


## Article

# TITF1 Screening in Human Congenital Diaphragmatic Hernia (CDH)

Maria Eugenia Gulino <sup>1,\*</sup>, Giuseppe Martucciello <sup>2,3</sup> , Elio Biffali <sup>4</sup>, Patrizia Morbini <sup>5</sup> , Roberta Patti <sup>6</sup>, Marco Borra <sup>4,\*</sup> and Maria Grazia Scuderi <sup>6</sup>

<sup>1</sup> UOC di Chirurgia Pediatrica, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

<sup>2</sup> Pediatric Surgery DiNOGMI, University of Genova, 16147 Genoa, Italy; martucciello@yahoo.com

<sup>3</sup> Department of Paediatric Surgery, IRCCS Giannina Gaslini Children's Hospital, 16147 Genoa, Italy

<sup>4</sup> Stazione Zoologica "A. Dohrn", 80121 Naples, Italy; elio.biffali@szn.it

<sup>5</sup> UOC Anatomia Patologica, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy; patrizia.morbini@unipv.it

<sup>6</sup> UOC di Chirurgia Pediatrica, Ospedale Policlinico San Marco, 95121 Catania, Italy; roberta.patti92@gmail.com (R.P.); mgscuderi@gmail.com (M.G.S.)

\* Correspondence: mariaeugeniagulino@gmail.com (M.E.G.); marco.borra@szn.it (M.B.)

**Abstract:** TITF1 (Thyroid Transcription Factor-1) is a homeodomain-containing transcription factor. Previous studies showed that *Titf1* null mice are characterized by failure of tracheo-oesophageal separation and impaired lung morphogenesis resulting in Pulmonary Hypoplasia (PH). In this study, we aim to evaluate the role of *TITF1* in the pathogenesis of congenital diaphragmatic hernia (CDH) in humans. We investigated *TITF1* expression in human trachea and lungs and performed direct mutation analysis in a CDH population. We studied 13 human fetuses at 14 to 24 weeks of gestation. Five µm sections were fixed in paraformaldehyde and incubated with anti-TITF1 primary antibody. Positive staining was visualized by biotinylated secondary antibody. We also performed *TITF1* screening on genomic DNA extracted from peripheral blood of 16 patients affected by CDH and different degrees of PH, searching for mutations, insertions, and/or deletions, by sequencing the exonic regions of the gene. Histochemical studies showed positive brown staining of fetal follicular thyroid epithelium, normal fetal trachea, and normal fetal lung bronchial epithelium. Fetal esophageal wall was immunohistochemically negative. Molecular genetic analysis showed complete identity between the sequences obtained and the Wild Type (WT) form of the gene in all cases. No mutation, insertion and/or deletion was detected. Although *TITF1* is expressed in the human fetal lung and has been considered to have a role in the pathogenesis of PH in CDH, the results of our study do not support the hypothesis that *TITF1* mutations play a key role in the etiopathogenesis of CDH.

**Keywords:** congenital diaphragmatic hernia (CDH); pulmonary hypoplasia (PH); molecular genetics; histochemistry; *TITF1*



**Citation:** Gulino, M.E.; Martucciello, G.; Biffali, E.; Morbini, P.; Patti, R.; Borra, M.; Scuderi, M.G. *TITF1* Screening in Human Congenital Diaphragmatic Hernia (CDH). *Children* **2022**, *9*, 1108. <https://doi.org/10.3390/children9081108>

Academic Editor: Christian Aplitz

Received: 9 May 2022

Accepted: 16 July 2022

Published: 23 July 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Congenital Diaphragmatic Hernia (CDH) is a developmental defect characterized by the partial or complete absence of the diaphragm, the muscle that separates the chest cavity from the abdominal cavity, followed by the herniation of the abdominal organs into the thorax. CDH can occur as an isolated defect, in combination with multiple congenital anomalies, or as part of a well-defined syndrome [1]. According to the current literature, the incidence of CDH ranges from 0.8–5/10,000 newborns and varies across the population [2–5]. This defect is usually associated with bilateral Pulmonary Hypoplasia (PH), characterized by a reduction in the airway branching with smaller airspaces and pulmonary vascular abnormalities including a reduced number of vascular branches and thicker pulmonary arterial walls [6]. The hypermuscularization of the pulmonary bed clinically translates into pulmonary hypertension. In addition, an inadequate surfactant

production is present [7]. Over time, the improvement of prenatal assessment, antenatal interventions, and postnatal management have allowed to improve the survival rate in CDH [8], yet the mortality rate remains high for patients affected by pulmonary complications related to pulmonary hypoplasia and persistent pulmonary hypertension [9,10]. Therefore, prevention of lung maldevelopment in CDH remains a priority. To this end, the comprehension of the underlying causes of PH is critical.

Although PH in CDH was initially thought to be merely secondary to the diaphragmatic defect [11,12], its embryologic origins are still not clearly understood. More recent studies suggest that PH is not determined by the mass effect caused by the herniation of abdominal viscera in the chest [13–15] and that it can be associated with—rather than secondary to—diaphragmatic defects [16,17].

A genetic relation is found in 30% of CDH cases. Within this group, 1–2% are familial, while most of the cases are sporadic [18], leading to the hypothesis that de novo variants are an important etiological mechanism [19]. Because of its complexity, CDH is assumed to be a multifactorial disease involving genetic, environmental, and dietary variables [18,20,21].

Among the possible candidate genes for a predisposing role in CDH is *TITF1* (Thyroid Transcription Factor-1) [16]. *TITF1* is a homeodomain-containing transcription factor that was first identified as a nuclear protein able to bind to specific DNA sequences present in the thyroglobulin gene promoter. The *TITF1* protein is encoded by a single gene in mice and humans. In mice, *Titf1* is located on chromosome 12, whereas in humans it is on chromosome 14q13. The distribution of the *TITF1* protein and of the corresponding mRNA has been exhaustively studied in rodents [22]. *TITF1* is expressed in the foregut and in the thyroid anlage during mammalian development and continues to be expressed in the thyroid follicular cells (TFC) in adulthood. *TITF1* is also present in the trachea and lung bronchial epithelium and in selected areas of the forebrain, including the developing posterior pituitary. After birth and in adult animals, *TITF1* is still present in the thyroid and lung epithelium and in the posterior pituitary, whereas its expression in the brain is restricted to periventricular regions and some hypothalamic nuclei. Gene inactivation experiments have revealed some important functions of *TITF1* in vivo. The phenotype of mice homozygous for targeted disruption of the *Titf1* gene is rather complex, in accordance with the wide expression of this gene. *Titf1* null mice are characterized by impaired lung morphogenesis, lack of thyroid and pituitary glands, severe alterations in the ventral region of the forebrain, and death at birth [23]. Null mutation of *Titf1* also determines the failure of branching morphogenesis, resulting in PH [23,24]. Since *Titf1* is essential for lung development [25–29] and null mutation can cause PH in the mouse model, the authors undertook two lines of research to investigate the possible role of *TITF1* in the development of CDH in humans, namely: (a) an examination of *TITF1* expression in the trachea and lungs of human fetuses which died for causes other than CDH; and (b) the first direct *TITF1* mutation analysis of the genes of CDH patients.

## 2. Materials and Methods

### 2.1. Immunohistochemistry: Fetuses

We studied fetal foregut from 13 human fetuses without CDH at 14 to 24 weeks of gestation. All specimens were fixed in paraformaldehyde, embedded in paraffin and sectioned at 5 µm. Paraffin sections were dewaxed, rehydrated, and boiled by microwave oven (3 MW) with citrate buffer for 3 cycles of 5 min each. The sections were incubated overnight at 4 °C with anti-*TITF1* primary antibody (1:100; clone 8G7G3/1; DakoCytomation). Staining was carried out with biotinylated secondary antibody (Biotinylated Link Universal) and streptavidin peroxidase conjugate (Streptavidin-HRP; Kit Lsab + System HRP, K0690; DakoCytomation), and DAB (Diaminobenzidine, K3466; DakoCytomation) as chromogen. Slides were counterstained with hematoxylin.

## 2.2. Molecular Genetics: CDH Patients

Screening for mutations, insertions, and/or deletions was performed on genomic DNA from 16 patients (aged 2–7 days) affected by CDH (Table 1). Human *TITF1* is located on chromosome 14q13. We targeted exons II and III (Figure 1). DNA was extracted from frozen blood samples with Amersham Nucleon BAC3. We selected and synthesized two oligonucleotide pairs in the 5' and 3' external regions of the exon II and III, respectively, of the WT gene. We synthesized another set of nested oligos to obtain the complete exon sequences (Table 2). These oligonucleotides were used for PCR amplification of the two exons (Figure 2), and the resulting product was purified by gel electrophoresis. Sequences were performed in quadruplicate. Sequence reactions were purified automatically with a robotic station Biomek FX (Beckman Coulter, Brea, CA, USA) and obtained with a capillary electrophoresis sequencer 3730 DNA Analyzer (Applied Biosystems, Waltham, MA, USA). This procedure allowed us to obtain the entire sequences of the two exons in both strands, so each single nucleotide was sequenced several times in both directions. The sequences obtained were manually controlled and cleaned and then processed using bioinformatic tools to reconstruct the entire exon sequence like Blastn and Bioedit.

Finally, the reconstructed sequences of the two exons for each subject were aligned with the WT.

Table 1. CDH patients.

	Male/Female	Sporadic/Familial	Disease during Pregnancy	Left/Right Broad	Pulmonary Hypoplasia	Associated Anomalies	Survivor
CDH01	M	Familial	-	Right	Severe	-	not
CDH02	M	Sporadic	-	Right	Severe	Pectus excavatum	yes
CDH03	F	Familial	-	Left	Mild	-	-
CDH04	M	Familial	-	Left	Mild	-	-
CDH05	F	Familial	-	Left	Mild	Tricuspid regurgitation	yes
CDH06	M	Sporadic	-	Left	Mild	-	yes
CDH07	F	Sporadic	-	Left	Mild	-	yes
CDH08	F	Sporadic	-	Left	Mild	-	yes
CDH09	F	Sporadic	-	Left	Mild	-	yes
CDH10	M	Sporadic	-	Left	Mild	-	-
CDH11	M	Sporadic	-	Right	Mild	-	yes
CDH12	F	Sporadic	-	Left	Mild	-	-
CDH13	M	Sporadic	-	Left	Mild	-	yes
CDH14	M	Sporadic	-	Left	Mild	-	yes
CDH15	F	Sporadic	-	Right	Severe	Pectus excavatum	yes
CDH16	M	Sporadic	-	Left	Mild	-	yes

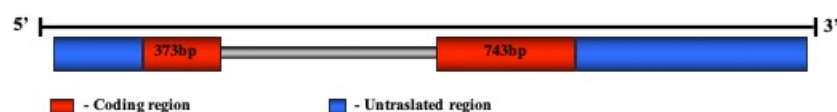
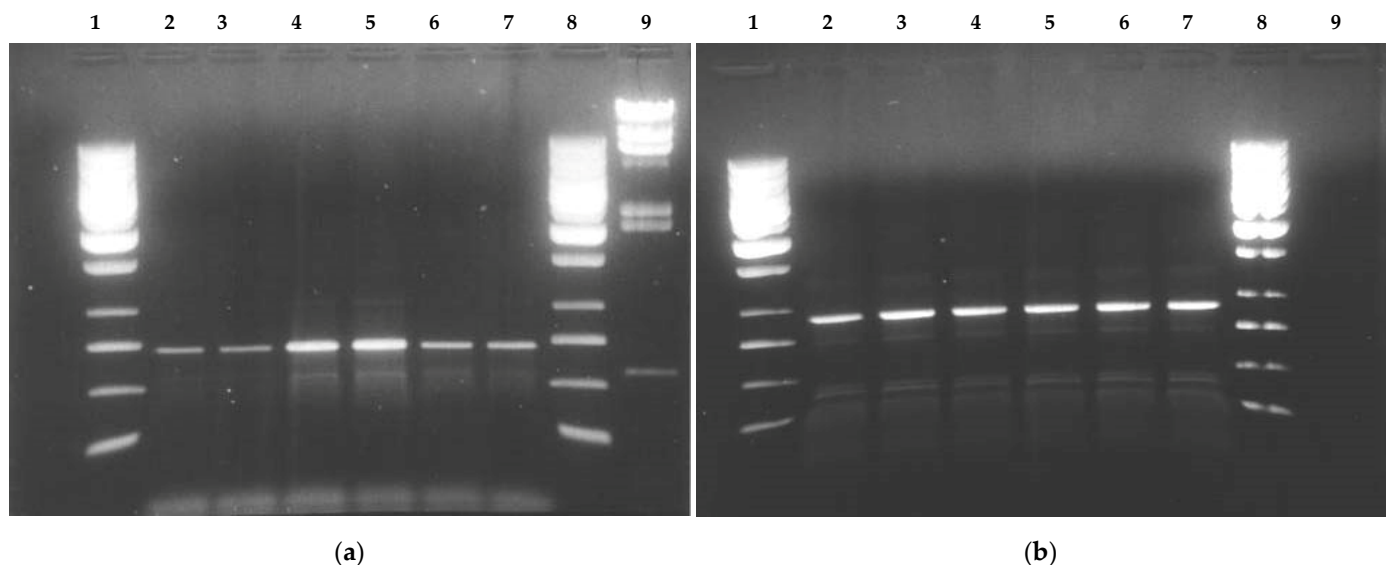


Figure 1. Exon II and III of *TITF1* gene targeted for genetic screening.

**Table 2.** Oligonucleotides for PCR and/or sequencing.

Name	Type	Sequence 5'→3'
hTiTF1 exIIF	Exon II PCR/Seq Forward	TGG CTG CCT AAA ACC TG
hTiTF1 exIIR	Exon II PCR/Seq Reverse	GCC CTC CCT GAT GC
hTiTF1 exIIF2	Exon II Seq Forward	GGA AAG CTA CAA GAA AGT GGG
hTiTF1 exIIR2	Exon II Seq Reverse	CTG TTC CTC ATG GTG TCC TGG
hTiTF1 exIIPCRUP	Exon II PCR/Seq Forward	GAG GAC TCG GTC CAC TCC GTT AC
hTiTF1 exIIPCRDw	Exon II PCR/Seq Reverse	AGC GCT ACC AAG TGC CTG TTC TTG
hTiTF1 exIIIF	Exon III PCR/Seq Forward	AGG GTT GGG GCT GTG AG
hTiTF1 exIIIR	Exon III PCR/Seq Reverse	GGA TGG TCT GTG TGG
hTiTF1 exIIIF2	Exon III Seq Forward	ATG GCG CGG AAA ACA GG
hTiTF1 exIIIR2	Exon III Seq Reverse	GCG GTG GAT GGT CA
hTiTF1 exIIIF3	Exon III Seq Forward	GCT TCA AGC AAC AGA AGT ACC
hTiTF1 exIIIR3	Exon III Seq Reverse	ACG GTT TGC CGT CTT TCA CC
hTiTF1 exIIIF4	Exon III Seq Forward	AAC AGG CTC AGC AGT CG
hTiTF1 exIIIR4	Exon III Seq Reverse	GTC AGG TGG ATC ATG CTG G

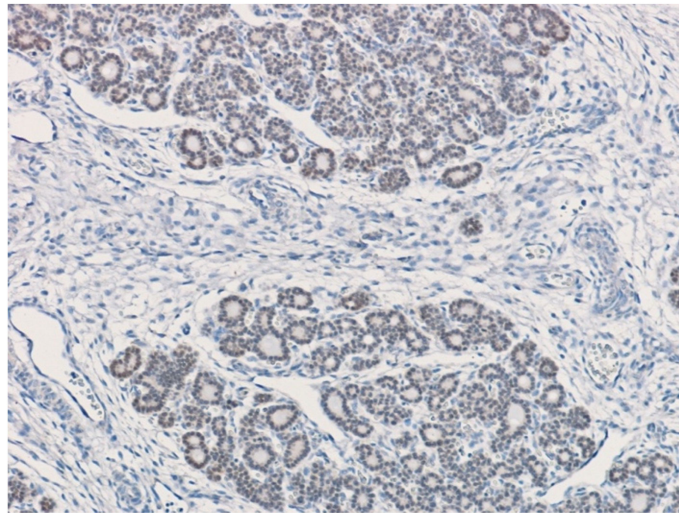


**Figure 2.** PCR amplification of exons, representative samples: (a) from left: lane 1 size molecular marker, lanes 2 to 7 samples amplification of the coding region exon II, lane 8 size molecular marker, lane 9 size and quantitative molecular marker; (b) from left: lane 1 size molecular marker, lanes 2 to 7 samples amplification of the coding region exon III, lane 8 size molecular marker.

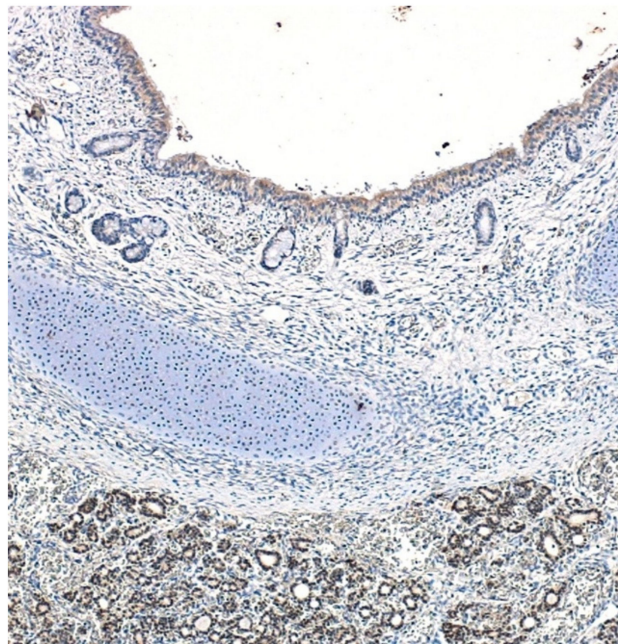
### 3. Results

#### 3.1. Fetuses

We evaluated the distribution of TITF1 in 13 normal human fetuses at 14 to 24 weeks of gestation, spanning from early TITF1 expression in epithelial cells of human lung to its pronounced expression in epithelial cells of terminal airways [30]. Positive staining for TITF1 was found in follicular thyroid epithelium (Figure 3), tracheal epithelium (Figure 4), and lung bronchial epithelium of all subjects. At these gestational stages, the fetal esophageal wall was immunohistochemically negative.



**Figure 3.** TITF1-positive nuclei (brown) of normal thyroid epithelium of a fetus at 14th week of gestation.



**Figure 4.** TITF1-positive nuclei (brown) are detected in both normal tracheal and thyroid epithelium at 14th week of gestation.

### 3.2. CDH Patients

Comparison of *TITF1* from CDH patients with WT revealed no differences. No mutations, insertions, and/or deletions were detected.

## 4. Discussion

Although significant efforts have been made to explain the pathophysiology of CDH, our current understanding of the etiology remains unclear. CDH is a multifactorial and multigenic condition, and several genes have been identified and proposed as possible candidates. The Wnt pathway is required in diaphragm development [31], and accordingly in CDH patients, both copy number variations (CNVs) and single nucleotide variations (SNVs) have been detected in genes associated with it such as *WT1* [31,32] and *FZD2* [33]. There is evidence for a pivotal role of vitamin A signaling in the developing lung and diaphragm [34,35]. In CDH patients, CNVs of and SNVs in *STRA6*, which encodes a

membrane receptor involved in the uptake of vitamin A [36], have been detected [37,38]. In CDH patients, CNVs have also been discovered in *ALDH1A2*, whose product is an enzyme that catalyzes the synthesis of retinoic acid from retinaldehyde, and in *RARA* and *RXRA*, which encode for retinoic acid receptor alpha and for Retinoid X Receptor Alpha, respectively, [39,40]. The region between 15q24 and 15q26 plays a critical role in the development of CDH [41]. *NR2F2* (*COUPTFII*), a member of a nuclear receptor superfamily, is highly expressed in the foregut [42] and resides within this minimal region. *NR2F2* may modulate the vitamin A pathway [43], and it is often deleted in CDH patients [41,44]. Consistent with this, *Nr2f2*-knockout mice develop diaphragmatic hernia [42]. In addition to mutations of genes associated with relevant signaling pathways, CNVs and SNVs have also been detected in other genes crucial during lung and diaphragm development. A list of candidate genes exists for CDH [45]. Because of the complexity of lung and diaphragm organogenesis and of CDH genetics, it is reasonable to consider the reported genes as predisposing to CDH rather than causative of CDH [45].

*TITF1* has long been considered a possible candidate gene in CDH pathogenesis because (i) it is expressed in lung endoderm and epithelium throughout lung development starting from 10 days of gestation, (ii) null mutation of *Titf1* in murine models determines a failure of tracheo-esophageal separation and branching resulting in PH [23,24], and (iii) the epithelial cells of these hypoplastic lungs do not undergo proper differentiation [23].

Our aim was to clarify whether *TITF1* gene plays a role in the complex etiology of CDH in humans. Although we confirmed the presence of *TITF1* in follicular thyroid epithelium, normal trachea, and bronchial epithelium of human fetuses at 14 to 24 weeks of gestation, we did not detect any mutation, insertion, or deletion on the human *TITF1* gene in our series of CDH cases. Our results, represent the first direct mutation analysis of *TITF1* in a human CDH population. In conclusion, our screening showed no differences in *TITF1* sequencing between CDH patients and the WT gene. Our results do not support the hypothesis that *TITF1* mutations play a key role in the etiopathogenesis of CDH.

Interestingly, Chapin and associates [46] showed that in a rodent model of nitrofen-induced CDH, lung hypoplasia was associated with an increased expression of *Titf1*. They demonstrated that stimulation of lung growth through tracheal occlusion was followed by restoration of *Titf1* expression to levels comparable to non-hypoplastic lung and by lung maturation and weight increase. Their study suggests that misregulation of *Titf1* expression may be central in the disruption of branching morphogenesis and proximal-distal patterning in the lung. Further investigations are required to obtain additional information on genes (and their mechanisms of action) involved in CDH. This will impact the availability of fetal therapy and will allow to correlate genetic variants with clinical outcome for providing personalized counselling and therapies.

**Author Contributions:** Molecular analysis, M.B. and E.B.; acquisition of data and immunohistochemical interpretation, M.E.G., G.M. and P.M.; original draft preparation M.E.G. and G.M.; review and editing M.G.S. and R.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Italian Ministry of Scientific Research, grant number RC 2005.

**Institutional Review Board Statement:** According to the Dutch law, material can be stored in a tissue bank under number and is freely available for researchers of ErasmusMC.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Acknowledgments:** The authors express their sincere thanks to Roberto Di Lauro, Elvira Mauriello, Raimondo Pannone and the Molecular Biology Service (Stazione Zoologica "A. Dohrn", Naples), Claire Archibald and Anna Capurro for the revision of the text and all clinicians for their precious collaboration. Dick Tibboel provided CDH samples for our analysis and critically reviewed the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Torfs, C.P.; Curry, C.J.R.; Bateson, T.F.; Honoré, L.H. A population-based study of congenital diaphragmatic hernia. *Teratology* **1992**, *46*, 555–565. [\[CrossRef\]](#)
2. McGivern, M.R.; Best, K.; Rankin, J.; Wellesley, D.; Greenlees, R.; Addor, M.-C.; Arriola, L.; De Walle, H.; Barisic, I.; Beres, J.; et al. Epidemiology of congenital diaphragmatic hernia in Europe: A register-based study. *Arch. Dis. Child.-Fetal Neonatal Ed.* **2015**, *100*, F137–F144. [\[CrossRef\]](#)
3. Yang, W.; Carmichael, S.L.; Harris, J.A.; Shaw, G.M. Epidemiologic characteristics of congenital diaphragmatic hernia among 2.5 million California births, 1989–1997. *Birth Defects Res. Part A Clin. Mol. Teratol.* **2006**, *76*, 170–174. [\[CrossRef\]](#)
4. Gallot, D.; Boda, C.; Ughetto, S.; Perthus, I.; Robert-Gnansia, E.; Francannet, C.; Laurichesse-Delmas, H.; Jani, J.; Coste, K.; Deprest, J.; et al. Prenatal detection and outcome of congenital diaphragmatic hernia: A French registry-based study. *Ultrasound Obstet. Gynecol.* **2007**, *29*, 276–283. [\[CrossRef\]](#)
5. Colvin, J.; Bower, C.; Dickinson, J.E.; Sokol, J. Outcomes of congenital diaphragmatic hernia: A population-based study in Western Australia. *Pediatrics* **2005**, *116*, e356–e363. [\[CrossRef\]](#)
6. Chandrasekharan, P.K.; Rawat, M.; Madappa, R.; Rothstein, D.H.; Lakshminrusimha, S. Congenital Diaphragmatic hernia—A review. *Matern. Health Neonatol. Perinatol.* **2017**, *3*, 6. [\[CrossRef\]](#)
7. Nakamura, Y.; Yamamoto, I.; Fukuda, S.; Hashimoto, T. Pulmonary acinar development in diaphragmatic hernia. *Arch. Pathol. Lab. Med.* **1991**, *115*, 372–376.
8. Morini, F.; Lally, K.P.; Lally, P.A.; Crisafulli, R.M.; Capolupo, I.; Bagolan, P. Treatment Strategies for Congenital Diaphragmatic Hernia: Change Sometimes Comes Bearing Gifts. *Front. Pediatr.* **2017**, *5*, 195. [\[CrossRef\]](#)
9. Deprest, J.; Brady, P.; Nicolaides, K.; Benachi, A.; Berg, C.; Vermeesch, J.; Gardener, G.; Gratacos, E. Prenatal management of the fetus with isolated congenital diaphragmatic hernia in the era of the TOTAL trial. *Semin. Fetal Neonatal Med.* **2014**, *19*, 338–348. [\[CrossRef\]](#)
10. Coughlin, M.A.; Werner, N.L.; Gajarski, R.; Gadepalli, S.; Hirschl, R.; Barks, J.; Treadwell, M.C.; Ladino-Torres, M.; Kreutzman, J.; Mychaliska, G. Prenatally diagnosed severe CDH: Mortality and morbidity remain high. *J. Pediatr. Surg.* **2016**, *51*, 1091–1095. [\[CrossRef\]](#)
11. Wells, L.J. A study of closure of the pleuropericardial and pleuroperitoneal canals in the human embryo. *Anat. Rec.* **1947**, *97*, 428. [\[CrossRef\]](#)
12. Harrison, M.R.; Jester, J.A.; Ross, N.A. Correction of congenital diaphragmatic hernia in utero. I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. *Surgery* **1980**, *88*, 174–182.
13. Iritani, I. Experimental study on embryogenesis of congenital diaphragmatic hernia. *Anat. Embryol.* **1984**, *169*, 133–139. [\[CrossRef\]](#)
14. Guilbert, T.W.; Gebb, S.A.; Shannon, J.M. Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia occurs early in development. *Am. J. Physiol. Cell. Mol. Physiol.* **2000**, *279*, L1159–L1171. [\[CrossRef\]](#)
15. Jesudason, E.C.; Connell, M.; Fernig, D.G.; Lloyd, D.A.; Losty, P.D. Early lung malformations in congenital diaphragmatic hernia. *J. Pediatr. Surg.* **2000**, *35*, 124–127, discussion 128. [\[CrossRef\]](#)
16. Keijzer, R.; Liu, J.; Deimling, J.; Tibboel, D.; Post, M. Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. *Am. J. Pathol.* **2000**, *156*, 1299–1306. [\[CrossRef\]](#)
17. Kunisaki, S.M.; Jiang, G.; Biancotti, J.C.; Ho, K.K.Y.; Dye, B.R.; Liu, A.P.; Spence, J.R. Human induced pluripotent stem cell-derived lung organoids in an ex vivo model of the congenital diaphragmatic hernia fetal lung. *Stem Cells Transl. Med.* **2021**, *10*, 98–114. [\[CrossRef\]](#)
18. Kardon, G.; Ackerman, K.G.; McCulley, D.J.; Shen, Y.; Wynn, J.; Shang, L.; Bogenschutz, E.; Sun, X.; Chung, W.K. Congenital diaphragmatic hernias: From genes to mechanisms to therapies. *Dis. Model. Mech.* **2017**, *10*, 955–970. [\[CrossRef\]](#)
19. Yu, L.; Sawle, A.D.; Wynn, J.; Aspelund, G.; Stolar, C.J.; Arkovitz, M.S.; Potoka, D.; Azarow, K.S.; Mychaliska, G.B.; Shen, Y.; et al. Increased burden of de novo predicted deleterious variants in complex congenital diaphragmatic hernia. *Hum. Mol. Genet.* **2015**, *24*, 4764–4773. [\[CrossRef\]](#)
20. Beurskens, L.W.J.E.; Tibboel, D.; Lindemans, J.; Duvekot, J.J.; Cohen-Overbeek, T.E.; Veenma, D.C.M.; de Klein, A.; Greer, J.J.; Steegers-Theunissen, R.P.M. Retinol status of newborn infants is associated with congenital diaphragmatic hernia. *Pediatrics* **2010**, *126*, 712–720. [\[CrossRef\]](#)
21. Beurskens, L.W.; Tibboel, D.; Steegers-Theunissen, R.P. Role of nutrition, lifestyle factors, and genes in the pathogenesis of congenital diaphragmatic hernia: Human and animal studies. *Nutr. Rev.* **2009**, *67*, 719–730. [\[CrossRef\]](#)
22. Lazzaro, D.; Price, M.; de Felice, M.; Di Lauro, R. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **1991**, *113*, 1093–1104. [\[CrossRef\]](#)
23. Kimura, S.; Hara, Y.; Pineau, T.; Fernandez-Salguero, P.; Fox, C.H.; Ward, J.M.; Gonzalez, F.J. The T/ebp null mouse: Thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **1996**, *10*, 60–69. [\[CrossRef\]](#)
24. Kimura, S.; Ward, J.M.; Minoo, P. Thyroid-specific enhancer-binding protein/thyroid transcription factor 1 is not required for the initial specification of the thyroid and lung primordia. *Biochimie* **1999**, *81*, 321–327. [\[CrossRef\]](#)
25. Minoo, P.; Hamdan, H.; Bu, D.; Warburton, D.; Stepanik, P.; deLemos, R. TTF-1 regulates lung epithelial morphogenesis. *Dev. Biol.* **1995**, *172*, 694–698. [\[CrossRef\]](#)

26. Minoo, P.; Li, C.; Liu, H.B.; Hamdan, H.; DeLemos, R. TTF-1 is an epithelial morphoregulatory transcriptional factor. *Chest* **1997**, *111*, 135S–137S. [[CrossRef](#)]
27. Minoo, P.; Su, G.; Drum, H.; Bringas, P.; Kimura, S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. *Dev. Biol.* **1999**, *209*, 60–71. [[CrossRef](#)]
28. Snyder, E.L.; Watanabe, H.; Magendantz, M.; Hoersch, S.; Chen, T.A.; Wang, D.G.; Crowley, D.; Whittaker, C.A.; Meyerson, M.; Kimura, S.; et al. Nkx2-1 represses a latent gastric differentiation program in lung adenocarcinoma. *Mol. Cell* **2013**, *50*, 185–199. [[CrossRef](#)]
29. Little, D.R.; Gerner-Mauro, K.N.; Flodby, P.; Crandall, E.D.; Borok, Z.; Akiyama, H.; Kimura, S.; Ostrin, E.J.; Chen, J. Transcriptional control of lung alveolar type 1 cell development and maintenance by NK homeobox 2-1. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 20545–20555. [[CrossRef](#)]
30. Stahlman, M.T.; Gray, M.E.; Whitsett, J.A. Expression of thyroid transcription factor-1 (TTF-1) in fetal and neonatal human lung. *J. Histochem. Cytochem.* **1996**, *44*, 673–678. [[CrossRef](#)]
31. Paris, N.D.; Coles, G.L.; Ackerman, K.G. Wt1 and  $\beta$ -catenin cooperatively regulate diaphragm development in the mouse. *Dev. Biol.* **2015**, *407*, 40–56. [[CrossRef](#)]
32. Schwab, M.E.; Dong, S.; Lianoglou, B.R.; Lucero, A.F.A.; Schwartz, G.B.; Norton, M.E.; MacKenzie, T.C.; Sanders, S.J. Exome sequencing of fetuses with congenital diaphragmatic hernia supports a causal role for NR2F2, PTPN11, and WT1 variants. *Am. J. Surg.* **2022**, *223*, 182–186. [[CrossRef](#)]
33. Wat, M.J.; Veenma, D.; Hogue, J.; Holder, A.; Yu, Z.; Wat, J.J.; Hanchard, N.; Shchelochkov, O.A.; Fernandes, C.; Johnson, A.; et al. Genomic alterations that contribute to the development of isolated and non-isolated congenital diaphragmatic hernia. *J. Med. Genet.* **2011**, *48*, 299–307. [[CrossRef](#)]
34. Wilson, J.G.; Roth, C.B.; Warkany, J. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am. J. Anat.* **1953**, *92*, 189–217. [[CrossRef](#)]
35. Mendelsohn, C.; Lohnes, D.; Décimo, D.; Lufkin, T.; LeMeur, M.; Chambon, P.; Mark, M. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **1994**, *120*, 2749–2771. [[CrossRef](#)]
36. Kelly, M.; von Lintig, J. STRA6: Role in cellular retinol uptake and efflux. *Hepatobiliary Surg. Nutr.* **2015**, *4*, 229–242. [[CrossRef](#)]
37. Pasutto, F.; Sticht, H.; Hammersen, G.; Gillesen-Kaesbach, G.; FitzPatrick, D.R.; Nürnberg, G.; Brasch, F.; Schirmer-Zimmermann, H.; Tolmie, J.L.; Chitayat, D.; et al. Mutations in STRA6 cause a broad spectrum of malformations including anophthalmia, congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and mental retardation. *Am. J. Hum. Genet.* **2007**, *80*, 550–560. [[CrossRef](#)]
38. Van Esch, H.; Backx, L.; Pijkels, E.; Fryns, J.P. Congenital diaphragmatic hernia is part of the new 15q24 microdeletion syndrome. *Eur. J. Med. Genet.* **2009**, *52*, 153–156. [[CrossRef](#)]
39. Steiner, M.B.; Vengoechea, J.; Collins, R.T. Duplication of the ALDH1A2 gene in association with pentalogy of Cantrell: A case report. *J. Med. Case Rep.* **2013**, *7*, 287. [[CrossRef](#)]
40. Henriques-Coelho, T.; Oliva-Teles, N.; Fonseca-Silva, M.L.; Tibboel, D.; Guimarães, H.; Correia-Pinto, J. Congenital diaphragmatic hernia in a patient with tetrasomy 9p. *Pediatr. Surg.* **2005**, *40*, e29–e31. [[CrossRef](#)]
41. Klaassens, M.; van Dooren, M.; Eussen, H.; Douben, H.; Dekker, A.D.; Lee, C.; Donahoe, P.; Galjaard, R.; Goemaere, N.; de Krijger, R.; et al. Congenital diaphragmatic hernia and chromosome 15q26: Determination of a candidate region by use of fluorescent in situ hybridization and array-based comparative genomic hybridization. *Am. J. Hum. Genet.* **2005**, *76*, 877–882. [[CrossRef](#)]
42. You, L.-R.; Takamoto, N.; Yu, C.-T.; Tanaka, T.; Kodama, T.; DeMayo, F.J.; Tsai, S.Y.; Tsai, M.-J. Mouse lacking COUP-TFII as an animal model of Bochdalek-type congenital diaphragmatic hernia. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 16351–16356. [[CrossRef](#)]
43. Cooney, A.J.; Tsai, S.Y.; O'Malley, B.W.; Tsai, M.J. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTC A response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.* **1992**, *12*, 4153–4163. [[CrossRef](#)]
44. Brady, P.D.; DeKoninck, P.; Fryns, J.P.; Devriendt, K.; Deprest, J.A.; Vermeesch, J.R. Identification of dosage-sensitive genes in fetuses referred with severe isolated congenital diaphragmatic hernia. *Prenat. Diagn.* **2013**, *33*, 1283–1292. [[CrossRef](#)]
45. Schreiner, Y.; Schaible, T.; Rafat, N. Genetics of diaphragmatic hernia. *Eur. J. Hum. Genet.* **2021**, *29*, 1729–1733. [[CrossRef](#)]
46. Chapin, C.J.; Ertsey, R.; Yoshizawa, J.; Hara, A.; Sbragia, L.; Greer, J.J.; Kitterman, J.A. Congenital diaphragmatic hernia, tracheal occlusion, thyroid transcription factor-1, and fetal pulmonary epithelial maturation. *Am. J. Physiol. Cell. Mol. Physiol.* **2005**, *289*, L44–L52. [[CrossRef](#)]