.4 A). CRBP2 was also induced by RA treatment but its expression appeared later (24 hours), weaker (1.7 fold) and with an increasing trend as compared to CYP26B1 (Figure 8.4 B). The genes previously described as similarly expressed in our first qualitative analysis (Figure 8.2) were subsequently analyzed by quantitative PCR assays to investigate a difference in expression that could also contribute to an alteration of RA production in CDH versus normal lungs. From the 20 genes analyzed, we only found a significant difference in RALDH2 between CDH and normal lungs. RALDH2 levels were increased 5.9 times ($p=0.03$) in the CDH group compared to normal lungs (Figure 8.5). Further sequential RALDH2 analysis in each stage showed only a significant different increase between CDH and healthy lungs for the saccular stage ($p=0.02$) (data not shown).

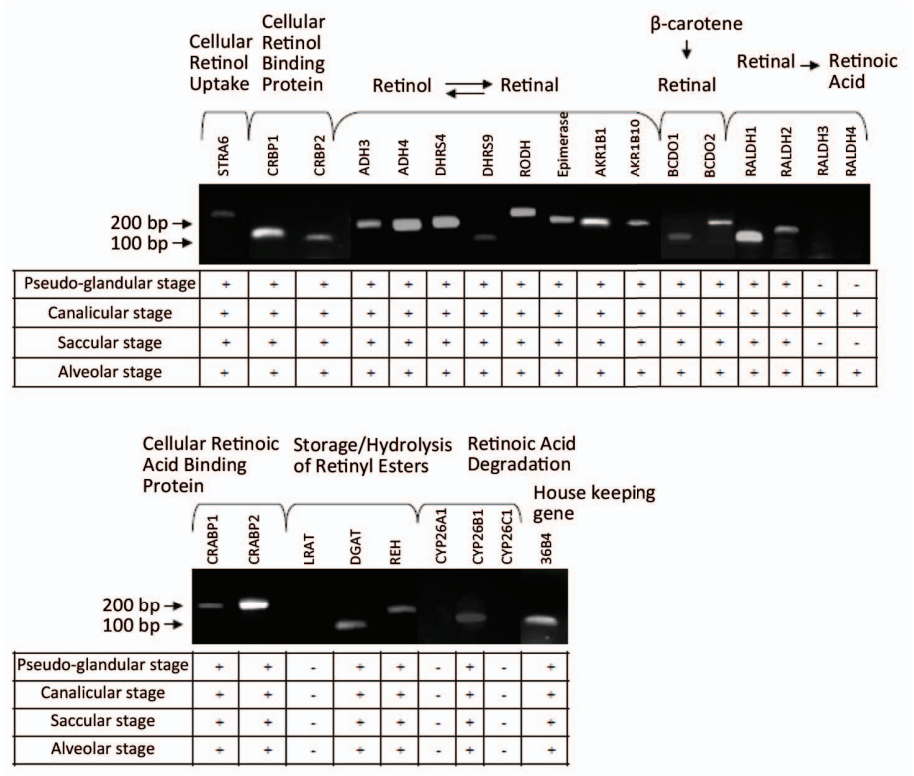


**Figure 8.2** | RT-PCR expression patterns of retinoid metabolism actors during normal human lung development. The expression patterns were determined by RT-PCR analysis on 13 samples of total RNA extracted from four stages of the normal human lung development: pseudoglandular (13.5, 14, 15, 17 and 17.5 Weeks of Gestation/WG), canalicular (21, 22 and 24 WG), saccular (2 samples at 27, 28.5 and 34 WG) and alveolar (37 WG) stages. All the different tissues tested for one developmental stage presented the same pattern. The agarose (2%) gel electrophoresis illustrates the results obtained for the pseudoglandular stage. The positive control (36B4) was performed under the same conditions using specific primers for the housekeeping gene 36B4 (199 base pairs). The negative control was performed in the absence of oligonucleotide or matrix.

Abbreviations: +: expression; -: no expression; STRA 6: membrane receptor of retinol; CRBP1-2: cellular retinol binding protein 1 and 2; ADH3 and 4: alcohol dehydrogenase 3 and 4; DHRS 4 and 9: short-chain dehydrogenase-reductase 4 and 9; RODH: retinol dehydrogenase; AKR 1B1 and 1B10: aldo keto reductase 1B1 and 1B10; BCDO1 and 2: beta-carotene 15-15' dioxygenase 1 and 2; RALDH 1 to 4: retinaldehyde dehydrogenase 1 to 4; CRABP 1 and 2: cellular retinoic acid binding protein 1 and 2; DGAT: diacylglycerol acyl transferase; LRAT: lecithin: retinol acyltransferase; REH: retinyl esters hydrolase; CYP26 A1, B1 and C1: cytochrome P450 A1,B1 and C1.


| | | ADH4 | ADH3 | DHRS4 | DHRS9 | RODH | Epim. | RALDH1 | RALDH2 | RALDH3 | RALDH4 | CYP26A1 | CYP26B1 | LRAT | DGAT | REG | BCDO1 | BCDO2 | CRBP1 | CRBP2 | CRABP1 | CRABP2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| canalicular | N | + | + | + | + | + | + | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + |
| | CDH | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + | + | + | - | + | + |
| saccular | N | + | + | + | + | + | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + |
| | CDH | + | + | + | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | - | + | + |
| alveolar | N | + | + | + | + | + | + | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + |
| | CDH | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + | + | + | - | + | + |

**Figure 8.3** | Differential expression patterns of retinoid metabolism actors in human normal and CDH lung tissue. Results from RT-PCR were obtained in 13 normal and 9 CDH lung samples from canalicular, saccular and alveolar stages. The upper part of the figure illustrates the expression pattern at the saccular stage after gel electrophoresis. The expression pattern for the three stages is summarized in the table (lower part). The positive control (36B4) was performed under the same conditions; using specific primers for the housekeeping gene 36B4 (199 base pairs). The negative control was performed in the absence of oligonucleotide or matrix.

Abbreviations: +: expression; -: no expression; CRBP1-2: cellular retinol binding protein 1 and 2; ADH3 and 4: alcohol dehydrogenase 3 and 4; DHRS 4 and 9: short-chain dehydrogenase-reductase 4 and 9; RODH: retinol dehydrogenase; BCDO1 and 2: beta-carotene 15-15' dioxygenase 1 and 2; RALDH 1 to 4: retinaldehyde dehydrogenase 1 to 4; CRABP 1 and 2: cellular retinoic acid binding protein 1 and 2; DGAT: diacylglycerol acyl transferase; LRAT: lecithin: retinol acyltransferase; REH: retinyl esters hydrolase; CYP26 A1, B1 and C1: cytochrome P450 A1,B1 and C1.

### Abnormal expression of retinoid metabolic actors in the CDH rabbit and rat model

In order to gain more insight in the observed changes in gene expression described in CDH lung tissues, we investigated two animal models for CDH, the surgical rabbit model and the nitrofen rat model. We started comparing the qualitative and quantitative RALDH2 expression in normal human and rabbit lung. For both species, RALDH2 expression was higher in the pseudoglandular and saccular stages compared to the canalicular and alveolar stage (Figure 8.6 A and B). Immunohistochemical staining showed a similar distribution of RALDH2 protein in normal rabbit and human developmental lung. Indeed, RALDH2 was

detected in sacculi, bronchi and alveolar parenchyme at the canalicular, saccular and alveolar stages in fetal human (Figure 8.7 A, C, I and K) and rabbit (Figure 8.7 E, G, M and N) lung. Using the surgical CDH rabbit lungs, we observed similar expression levels for CRBP2 and CYP26A1 and a significantly 10-fold increase ($p<0.001$) of RALDH2 levels as compared to normal lungs collected at the same saccular stage. This increase of RALDH2 was not found in the brain of a CDH fetus which served as a control tissue not involved in the CDH mechanism.

In the nitrofen model of CDH generation, we identified a lower expression of CYP26A1, CYP26B1 and LRAT in CDH lungs compared to control lungs. In contrast, DHRS4 expression was increased in the CDH lungs (Figure 8.8).

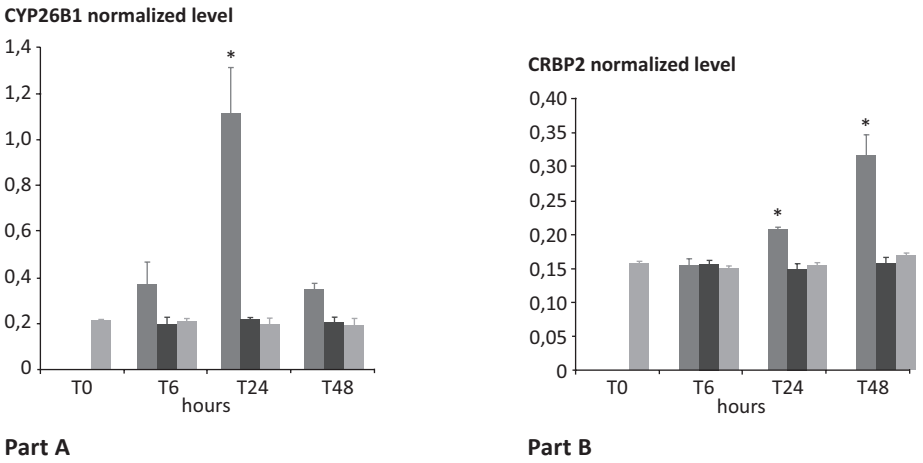


**Figure 8.4** | Expression levels of CYP26B1 and CRBP2 mRNA in human alveolar epithelial cell line A549 after retinoic acid treatment.

Human alveolar epithelial A549 cells were exposed to *all-trans* retinoic acid (ATRA) at $10^{-6}$ µmol/l during 6, 24 and 48 hours. Expression levels of CYP26B1 (part A) and CRBP2 (part B) were determined by qPCR (Light-Cycler®, Roche Diagnosis) on total RNA extracted from the cells exposition. Absolute quantification of CYP26B1 and CRBP2 was performed in triplicate (mean with standard deviations) and then normalized to the 36B4 house-keeping gene. Medium grey bar: ATRA; dark grey bar: DMSO; light grey bar: control (no treatment); *: statistical difference between basal (T0) and treatment (T6, T24 and T48) levels ($p<0.05$).

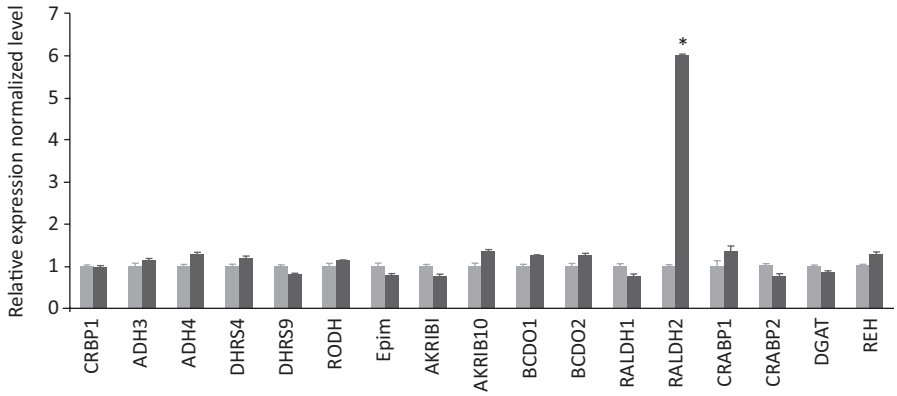


**Figure 8.5** | Differential expression levels of retinoid metabolism actors in human normal and CDH lung tissue. Results from quantitative RT-PCR were obtained in 13 normal and 9 CDH lung samples from canalicular, saccular and alveolar stages. Each quantification of specific gene was normalized using the quantification of the house keeping gene 36B4. A relative ratio for each gene was presented using the value of normal lung as 1. *: statistical difference between normal (light grey bar) and CDH (dark grey bar) lungs ($p<0.05$).

Abbreviations: CRBP1: cellular retinol binding protein 1 and 2; ADH3 and 4: alcohol dehydrogenase 3 and 4; DHRS 4 and 9: short-chain dehydrogenase-reductase 4 and 9; RODH: retinol dehydrogenase; Epim. : Epimerase; AKR 1B1 and 1B10: aldo keto reductase 1B1 and 1B10; BCDO1 and 2: beta-carotene 15-15' dioxygenase 1 and 2; RALDH 1 and 2: retinaldehyde dehydrogenase 1 and 2; CRABP 1 and 2: cellular retinoic acid binding protein 1 and 2; DGAT: diacylglycerol acyl transferase;; REH: retinyl esters hydrolase

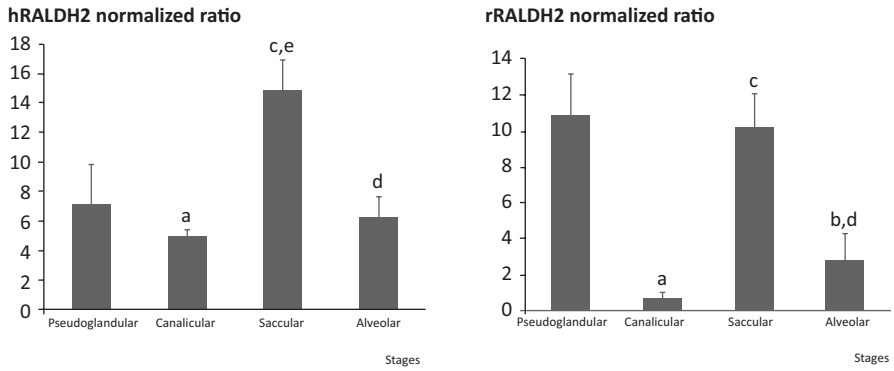


**Figure 8.6** | Quantitative expression of RALDH2 mRNA during human and rabbit lung development. ***Part A.*** The expression levels of RALDH2 were established in normal rabbit lung development (n=3). The absolute quantification of RALDH2 was performed in duplicate for each sample and then normalized with 36B4 (house-keeping gene). ***Part B.*** The expression levels of RALDH2 were studied in normal human lung development (n=13). Pseudoglandular: 13.5, 14, 15, 17 and 17.5 weeks of gestation (WG); canalicular: 21, 22 and 24 WG; saccular: 27, 27, 28.5 and 34 WG; alveolar: 37 WG. The absolute quantification of RALDH2 was performed in duplicate for each sample and then normalized with 36B4 (house-keeping gene). Statistically significant differences ($p<0.05$) were noted a between pseudoglandular and canalicular stages, b between pseudoglandular and alveolar stages, c between canalicular and saccular stages, d between saccular and alveolar stages, e between pseudoglandular and saccular stages.

*Abbreviations: hRALDH*2: human retinaldehyde dehydrogenase 2; *rRALDH2:* rabbit retinaldehyde dehydrogenase 2;

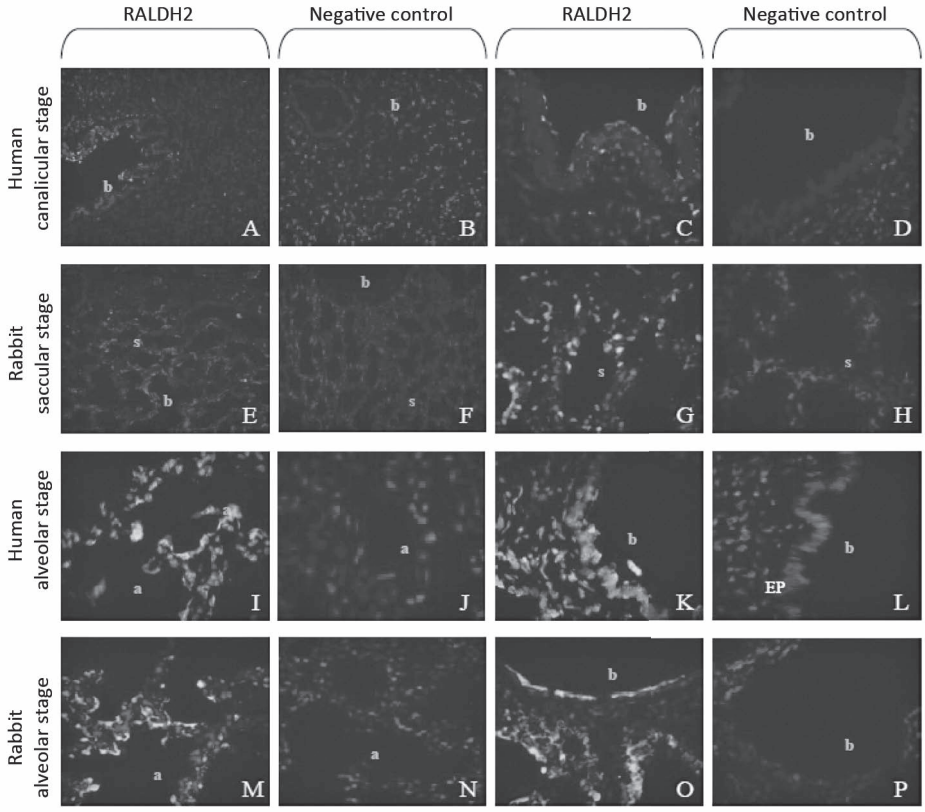


**Figure 8.7** | Immunohistochemical staining of RALDH2 in the developing human and rabbit lung. RALDH2 immunolocalization (green fluorescence) was realized in human canalicular (A to D) and alveolar (I to L) stages and in rabbit saccular (E to H) and alveolar (M to Q) stages. Negative controls (without secondary antibody) were illustrated in B, D, F, H, J, L, N and P. Nuclear localization (blue fluorescence) was visualized using a Hoetch staining. Magnification for A, B, E, F, I, J, M and N: 10x; C, D, G, H, K, L, O and P: 40x.

*Abbreviations:* a: alveoli; b: bronchi; s: sacculi

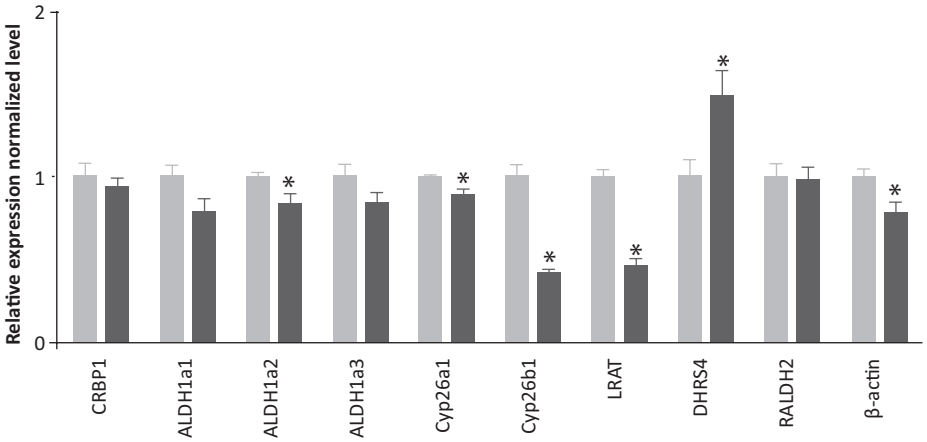


**Figure 8.8** | Differential expression levels of retinoid metabolism actors in rat normal and nitrofen lung tissue. Results from Q-PCR were obtained in 5 normal and 5 CDH lung samples from embryonic day 21. Each specific gene was run in duplicate and normalized to the house keeping gene RPS18. A relative ratio for each gene was presented using the value of normal lung as 1. *: statistical difference between normal (light grey bar) and CDH (dark grey bar) lungs ($p<0.05$).

*Abbreviations: CRBP1:* cellular retinol binding protein 1; *RALDH 1, 2 and 3*: retinaldehyde dehydrogenase 1, 2 and 3; *DHRS 4:* short-chain dehydrogenase-reductase 4; *Cyp26a1 and b1:* Cytochrome P450 subtype 26a1 and 26b1; *LRAT:* lecithin:retinyl acyl transferase.

## Discussion

Our study established an extensive analysis of factors involved in retinoid metabolism (Figure 8.1) during the 4 stages of human lung development, completing the data previously described for the molecular (nuclear receptors RARs and RXRs) signalling pathways.[18] So far, expression studies were only performed for a number of selective genes in mouse lung development: LRAT, DGAT, CRBP1 and 2, CRABP1, RALDH2 and 3, STRA6, CYB26A1 and B1.[8,24-27] Given the limited availability of human material, we correlated the expression of genes measured by quantitative PCR with protein function using a RARE gene reporter strategy in a representative lung cellular model, the A549 cell line.[28] This strongly suggests that the human developmental lung is able to produce active retinoids from foetal blood retinol throughout the different developmental stages. The developing lung also expresses

genes whose products are involved in other enzymatic processes: the degradation of retinoic acid and the production/hydrolysis of retinyl esters.

The intracellular binding of vitamin A and the degradation of retinoic acid are identified as critical steps in this metabolic pathway due to the presence of just a single bio-active molecule. Therefore, functional mutants of either member of this pathway could easily block this metabolic cascade. Indeed gene inactivation studies of STRA6 or Cyp26B1 displayed important lung phenotypes.[29,30] Stra6 plays an important role in the rodent lung retinoid homeostasis when expressed in neonatal rat lungs and is regulated by RA.[26] During mouse lung development, Cyp26B1 is also the most predominant member of the cytochrome P450 family that is able to oxidize RA in inactive metabolites.[31] The activation of Rol to RA could be considered as robust with the presence of several enzymes for Ral generation and two distinct metabolic ways with two different precursors (Rol and beta-carotene). The potential weakness of this metabolic pathway could be represented by the presence of only two retinal dehydrogenases (RALDH1and RALDH2) at the pseudoglandular and saccular period, with the well-known predominant mammalian developmental implications of RALDH2.[9] Nevertheless, we determined that the expression level of RALDH2 was the highest at these two stages. During the canalicular and alveolar stages, this level was determined as the lowest but may be compensated by the expression of the other 3 RALDHs, suggesting a fine adaptation between the level of the most important enzyme RALDH2 and the ability of redundancy with the 3 other enzymes. Using this mechanism, the strong need of RA during the prenatal period and more particularly for alveolarization could be ensured.

Throughout human lung development, we found the presence of 4 specific intracellular binding proteins: CRPB1 and 2, CRABP1 and 2. These proteins optimize the cellular trafficking of retinoids by increasing their solubility, but also by driving these molecules in the different metabolic potentialities.[32] Indeed, intracellular RA bound to CRABP1 is targeted for degradation in fetal tissues by Cyp26 members of the cytochrome P450 system. Intracellular RA may also bind to CRABP2, which facilitates delivery to the nucleus, where it activates the retinoid receptors and transcriptional regulation of target genes.[33] The CRBPs could direct retinol to RA production, but also to storage as retinyl esters (RE). During development, the lung is well known to accumulate retinyl esters in lipid droplets which may be an important local supply of retinol.[34] This function is performed by the lipid interstitial fibroblasts, one of the two populations of lung fibroblasts, which synthesize and secrete retinoic acid.[35] From the third trimester of fetal life, there is a significant accumulation of retinyl esters in the lung. During late gestation and early postnatal life the RE stores become quickly depleted.[36] Our results showed that in contrast to the murine model, DGAT (and not LRAT) is involved in RE generation during human lung development.

Based on human material and two complementary animal models, our results clearly confirm the important role of RALDH2 in normal and CDH lung development, as has already been shown for diaphragm tissue.[37,38] The genetic sequence of RALDH2 exhibits a strong homology among species: 65%, 67% and 89% between rat and rabbit, human and rabbit, rat and human nucleotide homology, respectively (Genomatix® analysis). Its developmental kinetic pattern is similar in terms of relative expression levels in human and rabbit: an early expression in the pseudoglandular period and an up-regulation during saccular period found in both studied species as in rodent models.[8] The last peak just before the alveolar stage is physiologically very important for alveolarization, which is RA-dependent. Expressed in the bronchus and the alveolar wall, the RALDH2 protein presents the same cellular localization during human and rabbit lung development. The bronchial expression has also been found in the rodent model, which only differs in the second expression site: pleura instead of alveolar wall.[39] Nevertheless, all 3 expression patterns support the idea that RALDH2 acts as a local developmental signal to induce the distal formation of alveoli. We also established that the RALDH2 expression is increased specifically in the lung after a surgical diaphragmatic hernia. From a developmental point of view, this pulmonary tissue is healthy in origin, no genetic, dietary or teratogenic disturbances have taken place. Therefore, this model is ideal to study the isolated impact of competition for space. The acute RALDH2 sensibility to mechanical stress (compression by the bowels) has to be linked to those similarly described in two other traumatic models: zebrafish[40] and rat.[41] Our data obtained from the toxic nitrofen rat model confirms the lung disturbances in term of retinoid metabolism. First molecular stigmas described by Nakazawa et al.[25] were completed and confirmed the depleted generation of retinoic acid, one of the two complementary hypothese with the more recent "apoptosis" one[42] to explain the mechanism by which nitrofen causes CDH. We also reported a downregulation of the pulmonary retinol storage enzyme LRAT and the RA-degrading enzyme Cyp26B1, while not affecting RALDH2.[25] We established that a second enzyme belonging to the Cyp26 family, *i.e*. Cyp26A1 is decreased, adding to the disturbance of this degradation pathway. We also showed that two related RALDHs (1 and 3) did not compensate the RALDH2 inhibition and that DHRS4 expression level is increased. The latter could produce more Ral to reverse the inhibition realized by the nitrofen on RALDH activity.[43] In the nitrofen model, the early diaphragmatic defect leads to a chronically altered development of the lung. This excludes acute lung injury and the related RALDH2 response as described in our rabbit surgical model, where the diaphragmatic defect is created relatively late in gestation. We demonstrated that CRBP2 and Cyp26B1 are transcriptionally regulated by RA in a lung cellular context, but their RA-induction is different with an early (Cyp26B1) and a delayed (CRBP2) response. This could be related to the direct and indirect mechanism of retinoid-induction, which has already been described in the human intestinal Caco-2 cell line.[44,45]

The absence of Cyp26B1 and CRBP2 detection in human CDH lung could be linked with a weak decrease of Rol (and RA) in these patients as recently reported by Beurskens et al.[16] In contrast, no modifications of Cyp26B1 and CRBP2 levels could be detected in the rabbit model, which has a normal homeostasis and was not disturbed by a gift of retinol.

All together, based on these results we propose a model explaining the disturbances of retinoid metabolism in CDH. This model starts with a slight retinol deficiency in the foetal compartment. Indeed, our recent data confirms that the significant association between low levels of vitamin A in cord blood and CDH in the newborn, while the vitamin A levels of the mother are comparable.[15,16] In this recent study we established the highest risk for CDH in patients with the lowest 15% of vitamin A levels as determined at the end of the gestation. During pregnancy, retinol blood levels are maintained at the same level throughout all the trimesters, suggesting that the level at the end of pregnancy is comparable to the level in the first trimester.[46] As the choriovitelline and chorioallantoic placenta are strongly involved in retinoid transport and metabolism,[23] a placental problem in maternal-to-foetal transport could cause a retinol deficiency in the child, while mother has normal levels. This early, low retinol in foetal blood level is inadequate to support normal diaphragm development, leading to the first hit of the retinoid hypothesis in CDH generation.[7,47] In this respect it is imperative to realize that the defect in the diaphragm already exists before the placental circulation has commenced. However, primordial placental tissue, as well as the amniotic membranes may play an important role in the transfer of retinoids to the fetus.[48] The second hit is caused by the herniation of the abdominal viscera into the thorax due to disrupted closure of the diaphragm. This process occurs in the context of fetal vitamin A deficiency. This leads to abnormal lung development (hypoplasia and disturbed alveolarization) associated with a local deficiency in retinoic acid. It has been shown that retinoic acid is involved in injury-related tissue responses. In an adult rat model, it was shown that spinal cord injury induces the activity of RALDH2 in the first or second week after the injury. This excludes RA as a trigger in the immediate inflammatory reaction and suggests a function as a regenerative actor, at least in spinal cord injury.[49] Whether there is a comparable subpopulation cells that generates RA after injury (hits) in the foetal lung, needs to be established. In the second phase of development of CDH, the growth of the lung is severely hampered by the space occupying effect of mainly the liver in early stages of development, later in combination with the growing intestines. An adaptive mechanism tries to repair the damage by an increase in RALDH2. As it appears, this mechanism to produce more RA is insufficient as was shown by absent expression of RA-sensible target genes CRBP2 and CYP26B1, an indirect marker of RA deficiency. This organ-specific RA deficiency is one of the explanations for the lung hypoplasia and the delayed alveolar maturation found in human CDH lungs. Recent advances in prenatal diagnostic procedures allow clinicians to check the veracity

of this foetal Rol and/or RA deficiency during the second trimester of pregnancy, *i.e.* at the beginning of the canalicular period. If a retinoid deficiency is confirmed, one might suggest supplementing retinoic acid or vitamin A outside the teratogenic period. Retinoids could be supplemented using maternal or foetal (during foetoscopic endoluminal tracheal occlusion) ways as already proposed by our group and others.[4,50] In animal models RA treatment can induce alveolar regeneration after injury by dexamethasone or oxygen[51-53] and we recently reported that RA could normalize the altered pneumocytes I/II CDH ratio.[19]

In conclusion, based on the analysis of human lung samples and two complementary animal models, our study clearly established disturbances of the retinoid metabolism in congenital diaphragmatic hernia lungs. This new pathophysiological mechanism confirms the "retinoid hypothesis" in CDH. It provides new perspectives for diagnosis and therapy for multidisciplinary clinical teams involved into the management of this severe developmental lung disease.

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# Chapter 9

## General discussion

The overall aim of the research described in this thesis was to investigate the role of etiologic factors, especially vitamin A, in Congenital Diaphragmatic Hernia (CDH) and lung development.

The results support the hypothesis that vitamin A is one of the factors implicated in the aetiology of human CDH.

In part I, the epidemiological studies (chapter 3 to 5), we showed that newborns with CDH have a 25% lower level of retinol and retinol-binding protein (RBP) in cord blood as compared to healthy newborns. The adjusted odds ratio (OR) for retinol concentrations <p15 (<0.61 µmol/l) was 11.11 (95% confidence interval [CI] 2.54 to 48.66; p=0.001) and for RBP < p15 (<4.54 mg/l) 4.00 (95%CI 1.00 to 15.99; p=0.05). We observed that a vitamin A intake lower than the daily recommended intake (DRI; 800 µg retinol activity equivalents [RAE] / day) was significantly associated with an increased risk estimate for CDH (OR 4.55; 95%CI 1.05 to 19.61; p=0.04). The retinol and RBP concentrations, however, were comparable in case and control mothers at delivery. This is in contrast to the findings in animal models, in which a maternal disturbance in vitamin A homeostasis leads to CDH in the offspring.

CDH and neural tube defects may have a mutual origin of the involvement of the neural crest cells in the embryogenesis (chapter 2). Neural crest cells are sensitive for homocysteine and vitamin A affects the homocysteine pathway. The homocysteine pathway is essential in one carbon metabolism in which methyl groups are used for DNA and histon methylation. Therefore, we investigated the biomarkers of the homocysteine pathway in association with CDH. The results in a small group of case and control newborns did not show significant differences.

In the molecular biological studies described in part II, we showed that human lung can be successfully cultured to investigate human lung development (chapter 7). Furthermore, in chapter 8 we demonstrated that there is a difference in the expression of vitamin A pathway genes between normal and CDH-affected lungs, both in humans as in two animal models of CDH. In these models, the development of the diaphragm defect was modeled at two distinct phases in development. The increased expression of RALDH2 suggests a compensatory reaction to a developmental hit. In the rat model this reaction is blocked by nitrofen, as indicated by the observation that RALDH2 expression was not changed.[1] The results of this study indicate that the vitamin A pathway is also disturbed in human CDH, but probably at a level more downstream than in the toxicology based nitrofen rat model.

In summary, the studies described in this thesis make an important step in human CDH research by providing evidence that disturbances in vitamin A homeostasis are involved in human CDH.

## The role of Vitamin A in lung and diaphragm development

As discussed in chapter 2, the lungs and diaphragm develop in the same time frame and in close proximity during embryogenesis. It is therefore feasible to consider lung and diaphragm development as two correlating processes, guided by similar pathways. Clarification of the pathogenesis of the associated lung hypoplasia might explain the pathogenesis of the diaphragm defect and vice versa.[2] In lung development, the local concentration of retinoic acid (RA) provides the developing lung with positional clues and determines whether the tubuli extend or branch, a process called branching morphogenesis.[3] At the moment of first outgrowth of the lungs from the posterior foregut (lung bud), ubiquitously present RA stimulates the posterior foregut endoderm to a lung fate in the mouse,[4,5] probably by regulation of the Wnt- and TGF-β-pathways.[6] During branching morphogenesis, a proximo-distal gradient of RA is constituted. The high proximal concentration stabilizes the already formed tubes and the lower concentration in the distal mesenchyme allows additional lung bud formation. The local RA regulation is controlled by a complex interplay of generation and degradation of RA. Indeed, embryos lacking the main RA-generating enzyme (RALDH2) lack RA signalling in the foregut and fail to develop lungs.[7] The high RA demand is reflected in the presence of local retinyl ester storages in the lung. Further, the expression pattern of the nuclear retinoic acid receptors (RAR, RXR) varies during the different stages of lung development.[8] It is therefore plausible to assume that a disturbance in the (local) availability of RA or other retinoids can lead to defective lung development. Some proof for this assumption may be found in the associated pulmonary hypoplasia and the delayed differentiation of the lungs in CDH. These lungs are not similar to the lungs in premature born infants, as, although suggested otherwise, in human CDH no primary surfactant deficiency is documented.[9] Several genes regulated by retinoic acid are located on frequently altered regions on the chromosomes of CDH patients (chapter 6). However, except for STRA6 in a complex congenital anomaly phenotype,[10] no isolated CDH cases have been described with mutations in specific RA-regulated candidate genes.

In diaphragm development, the role of retinoids is much less investigated. A complicating factor is the fact that there is still debate on how the human diaphragm develops, especially in relation to the defects seen in CDH. Some authors even suggest that the classical view of diaphragm development has to be revised.[11] Some progress has been made by the use of knock-out mice models. The manipulation of retinoid

regulated genes such as Coup-tfII, RAR, Gata4 and Fog2 leads to diaphragm defects with a varying phenotype. Clugston et al.[12] demonstrated that genes from CDH-critical regions on chromosomes 8 and 15 are expressed in the rodent diaphragm. However, the gene expression in human diaphragm is unclear and detailed genetic analyses of fibroblast cell lines in large cohorts of human CDH have never revealed mutations to be present in individual cases.

Before we elaborate on the impact and relevance of our findings we discuss some methodological issues of our studies. Finally we end this chapter with recommendations for further research and implications for the clinical practice.

## Methodological issues

The epidemiological studies described in this thesis were conducted within the HERNIA-study, Erasmus MC, Rotterdam, the Netherlands. The HERNIA-study was designed to investigate CDH and the role of Environmental factors, Retinoids, Nutrition, Inheritance and other Associations and is worldwide the largest case-control family study to investigate the role of etiologic factors in CDH. The HERNIA-study was originally designed as an international study ("retinoid study"). The power calculation for vitamin A status at pregnancy and birth was based on the data described by Major et al.[13] Based on these data and a type I error of 0.05, the inclusion of 49 case and 49 control patients should enable us to identify a 30% difference between the two groups with a power of 90%. Unfortunately, the international component of the study was aborted because it was not feasible to include participants in the selected centers. Fortunately, we have succeeded in including enough participants in the Rotterdam protocol (HERNIA) to answer the main study questions. The design of the HERNIA study has been described in chapter 1. In short, the HERNIA study was designed as a birth cohort study in which at several moments a nested case-control study was conducted. In this thesis, the results of two studies are described:

- T1: immediately after prenatal diagnosis of CDH
- T2: at birth.

The case-control design was based on the fact that CDH is a rare disease. A periconceptional cohort study would have been more appropriate to study causal relationships. However, this is less efficient and would have required a very large population base (approximately 150,000 inclusions, nearly all births in the Netherlands per year) to include the same number of CDH patients as described in chapter 3. This is obviously not feasible.

In the molecular biological studies (chapters 7 and 8), human lung tissue has been used to study lung development and the role of oxygen and metabolic actors of the vitamin A pathway. In chapter 7 we describe the successful culture of human foetal lung to study the expression of oxygen related factors during lung development. Foetal lung explants maintained *in vitro* are a well-characterized model for studying foetal lung development. This model allows us to study *in vitro* the effect of external factors (teratogens, medication) and internal factors (signalling molecules such as retinoic acid) on human branching morphogenesis. In separate pilot experiments we have tried to culture the lungs of CDH patients. Although the number of experiments is limited, the lungs of CDH patients seem to have less growth capacity *in vitro*. This may be suggestive for the dual-hit hypothesis,[2] stating that the lung development in CDH patients is disturbed by a separate mechanism, apart from the mechanical effects from the diaphragm defect. This mechanism might be influenced by the vitamin A pathway, as indicated in Chapter 8, in which we studied tissue samples of foetal lung of human, rat and rabbit origin.

A general intrinsic problem with models is the limited translation to the human situation. We tried to tackle this problem by including two different animals models and comparing them with human tissue. This method also compensates in part for the general drawback of the use of human foetal tissue: the difficulty to ensure sufficient tissue quality to perform experiments. For culture, the tissue needs to be as fresh as possible to maintain viability. This can vary between tissue samples. Further, in contrast to most animal models, the genetic and phenotypic heterogeneity of the tissue is much larger, and can lead to possible under- or overestimation of the effects studied. However, this remains inherent to human tissue, and especially foetal tissue. The use of alternative tissues, such as cell lines, umbilical cord and liver may solve part of this problem. Further, the use of patient and control cell lines makes it possible to study the effects of increased or decreased retinoid levels on tissue differentiation and the expression of RA-regulated genes. This might particularly be interesting in tissues of patients with CDH and a genetic defect.

**Ascertainment of the cases and controls**

Case families were identified after the diagnosis was made by prenatal ultrasound and/or after birth at Erasmus MC. Due to the centralization of CDH in two centers, the patient population at Erasmus MC is derived largely from the western part of the Netherlands. Nearly all case families (father, mother and child) that were diagnosed and treated at Erasmus MC participated in the study at one or both study moments. The cases described in this thesis were all diagnosed during pregnancy. Due to variation in the time of diagnosis and the referral from other clinics, the time of inclusion varied roughly from 20 weeks after the last menstrual period, i.e. gestational age, to nearly term. This resulted in missing data and maybe some recall bias of exposures. Furthermore, some CDH patients were

born premature. To prevent distortion of the association between vitamin A and CDH by prematurity and the lack of premature controls we excluded all premature patients.

Control families were enrolled at the outpatient clinic of Erasmus MC covering the same population of the western part of the Netherlands. Control families were eligible if there were no major congenital anomalies in the foetus. Pregnant control women were selected on comparable gestational age, age, parity, fertilization method and ethnicity. The case and control families were not related.

**Comparability of cases and controls**

Although the sample population is largely the same for cases and controls, a subgroup of cases was derived outside the Western part of the Netherlands. Further, the need for cases to deliver at Erasmus MC and immediate preparation of the samples made it essential to select controls from the population visiting the outpatient clinic. Although these factors may have introduced some heterogeneity, the general characteristics of cases and controls were comparable. It is not to be expected that geographical variations affected the (in)dependent variables. However, the maternal age in the control group was slightly higher which can partially be explained by the women with high maternal age (>36 years) that were included to collect amniotic fluid to investigate the vitamin A status of the child during pregnancy.

**Validity of information**

Differential recall bias in cases and controls cannot be excluded. Case families may have a better recall of the pregnancy period, because they actively seek an explanation for the malformation in their child. Moreover, case families may have a different level of motivation for filling out the questionnaires. These two differences may have lead to underreporting of exposures in the control group. Further, case mothers may have changed their diet after diagnosis of CDH in their child. We therefore aimed to complete the questionnaires as soon as possible after diagnosis, to minimize the influence of changing nutritional habits. The questionnaires were completed largely within one month after inclusion, minimizing the effect of the diagnosis on the diet. The questionnaires were checked for completeness by the researcher and, if necessary, completed by telephonic consultation of the participant. The FFQ has been shown to produce valid dietary information, as has been proven by others.[14]

The samples for determination of the biomarkers were collected and processed in a highly standardized manner. This minimized the influence of sampling, processing and storage, which is underlined by the fact that the concentrations of the biomarkers were not related to the processing time or storage at -80°C. The samples were taken after the diaphragm defect has developed. The comparability of the concentrations after diagnosis

and at birth to the levels in the periconceptional period, has to be investigated.[15] However, definite proof of a causal relationship is still needed.

The premature CDH patients we excluded from analysis (chapter 3) had comparable concentrations compared to the term patients. However, it is difficult to compare the values between two different time-points. In the CDH patients admitted after birth, no cord blood was available. In these patients, a blood sample from the arterial line was taken. The values of retinol and RBP in these patients were lower compared to the values in cord blood. Since these patients already received fluids and medication, it is difficult to determine the value of these determinations.

## Inferences and elaborations on our findings

The studies described in this thesis have generated significant data on the involvement of vitamin A in CDH. Based on the results of our studies, we hypothesize that human CDH is associated with low vitamin A in the child. Low maternal vitamin A intake during pregnancy might contribute to the CDH risk, but this is not reflected in maternal vitamin A status as determined by measurement of retinol and RBP at birth. The origin of the low vitamin A status is unclear, which will be discussed in detail in the following paragraphs:

### Low vitamin A status: origin in the mother

The hypothesis that CDH is caused by a maternal vitamin A deficiency is not supported by the comparable retinol and RBP levels found at birth. However, the comparison was made after the defect in the diaphragm has developed. Although maternal vitamin A levels at birth are strongly correlated to the periconception period,[15] it is still possible that the maternal vitamin A levels change after the development of CDH in the child.[13,16] The latter is supported by the increased risk estimate for CDH we found in normal weight mothers with a vitamin A intake under the daily recommended intake. Although not significantly, serum levels were lower in the case group. These data suggest that a low-normal maternal vitamin A status may be a risk factor for CDH. In this respect, it is unfortunate that serum levels of retinol and RBP have not been determined in the mother rat in the vitamin A deficient rodent model.

During diaphragm development, a comparable peak in retinol utilization may exist in humans as has been observed in rodents.[17] A low-normal maternal vitamin A status in this critical period may lead to a short, but relevant insufficient supply of retinoids to the foetus. In this respect, we assume that the liver stores are depleted after multiple pregnancies. It follows that we would expect a higher incidence of CDH in mother with high parity. This has not been proven in epidemiological studies.[18,19]

Although retinol and RBP are the optimal method to study vitamin A status,[20] the diet contains other forms of vitamin A such as retinyl esters and carotenoids. These may have additive and unique functions apart from the potential to be converted to retinol and RA. These roles should be investigated and explored further. Because of the strong relationship with levels of these nutrients and the previous meal, the biochemical analysis may be improved by collecting samples during a fasting state. In the HERNIA-study we collected random samples. It is not to be expected that values differ between cases and controls.

**Low vitamin A status: origin the maternal-to-foetal transport**

At the moment of primordial diaphragm formation, before the placental circulation is functional, the foetal supply of nutrients is mainly by passive diffusion into the coelomic fluid, by metabolic functions of the amniotic membranes or by the "uterine milk".[21-24] The nutrients that are passed during this timeframe are largely unknown but might contain retinol-RBP, retinyl esters and RA bound to albumin. In fact, amniotic membranes express LRAT, the enzyme converting retinol to retinyl esters.[22] Although it is not feasible to determine embryonic retinol levels, sampling of amniotic fluid might give additional information on the foetal vitamin A status.

The placental circulation commences after the defect in the primordial diaphragm has developed. This rules out that the placenta is an etiologic factor in CDH. However, the placental metabolism influences the vitamin A concentration during pregnancy. Furthermore, the placental vitamin A-related metabolic functions in the 2$^{nd}$ and 3$^{rd}$ trimesters may influence the foetal lung development and the severity of pulmonary hypoplasia. The changes in vitamin A metabolism associated with CDH, might also have an effect on placental tissue. Studying the placenta of healthy and CDH newborns might therefore provide us with clues on vitamin A metabolism, the placental expression of vitamin A transport genes or genetic markers related to diaphragm development. In this respect, it is important to realize that the placental transfer of retinoids differs between rodents and primates, which could also explain the difference in teratogenic capacity of certain retinoids between these species.[25]

**Low vitamin A status: origin in the child**

Our data that newborns with CDH have lower levels of retinol and RBP support the hypothesis that vitamin A is associated with CDH. However, a causal relationship cannot be determined by a case-control study and it is possible that low vitamin A is an epiphenomenon. Still, given the extensive body of evidence from animal models, it is very likely that a causal relationship exists.

The embryonic and foetal vitamin A status is strongly dependent on the supply by the mother, since liver stores are not available until late pregnancy. Although the maternal-to-foetal transport of retinoids is strictly regulated, this dependence makes the embryo very sensitive to variations in maternal retinoid levels, especially in periods of high retinoid utilization. Apart from maternal levels and disturbances in transport, the embryonic vitamin A status might be affected by changes in receptors, converting enzymes and binding proteins. As we showed in chapter 8, the expression of vitamin A pathway genes is indeed different in human CDH lungs. Whether this difference is also reflected in other tissues of these patients, needs to be elucidated. However, it is difficult to consider vitamin A activity and gene transcription separately because vitamin A itself is an important regulator of gene transcription through its metabolite retinoic acid (RA). Furthermore, the role of RA may extend beyond the regulation of gene transcription because a large number of noncoding RNAs also are regulated by RA. Additionally, extranuclear mechanisms that are influenced by retinoids are being identified.[26]

In conclusion, disturbed levels of vitamin A and its derivatives or a slight reduction in its signalling properties may be harmful to the embryo and foetus, possibly in more subtle ways than can be found by gene expression analyses alone.[27] Further, the timing of events is critical. The complex interplay between these highly variable parameters might account for the phenotypic variation seen in CDH patients. Moreover, other (un)related causative factors play a role. It will be difficult to analyze all these factors separately. In the next paragraph we will suggest some elements for future research to focus on.

## Further perspectives

### On the aetiology

The studies described in this thesis have substantiated that vitamin A is an etiological factor in human CDH. However, the exact relationships and mechanisms remain to be elucidated. Given the important role of retinoids and retinoid-related genes in lung and diaphragm tissue, it is conceivable that a correct maternal and embryonic / foetal vitamin A status is prerequisites for these tissues to develop normally. However, considering the multiple functions of vitamin A, a general disturbance of vitamin A would lead to more malformations than just a diaphragm defect and lung hypoplasia. Moreover, the phenotype of CDH is very variable. Therefore, it is reasonable to assume that the aetiology of CDH is multifactorial, and that the lung and diaphragm are sensitive to a retinoid disturbance in a specific period of embryonic development. Epidemiological and molecular biological studies should therefore focus on the identification of these factors in the critical period in diaphragm development. It is obvious that this is impossible for CDH, because the

diagnosis can only be made several weeks after the initial defect developed. However, focus on the preconception period in cohort studies might identify factors that add to the risk of developing CDH in the child.

To understand the processes in defective diaphragm development, diaphragm tissue from healthy and CDH-affected newborns and foetuses should be analyzed for differential gene expression patterns. Moreover, the additional analysis of lung tissue may provide us with important clues on the aetiology of CDH and the associated or iatrogenic pulmonary defects. It is imperative to prospectively collect these tissues and standardize handling and storage.

Further specification of the diaphragm defects *in vivo* by paediatric surgeons may enhance our knowledge on the various types of diaphragm defects,[28] and may help to understand the variety in clinical presentation of CDH patients. The scrutinous description of these diaphragm defects may enable us to link several types of diaphragm defects to different aetiologies, treatment algorithms and prognostic figures.

The analysis of placental tissue, both fresh frozen as well as formalin fixed paraffin embedded (FFPE), might provide us with more insight in the vitamin A transport between mother and child and affected metabolic pathways. Combined with vitamin A concentrations, phenotypic features and genetic data, the analysis of placental tissue from case and control pregnancies could provide us with these insights.

The collection of genetic material of the child, father and mother has been an ongoing study protocol at the department of paediatric surgery and clinical genetics. Now this information can be combined with the exposure data from the questionnaires and biochemical markers. Single-nucleotide polymorphisms (SNPs) in genes that are involved in the vitamin A pathway,[29] embryonic lung and diaphragm development should be investigated and combined with the exposure data and biochemical analyses.

These research questions require large numbers of cases and controls, which can only be achieved in projects in which patient care and research are combined. These projects (like the “Predict study” at Erasmus MC) will provide an ideal environment to study preconception exposures, risk factors during pregnancy and pregnancy outcome. Only in large-scale prospective projects it will be possible to investigate vitamin A status before CDH is diagnosed and to prove definite causal relationships. International collaborative networks such as the CDH study group and CDH Euro-consortium have been valuable tools to study characteristics of CDH patients.[30] Further, they provide a collaborative network for future research and development of treatment protocols.[31]

**Primary and secondary prevention**

With increasing knowledge of the aetiology of CDH, primary prevention of CDH may ultimately become available. Based on the current knowledge and the data presented in

this thesis, it can be hypothesized that increasing maternal retinoid intake could provide the child with optimal vitamin A concentrations for normal embryonic diaphragm and lung development. However, vitamin A has a narrow pharmacological window, both high and low levels are teratogenic.[32-35] The current policy is to limit the vitamin A intake in pregnant women, with a recommended daily intake (RDI) of 800 µg retinol equivalents (RAE) per day.[36] This advice should not be changed as high retinoid levels are surely teratogenic. Human experiments of maternal retinoic acid use have unfortunately proven this phenomenon resulting in major congenital anomalies such as craniofacial anomalies and outflow tract anomalies of the heart, but not CDH.[35] However, the drawback of the advise to limit vitamin A is that currently the vitamin A intake in pregnant women is often too low.[37] Based on the data in our studies, this may be a risk factor for developing CDH in the child. This stresses once more the need to advice future parents about healthy lifestyle and nutrition, preferably before conception. Initiatives such as www.zwangerwijzer.nl and the preconception nutrient and lifestyle counselling at Erasmus MC provide in this need and may add to the prevention of congenital anomalies.

Given the important role of vitamin A in lung development and the low levels of vitamin A in cord blood, it might be conceivable to supplement extra vitamin A to newborns with CDH. Retinol levels in blood drawn from the arterial line after birth contained even lower levels of retinol as compared to cord blood. Vitamin A has already been used successfully to prevent chronic lung damage in premature or very low birth weight newborns.[38-40] In a chronically undernourished population, maternal repletion with vitamin A at recommended dietary levels before, during, and after pregnancy improved lung function in normal weight offspring.[41] Therefore, the effect of vitamin A supplementation on the survival and indicators of pulmonary function of CDH patients should be studied in the future.

The studies described in this thesis provide evidence for an important role of vitamin A in the multifactorial aetiology of CDH. To gain a better understanding of the role of vitamin A and other etiologic factors that interact in the multifactorial aetiology of CDH, it is essential to perform large-scale, international studies. National and international collaborations such as the CDH-EURO consortium and the CDH Study group are valuable tools to study a disease with one name but a large variety in clinical presentation.

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Summary

Samenvatting

## Summary

Congenital Diaphragmatic Hernia (CDH) is a severe birth defect that affects approximately 1 in 3,000 newborns. The aetiology of CDH is unknown in most patients, only a minority of patients is diagnosed with a genetic defect or a syndrome. The clinical presentation of newborns with CDH is variable, but is usually characterized by cardiopulmonary distress caused by underdevelopment of the lungs and the pulmonary vasculature. The pulmonary hypoplasia and persistent pulmonary hypertension are the primary causes of mortality and morbidity in CDH patients. Our understanding of the pathophysiological mechanisms in CDH is still limited. The studies described in this thesis have provided new insights in the aetiology factors of human CDH.

### Part I

In chapter 2 we review the current knowledge on the role of nutrition, life style factors and genes in human CDH. In this chapter we further elaborate on the evidence that is available on the role of vitamin A which provides a basis for the study in chapter 3. In this study we demonstrate that newborns with CDH have approximately 25% lower levels of retinol and retinol-binding protein (RBP) in cord blood as compared to healthy newborns. In a multivariable logistic-regression model the odds ratio for retinol levels < 0.61 µmol/l was 11.11. In contrast to the animal models of CDH, we did not find a difference in vitamin A levels in the mothers of these children. In chapter 4 dietary intake and the biomarkers retinol and RBP are investigated during pregnancy. In this study we found a lower intake of vitamin A in case mothers with normal weight, as compared to normal weight control mothers. This intake was significantly lower than the recommended daily intake (800 µg) for pregnant women, and was associated with a 7.2 times increased risk on CDH. The relationship between vitamin A and homocysteine that was suggested in chapter 2 has been investigated in chapter 5 where we describe the levels of homocysteine, s-adenosyl methionine (SAM) and S-adenosyl homocysteine (SAH) in the cord blood of healthy and CDH-affected newborns. We did not identify a difference between case and control newborns, nor could we identify (maternal) determinants of methylation state, as determined by SAM/SAH levels.

## Part II

Part II is introduced by chapter 6 in which we review the current knowledge on the aetiology of CDH that is based on various animal models and genetic studies in human. The most important theories on defective diaphragm development are discussed. In chapter 7 we describe a model to study human lung development and the role of hypoxia–related factors in lung development. The data in this chapter suggests that this model is useful, and can be used to manipulate lung growth by pharmacological agents or signalling molecules such as the vitamin A derivative retinoic acid (RA). The expression of metabolic actors of the vitamin A pathway in human, rat and rabbit lung is described in chapter 8. In this study, we demonstrate that the vitamin A pathway is disturbed in the lung of human CDH, but probably at a different level than in the animal models.

The general discussion in Chapter 9 elaborates on the strengths and weaknesses of the studies and provides new insights and suggestions for future research on the aetiology of CDH.

# Samenvatting

Congenitale Hernia Diafragmatica (CDH) is een ernstige aangeboren aandoening die optreedt bij ongeveer 1 op 3000 pasgeborenen. De etiologie van CDH is bij de meeste patiënten onbekend, bij slechts een minderheid wordt een genetisch defect of syndroom gediagnosticeerd. Het klinisch beeld van pasgeborenen met CDH is variabel, maar wordt meestal gekenmerkt door cardiopulmonale instabiliteit op basis van onderontwikkeling van de long en het longvaatbed. De onderontwikkeling van de long en pulmonale hypertensie bepalen voor een groot deel de mortaliteit van CDH. De kennis van de pathofysiologische mechanismen die leiden tot CDH is nog steeds beperkt. De studies beschreven in dit proefschrift geven nieuwe inzichten in the oorzakelijke factoren van CDH bij de mens.

## Deel I

In hoofdstuk 2 beschrijven wij de huidige kennis op het gebied van voeding, leefstijlfactoren en genen die betrokken zijn bij het ontstaan van CDH bij de mens. In dit hoofdstuk beschrijven wij de resultaten die hebben geleid tot de hypothese dat de vitamine A stofwisseling is betrokken bij CDH. Deze resultaten vormen de basis voor het onderzoek in hoofdstuk 3. In dit onderzoek tonen wij aan dat pasgeborenen met CDH een 25% lager gehalte aan vitamine A en vitamine A-bindend eiwit (RBP) in het navelstrengbloed hebben, in vergelijking met gezonde pasgeborenen. Retinolwaarden < 0.61 µmol/l zijn geassocieerd met een odds ratio van 11.11. In tegenstelling tot de diermodellen van CDH is er geen verschil tussen de moeders van deze kinderen. In hoofdstuk 4 worden maternale voeding en de biomarkers retinol en RBP onderzocht tijdens de zwangerschap. In dit onderzoek vonden wij een significant lagere inname van vitamine A bij de CDH-moeders met een normaal gewicht ten opzichte van controle moeder met een normaal gewicht. De inname bij de CDH-moeders was significant lager dan de aanbevolen dagelijkse hoeveelheid voor zwangere vrouwen (800 µg) en was geassocieerd met een 7.2 keer verhoogd risico op CDH.

De relatie tussen vitamine A en homocysteine, zoals die werd gesuggereerd in hoofdstuk 2, wordt onderzocht in hoofdstuk 5, waarin het gehalte aan homocysteine, SAM en SAH wordt beschreven in navelstrengbloed van gezonde pasgeborenen en pasgeborenen met CDH. Wij vonden geen verschil tussen de case en de controle pasgeborenen, noch konden wij (maternale) determinanten bepalen van de methyleringsstatus van het kind, uitgedrukt in SAM/SAH waarden.

## Deel II

In hoofdstuk 6 beschrijven we de huidige kennis over de etiologie van CDH die is gebaseerd op verschillende diermodellen en erfelijkheidsonderzoek bij de mens. De belangrijkste theorieën worden besproken. In hoofdstuk 7 beschrijven we een model om de humane longontwikkeling te bestuderen en de rol van zuurstof-gerelateerde factoren in de longontwikkeling. De gegevens in dit hoofdstuk tonen dat dit model bruikbaar is en kan worden gebruikt om longgroei te manipuleren met farmacologische stoffen of signaalmoleculen zoals het vitamin A-afgeleide retinoinezuur (RA). In hoofdstuk 8 beschrijven wij de expressie van genen die onderdeel zijn van de vitamine A stofwisseling in longweefsel van de mens, rat en konijn. In dit onderzoek tonen wij aan dat op verschillende niveaus in de vitamine A stofwisseling de expressie van genen in humane CDH anders is dan in de gezonde long.
In de algemene discussie in hoofdstuk 9 wordt uitgeweid over de sterke en zwakke punten van de klinische studies. Wij beschrijven nieuwe inzichten en suggesties voor verder onderzoek naar de oorzaak van CDH.

# Dankwoord

Een proefschrift schrijf je niet alleen. Dit proefschrift was niet tot stand gekomen zonder de bijdrage van velen, van wie ik een aantal in het bijzonder wil noemen.

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# Curriculum Vitae

L.W.J.E. (Niels) Beurskens was born in Heel, the Netherlands, in 1980. After completing Gymnasium beta education at Scholengemeenschap St. Ursula, Horn, he started his medical study at the Radboud University Nijmegen in 1998 and graduated in 2004. In 2004 and 2005 he worked as a resident at the department of Neonatology of Radboud University Medical Center in Nijmegen. In 2005 he started his research training with prof.dr. D. Tibboel and prof.dr. R.P.M. Steegers-Theunissen on the project that has been described in this thesis. In march 2010 Niels started his training as a general practitioner at Maastricht University.

Niels and his wife Geertje have a son Lucas.

# Publications

Beurskens LW, Tibboel D, Lindemans J, Duvekot JJ, Cohen-Overbeek TE, Veenma DC, de Klein A, Greer JJ, Steegers-Theunissen RP. Retinol Status of Newborn Infants Is Associated With Congenital Diaphragmatic Hernia. Pediatrics. 2010 Oct;126(4):712-20

Rajatapiti P*, de Rooij JD*, Beurskens LW, Keijzer R, Tibboel D, Rottier RJ, de Krijger RR. Effect of oxygen on the expression of hypoxia-inducible factors in human fetal lung explants. Neonatology. 2010 Jun;97(4):346-54. *equal contribution

Beurskens LW, Tibboel D, Steegers-Theunissen RP. Role of nutrition, lifestyle factors, and genes in the pathogenesis of congenital diaphragmatic hernia: human and animal studies. Nutr Rev. 2009 Dec;67(12):719-30.

Beurskens N, Klaassens M, Rottier R, de Klein A, Tibboel D. Linking animal models to human congenital diaphragmatic hernia. Birth Defects Res A Clin Mol Teratol. 2007 Aug;79(8):565-72.

Beurskens LW, de Jonge R, Tibboel D, Steegers-Theunissen RP. Biomarkers of the methylation pathway in association with Congenital Diaphragmatic Hernia. Submitted

Beurskens LW, Schrijver LH, Tibboel D, Wildhagen MH, Knapen MF, Lindemans J, de Vries J, Steegers-Theunissen RP. Dietary vitamin intake during pregnancy and the risk of Congenital Diaphragmatic Hernia in the offspring. Submitted

Coste K*, Beurskens LW*, Gallot D, Tibboel D, Labbé A, Rottier RJ, Sapin V. Metabolic disturbances of the vitamin A pathway in congenital diaphragmatic hernia. Submitted *equal contribution

# PhD Portfolio

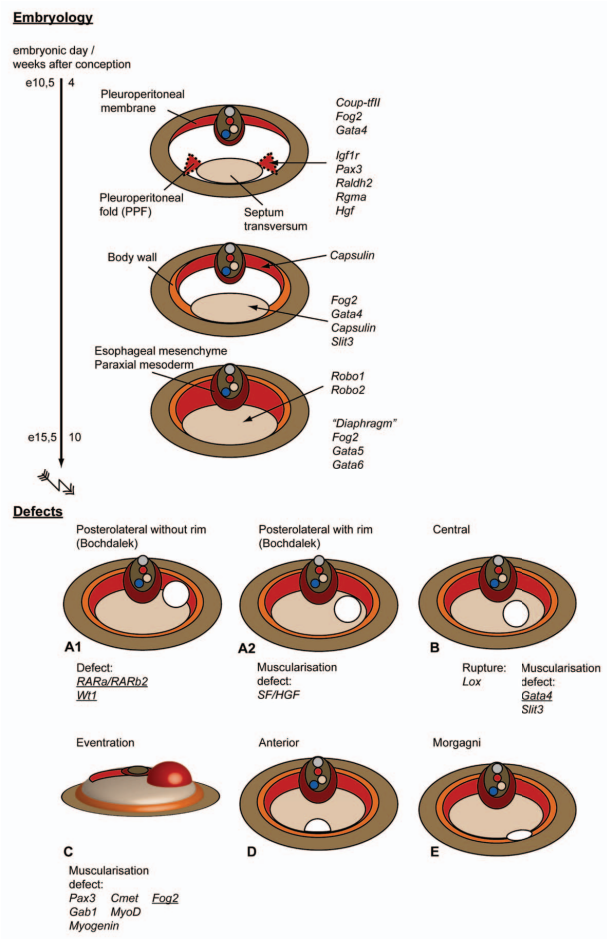
Summary of PhD training and teaching activities

| | |
|---|---|
| Name PhD student | **Leonardus Wilhelmus Josephus Elisabeth Beurskens** |
| Erasmus MC Department | **Pediatric Surgery** |
| PhD period | **November 1, 2005 – March 1, 2010** |
| Promotor's | **Prof.dr. D. Tibboel** |
| | **Prof.dr. R.P.M. Steegers-Theunissen** |

| **PhD training** | **Year** | **Workload (hours)** |
|---|---|---|
| **General academic skills** | | |
| Biomedical English Writing and Communication | 2008 / 2009 | 80 |
| Methodology of patient related research and preparation of funding | 2006 | 8 |
| Reading and discussing literature in molecular and cell biology | 2006 | 56 |
| Department journal club | 2008 / 2009 | 50 |
| **Research skills** | | |
| Classical methods for Data-analysis (CC02) | 2008 | 160 |
| Safe laboratory techniques | 2006 | 8 |
| Master course molecular and cell biology | 2007-2008 | 168 |
| From development to disease | 2007 | 40 |
| Regression analysis for clinicians (EWP23) | 2009 | 20 |
| Biomedical research techniques V | 2006 | 40 |
| Molecular diagnostics III | 2008 | 20 |
| Various research school courses and seminars | 2006 - 2009 | 40 |
| **International and national conferences** | | |
| International CDH meeting Houston, USA (presentation) | 2007 | 20 |
| International CDH meeting Mannheim, Germany (invited speaker) | 2009 | 20 |
| International meeting ESPR (abstract contribution) | 2009 | 2 |
| International meeting of the SGI, Orlando (poster contribution) | 2010 | 10 |
| National Congres of the NVOG (poster contribution) | 2010 | 10 |
| **Seminars** | | |
| Symposium perinatal lung development | 2007 | 8 |
| Success in research: learn from the experts | 2009 | 2 |

| | | |
|---|---|---|
| **Grant applications** | | |
| Presentation research grant Sophia Foundation for Medical Research | 2009 | 10 |
| | | |
| **Supervising Master's thesis** | | |
| "Maternal intake during pregnancy and the risk of having offspring with Congenital Diaphragmatic Hernia" - Lieske Schrijver | 2009 / 2010 | 7 months |

# Color figures

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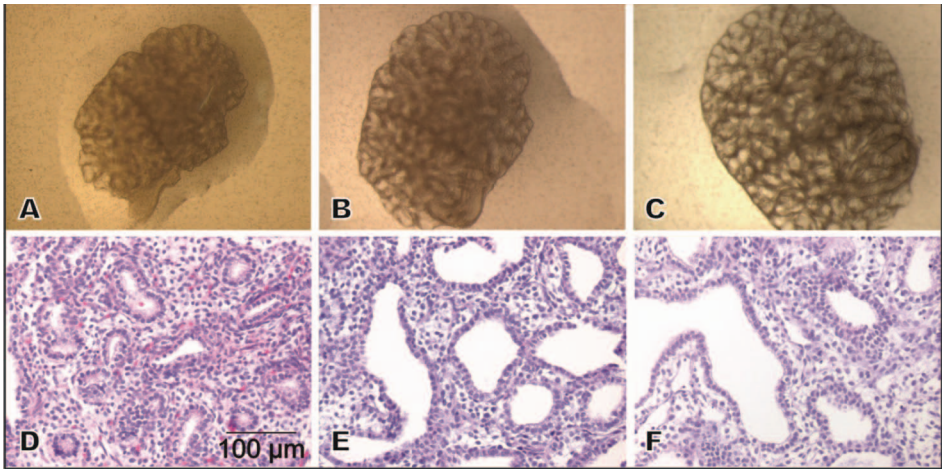
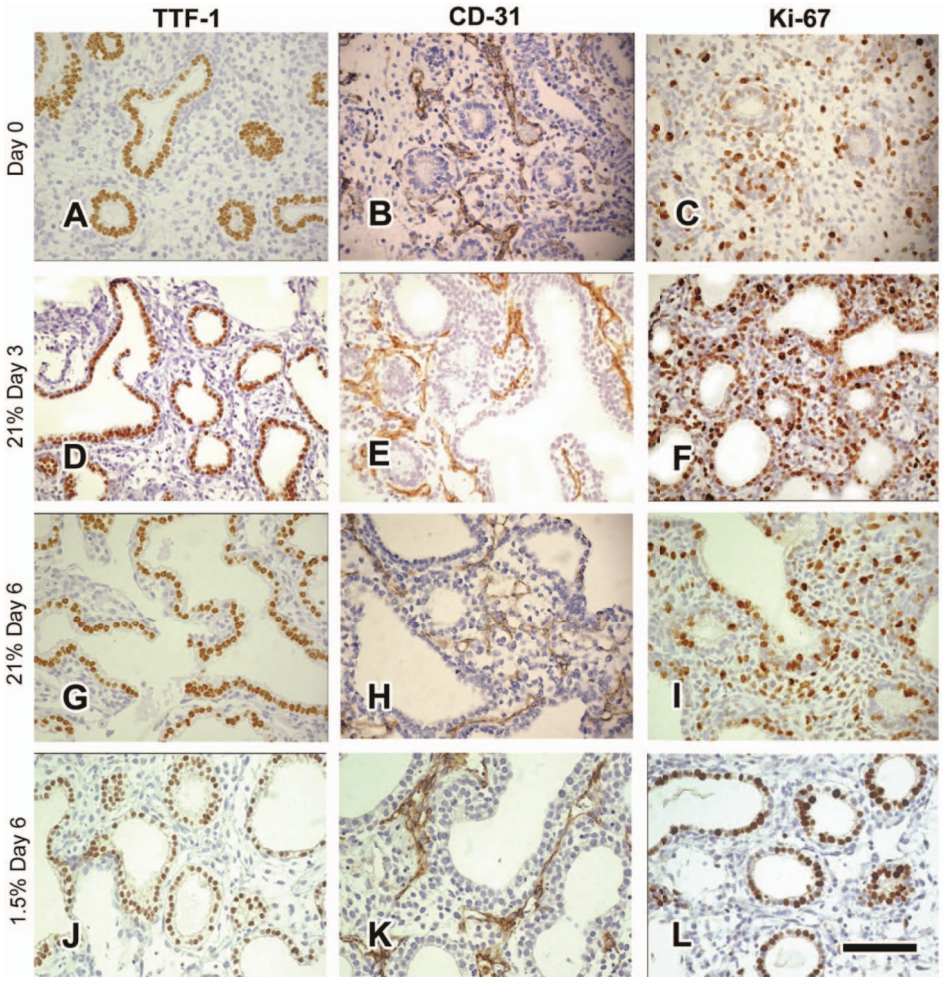
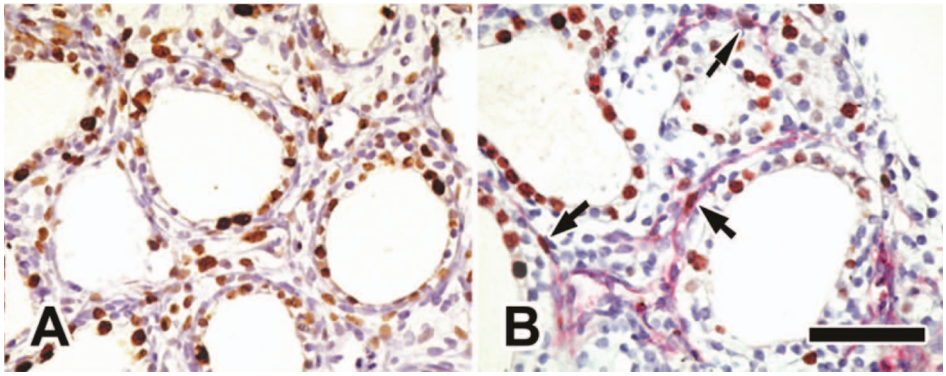



***Chapter 7:*** Figure 7.1 (page 99)




***Chapter 7:*** Figure 7.2 (page 100)

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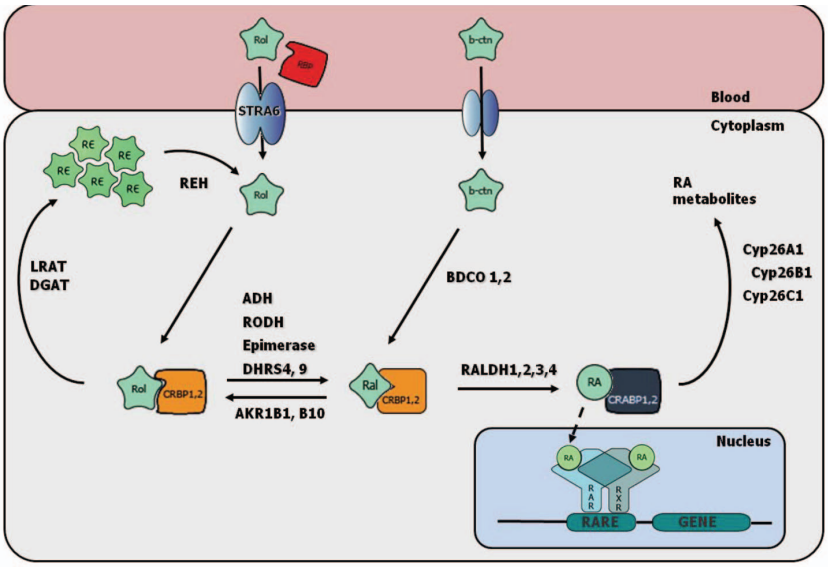


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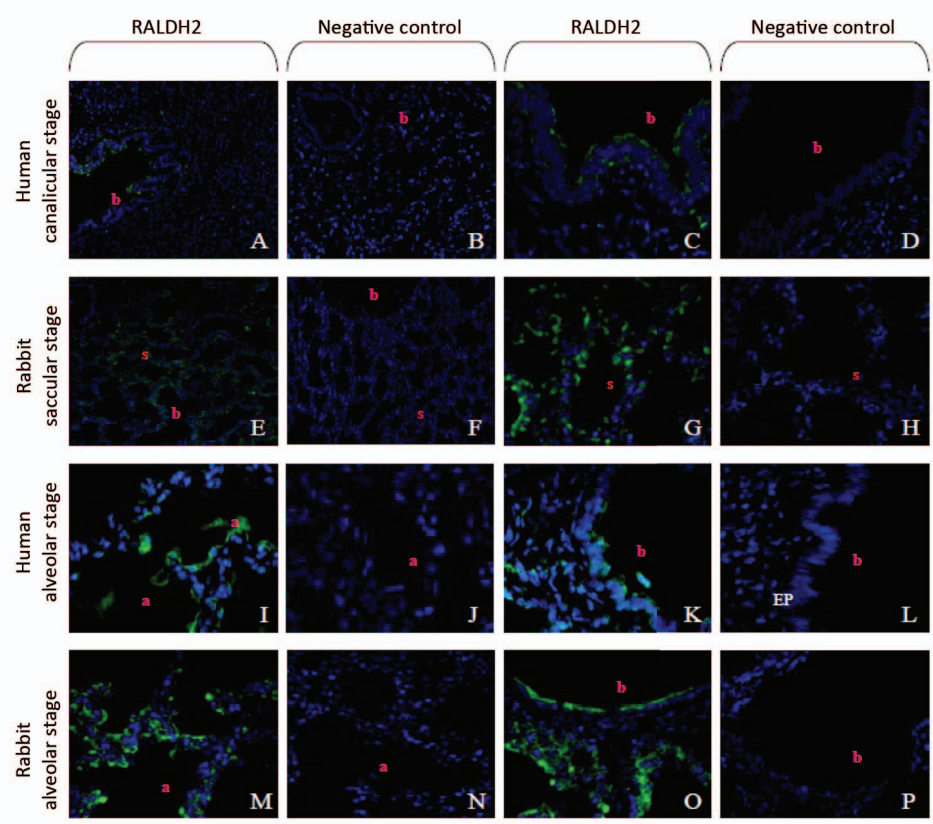


***Chapter 8:*** Figure 8.7 (page 124)