What basic/translational breakthroughs are going to change the way we understand CDH and care for these patients in the future?

Dick Tibboel; Robbert Rottier; Rene Wijnen

Departments of pediatric surgery; obstetrics; neonatology cell biology and molecular/clinical genetics

Erasmus MC – Sophia Children’s Hospital
Rotterdam the Netherlands

d.tibboel@erasusmc.nl
Congenital diaphragmatic hernia: “one disease” and a myriad of variability
Variability in: (epi)genetics/ etiology

Natural history during fetal development

Births and the first hours

Treatment sequences in particular pharmacotherapy related

Iatrogenic insults and specific responses of the lung

Microbiome effects
Insight in the black box of CDH

I. Sluiter, thesis ‘CDH: A vascular disease’
Genetics: What do I want to detect?

- Normal
- Polyplody
- Aneuploidy
- Balanced translocation
- Unbalanced or insertion
- Loss or Gain
- Inversions
- Uniparental disomy
- SNV/SNP/Indel

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- Yes
- If (depending on size, location etc)
- No

Adapted from Speicher & Carter: *The new cytogenetics: blurring the boundaries with molecular biology*
COUP-TF2 mouse model of CDH

- Tissue specific ablation

- Ablation in foregut mesoderm
  (incl. posthepatic mesenchymal plate)
  \(\rightarrow\) left-sided CDH

(You et al., PNAS, 2005)
Retinal dehydrogenase (Raldh2) expression in the developing diaphragm at E13.5 (A)
Robin D. Clugston, Wei Zhang, Susana Alvarez et al.
Am J Respir Cell Mol Biol 2010;42:276-285
Insights in molecular mechanisms

>450 chromosomal aberrations

- Involved in RA pathway
- Candidate genes??

Mutation analysis

**COUPTF II** (Tsai *et al*; KO mouse model)

-150 CDH pt for 15q gene **COUPTF II**

(total all research groups  >500 pt for COUPTFII, GATA4, FOG2, ROBO3/4...)

(STRA6 (Donnai-Barrow) & LRP2 (PDAC) : recessive mutation)

Only sporadic small (bp) changes!
Significance This study describes the results of a large-scale case control analysis of copy number variants (CNVs) in a cohort of patients with congenital diaphragmatic hernia (CDH) and a large number of healthy population-matched controls. Using a customized array comparative genomic hybridization system, we have identified six CNVs that are associated with CDH with statistical significance (P < 0.05). These regions validate several hypothesized CDH candidate genes and identify additional genes and pathways that contribute to the pathogenesis of CDH. The estimated frequency of pathogenic CNVs in this cohort is 13%, which underscores the critical contribution of CNVs in CDH. This study also provides a model approach that is broadly applicable to other structural birth defects and identifies candidates for future functional studies.
Zhu Q, High FA, Zhang C, et al. 2018
De novo variants in congenital diaphragmatic hernia identify MYRF as a new syndrome and reveal genetic overlaps with other developmental disorders

Hongjian Qi1,2,6, Lan Yu3,6, Xueya Zhou3,6, Julia Wynn3, Haoquan Zhao1,4, Yicheng Guo1, Na Zhu1,3, Alexander Kitaygorodsky3,4, Rebecca Hernan3, Gudrun Aspelund5, Foong-Yen Lim6, Timothy Crombleholme6, Robert Cusick7, Kenneth Azarow8, Melissa E. Danko9, Dai Chung9, Brad W. Warner10, George B. Mychaliska11, Douglas Potoka12, Amy J. Wagner13, Mahmoud ElFiky14, Jay M. Wilson15,16, Debbie Nickerson17, Michael Bamshad18, Frances A. High15,16,18, Mauro Longoni16,18, Patricia K. Donahoe16,18, Wendy K. Chung3,19,20+, Yufeng Shen1,4,21+

PLOS genetics, 2018
Deficiency of FRAS1-related extracellular matrix 1 (FREM1) causes congenital diaphragmatic hernia in humans and mice

Tyler F. Beck¹, Danielle Veenma³,⁴, Oleg A. Shchelochkov⁵, Zhiyin Yu¹, Bum Jun Kim¹, Hitisha P. Zaveri¹, Yolande van Bever⁴, Sunju Choi⁶, Hannie Douben⁴, Terry K. Bertin¹, Pragna I. Patel⁶, Brendan Lee¹,⁷, Dick Tibboel⁶, Annelies de Klein⁴, David W. Stockton⁸,⁹, Monica J. Justice¹ and Daryl A. Scott¹,²,*

Mouse model reveals the role of SOX7 in the development of congenital diaphragmatic hernia associated with recurrent deletions of 8p23.1

Margaret J. Wat¹, Tyler F. Beck¹, Andrés Hernández-García¹,⁵, Zhiyin Yu¹, Danielle Veenma⁶,⁷, Monica Garcia², Ashley M. Holder⁸, Jeanette J. Wat⁹, Yuqing Chen¹,⁴, Carrie A. Mohila⁴, Kevin P. Lally¹⁰, Mary Dickinson², Dick Tibboel⁶, Annelies de Klein⁷, Brendan Lee¹,³ and Daryl A. Scott¹,²,*
Figure 2. A portion of Sox7+/Δex2 mice develop retrosternal CDH that is similar to those seen in Gata4-/- mice.
Congenital Diaphragmatic Hernia

Defects

A1
- Posterolateral without rim (Bochdalek)
- Defect:
  - RARα/RARβ2
  - Wt1

A2
- Posterolateral with rim (Bochdalek)
- Muscularisation defect:
  - SF/HGF

B
- Central
- Rupture:
  - Lox
- Muscularisation defect:
  - Gata4
  - Slii3

C
- Eventration
- Muscularisation defect:
  - Pax3
  - Cmet
  - Fox2
  - Gab1
  - MyoD
  - Myogenin

D
- Anterior

E
- Morgagni

Adapted from Beurskens et al., 2009 Nutrition Reviews
‘Successful treatment of CDH is dependent on the integration of human genomic and genetic data with developmental expression profiling, mouse knockouts, and gene network and pathway modeling, which have generated a large number of candidate genes and pathways for follow-up studies.’
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<td>Lung hypoplasia, abnormal alveolus morphologic features</td>
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<td>Net proto-oncogene</td>
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<td>Abnormal seque morphologic features (conditional knockout in the respiratory epithelium)</td>
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**Table 1: Mouse Models with Both Diaphragm and Lung Abnormalities**

**Donahoe PK, Longoni M, High FA.**

*Am J Pathol* 2016, 186: 2532–2543
Molecular pathogenesis of congenital diaphragmatic hernia revealed by exome sequencing, developmental data, and bioinformatics

Mauro Longoni\textsuperscript{a,b,1}, Frances A. High\textsuperscript{a,c,1}, Meaghan K. Russell\textsuperscript{a,b,1}, Alireza Kashani\textsuperscript{a,d,1}, Adam A. Tracy\textsuperscript{a}, Caroline M. Coletti\textsuperscript{a}, Regis Hila\textsuperscript{a}, Ahmed Shamia\textsuperscript{a}, Julie Wells\textsuperscript{e}, Kate G. Ackerman\textsuperscript{f}, Jay M. Wilson\textsuperscript{g}, Carol J. Bult\textsuperscript{e}, Charles Lee\textsuperscript{b}, Kasper Lage\textsuperscript{a,b,d}, Barbara R. Pober\textsuperscript{a,g,i}, and Patricia K. Donahoe\textsuperscript{a,b,d,2}

Significance Congenital diaphragmatic hernia (CDH) is a common birth defect associated with high morbidity and mortality. Focusing on the coding sequence of 51 genes, discovered in human studies and in mouse models, we studied 275 CDH patients and identified multiple variants in CDH-causing genes. Information on gene expression in embryonic mouse diaphragms and protein interactions allowed us to prioritize additional compelling CDH associated genes. We believe that an improved understanding of the genetics of CDH will be important to design new therapeutic strategies for patients with diaphragmatic defects.

Proc Natl Acad Sci U S A. 2014;111(34):12450–12455
### Table

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### Pie Chart

- **Human Genes**
- **Mouse Genes**
- **CNV Candidates**
- **IBAS network and PPF expression**
- **Prioritized genes with ultra-rare variants**
MicroRNAs (miRNA) are small, non-coding RNAs that regulate gene expression through mRNA stability and translation. They are essential for development and homeostasis of organs. More than 1800 microRNAs have been identified in human. Research focusing on the role of microRNAs in lung development and disease is limited. We recently discovered that miR-200b is elevated in abnormal lungs of human CDH babies. In the same study, we found that higher miR-200b expression in the fetal tracheal fluid of CDH fetus is associated with a better response to fetoscopic endoluminal tracheal occlusion (FETO, a prenatal therapy to promote lung growth)
Conclusions: Our data indicate that miR-200b improves PH and decreases the incidence of CDH. Future studies will further exploit this newly discovered prenatal therapy for lung hypoplasia and CDH.
Koshgoo N, et al. 2019
CONCLUSIONS: Human fetal hypoplastic CDH lungs have a specific miR-200/miR-10a signature. Survival after FETO is associated with increased miR-200 family expression. miR-200b overexpression in CDH lungs results in decreased TGF-β/SMAD signaling.
Insight in the black box of CDH

I. Sluiter, thesis ‘CDH: A vascular disease’
Characteristic morphological findings

Normal vs. Pulmonary Hypertension
Oxygen and lung development

The master switch of life at birth?
Oxygen and lung development

Effect of Oxygen on the Expression of Hypoxia-Inducible Factors in Human Fetal Lung Explants

Prapapan Rajatapiti\textsuperscript{a,d} Jessica D. de Rooij\textsuperscript{a,b} Leonardus W.J.E. Beurskens\textsuperscript{a,b}
Richard Keijzer\textsuperscript{a} Dick Tibboel\textsuperscript{b} Robbert J. Rottier\textsuperscript{a,c} Ronald R. de Krijger\textsuperscript{b}

Representative images showing the morphology of human fetal lung explants (gestational age 16 weeks)
Lung Vascular Development

Vascular expansion through distal angiogenesis

Parera et al, Am J Physiol L141-149, 2005

Vascular growth guides epithelial branching

VSMCs are different in CDH


Working model

Mesenchymal
Epithelial

Growth factors

Hypoxia

Transcription factors

Normal in utero environment

Vegf

Hif-1a

Pulmonary vascular development

FGFs
TGFb
EGF
PDGF
BMP-4
Shh (morphogen)

Epithelial branching morphogenesis

HNF-3b
HFH4
GATA-6
TTF-1
Whole mount analysis of pulmonary vasculature

Lungs isolated at E15

Control

CDH

Capillaries in lungs of CDH pups appear less developed

NG2: pericytes
ACTA: smooth muscle cells
CD31: endothelial cells
Origin pulmonary perivascular cells?

Lineage tracing of pericytes:
- ex vivo lung explants, whole mount immuno staining,
- in vivo lineage tracing to reveal fate of perivascular cells
  c-Kit-CreERT/NG2-dsRed/eNOS-GFP mice reporter mouse lines, such as Rosa-mTmG, Rosa-Confetti, Rosa-YFP

24 Hours 48 Hours 72 Hours
Origin pulmonary perivascular cells

Model of pulmonary vascular disease:

Mouse CDH model (SSWO project 678)

A. Transcription factors up

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<tr>
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<th>EpCam+</th>
<th>Ng2+</th>
<th>Mes. Cells</th>
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B. Transcription factors down

SSWO projects 678, S15-11
RNA sequencing of different cell populations

E8.5
Control/CDH

E13
Isolation
Single cells
Staining

FACS sort

Biomics
Smart-seq2
full-length transcriptome

Analysis by Petros Kolvos

Analyzing data using Ingenuity
Top pathways overlap pericytes – endothelial cells
(Neo)-muscularization and perivascular cells?

Perivascular cells express differentiation markers in CDH

control  CDH

Premature or altered differentiation of pericytes in CDH

NG2
(perivascular cells)
SMA
(Smooth muscle cells)
CD31
(endothelial cells)
Pericytes express smooth muscle markers in CDH, independent of vessel size.

RA inhibition induces smooth muscle markers in vitro.

Increased pericyte coverage in CDH, and a reduced and impaired capillary bed.
Vascular abnormalities in human CDH

- Decreased collagen IV deposition (arrowheads)
- Increased pericyte coverage (arrows)
Pulmonary vascular development seems accelerated in CDH

Pericytes are different in CDH and may be the source of extensive muscularisation

Pericytes may be the origin of pulmonary hypertension in CDH

Increased pericyte coverage in CDH
Metabolic pathways of vascular tone

Daphne S Mous; Marjon J Buscop-van Kempen MSc et al European Resp Review 2018
Figure 3: Increased expression of both the ETA and ETB receptor and endothelin converting enzyme in human CDH.
Figure 3: Increased expression of both the ETA and ETB receptor and endothelin converting enzyme in human CDH.
Newborns with Congenital Diaphragmatic hernia: inhaled Nitric Oxide versus intravenous Sildenafil, an international randomized controlled trial

CoDiNOS Trial
Insight in the black box of CDH

I. Sluiter, thesis ‘CDH: A vascular disease’
Study human diseases using *in vitro* cultures

- Sufficient starting material
- Limited expansion (passage 2) + Air Exposure

Need only 1 cell to form an organoid
(Un)limited Expansion (Till P19)

No Air Exposure
We obtained material from 3 different sources:

- **Bronchial Tissue (BT)**
- **Broncho Alveolar Lavage (BAL)**
- **Tracheal Aspirate (TA)**

Department of Pulmonology
LUMC
Prof Hiemstra
Sander van Riet

Department of Neonatology
Sophia Children's' hospital
Prof Reiss
Dr Kroon
Tracheal aspirates are a good source for AEC differentiation
Generation of alveolar epithelial type 2 (ATII) cells from human induced pluripotent stems cells (hiPSC)

Stages of differentiation

- Induced Stem Cells
- Definitive Endoderm
- Foregut Endoderm
- Pulmonary Endoderm
- Lung Progenitor
- Alveolar type II

Gene expression of iPSC compared to generated ATII cells

- EpCAM
- SFTPC
- Merge

Staining of generated ATII cells

Sander van Riet, ms submitted
Airway organoids, an *in vitro* system to study airway diseases?

- The amount of primary tissue availability can be limited
  - An Organoid can be obtained from one single cell

- Can be obtained from
  - Conducting airways
  - Tracheal aspirates
  - Nose swaps

Model and study disease using a small amount of patient material – Patient specific cultures

Congenital Pulmonary Airway Malformations
3 types of lung organoids: Bronchiolar, Bronchoalveolar, Alveolar

- **Alveolar organoids**
  - Formed from Alveolar type II cells
  - Show presence of both Alveolar type I and type II cells

- **Bronchoalveolar organoids**
  - Formed from Bronchoalveolar stem cells
  - Show presence of Alveolar and Airway cells

- **Bronchiolar/Airway organoids**
  - Representing the airway by the presence of basal, ciliated and secretory cells
  - Defined medium to stimulate growth without losing phenotype

Scgb1a1: Secretory cells
Sftpc: Alveolar type II cells

Rock et al., 2010 Dis Mod & Mech
Choi et al., 2016 Dev Biol
Chip requirements:

Mimic the organ of interest
Recapitulate the organ’s physiology
Attainable read-out system(s)

For the lung:
• Stretchable (breathing)
• Air - and blood compartment
• Read out of both compartments (microscopy, O₂ sensor, TEER measurement, etc)
• Collect air and “blood” from the chip for analysis
• Recovery of cells post-chip for analysis
Epithelial differentiation - application

Dynamic 3D in vitro lung model:
- Mimics micro-anatomy of lung
- Recapitulates alveolar physiology
Lung-on-a-chip consortium

1. Develop advanced lung (alveolus) -on-a-chip.
2. Use the chip to study lung repair and regeneration, personalized medicine, etc.
3. Integrate several chip modules to start (partial) lung replacement.

microengineered 3D analogues of alveolar tissue for lung regeneration
What basic/ translational breakthroughs are going to change the way we understand CDH and care for these patients in the future?
CDH in 2025: prenatal

High-risk CDH patient: new risk assessment score integrating both pre- and postnatal characteristics.

Molecular genetic analysis incorporating next generation sequencing and adding this info to the international genetic biobank of CDH.

iPS cells will be generated for further identification of factors known to predict chronic lung disease using organoids and organ on chip techniques.

Single cell sequencing after differentiation will be performed to elucidate developmental abnormalities.

New experimental studies, like the use of microRNAs or stem cell therapy +/- PLUG.
CDH in 2025: perinatal

At birth, umbilical cord cells will be harvested for endothelial cells responses on vasoactive drugs using vessel-on-a-chip perfusion models.

To investigate potential biomarkers to identify at an early stage patients at risk for developing chronic lung disease as well as adverse comorbidities.

The best drug therapy for pulmonary hypertension based on in-vitro responses to a variety of drugs.

Drug dosing will be tailored based on phenotypic knowledge of drug metabolism as well as known influence of the disease state.
CDH in 2025: first admission and beyond

All data will be integrated using the infrastructure of the CDH-EURO Consortium; the CDH-registry and ERNICA taking the FAIR principle into account.

Markers of chronic lung disease extracted from tracheal aspirates during artificial ventilation will determine the ventilator settings and will be repeatedly re-evaluated.

Dense monitoring data are collected, notably with respect to brain-related parameters.

An MRI of the brain will be made with special attention for the hippocampus to predict dysfunction in executive functions enabling implementation in an intervention study.

The child and family are invited to join a tailor-made lifelong interdisciplinary follow-up program aiming to decrease long-term morbidity.
Acknowledgements

Overall supervisors: Dick Tibboel, Rene Wijnen, Robbert Rottier

Current Lab members: Anne Boerema-de Munck, Petra Burgisser, Marjon Buscop-van Kempen, Jennifer Collins, Evelien Eenjes, Heleen Kool, Daphne Mous, Koji Nagata

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Pediatrics: Prof I. Reiss, Ismé de Kleer

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Andre Poot (U-Twente)
Roman Truckenmuller (MERLN)
Irene Heijink (UMCG)
Reinoud Gosens (UMCG)
Machteld Hylkema (UMCG)

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Emma Rawlins (Cambridge)
Anne Hilgendorff (Munich)
Rory Morty (Bad Nauheim)
Jan DePrest (Leuven)
Richard Keijzer (Winnipeg)

Alumni lab-members: Niels Beurskens, Marike van Dooren, Janine Felix, Cristina Gontan, Irene van der Horst, Yadi Huang, Joshua Ochieng, Marta Canis Parera, Kim Schilders, Ilona Sluiter, Lalini Raghoebir, Prapapan Rajatapiti

Genomics: Jeroen Demmers, Wilfred v. IJcken

Clinic Pathology:

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Significant progress and shifts over time

- Prenatal risk stratification and trial design
- Improved survival and definition of standards of care

Comparative effectiveness trials CDH-EURO Consortium

- Developmental biology of the pulmonary vasculature
Significant progress and shifts over time

- Establishment of parent support groups in many countries
- Delayed surgical repair
- Gentle ventilation
- International collaboration such as the CDH registry and the DHREAMS initiative
European CDH Database

**MISSION** Better diagnosis, risk assessment and personalized treatment for CDH

**GOAL** Integration of clinical, molecular and cellular data, to understand the pulmonary vascularization in CDH

Conception → Prenatal → Perinatal → Postnatal

Collect Data/Material

European CDH database (WP1)

**ANALYSIS** Extract patient data/material for analysis:

- **Molecular:** GWAS/NGS studies, expression studies (-omics based), image analysis (WP1,3,4)
- **Cellular:** Isolate stem/progenitor cells, develop iPS cells, differentiation of cells, LCM (WP1,3)
- **Clinical:** Prediction model, pathology, link prenatal imaging with postnatal management (WP1,2,4)
Acknowledgements

Pulmonary medicine Erasmus MC
Professor Rudi Hendriks
Ingrid Bergen

Erasmus Centre for Optical Imaging
Professor Adriaan Houtsmuller
Gert-Jan Kremers
Gert van Capellen

Pediatrics Erasmus MC
Isme de Kleer

Cell Biology Erasmus MC
Frank Grosveld
Danny Huylebroeck

Regenerative Medicine & Stem Cells UMC Utrecht
Caroline Cheng
Maarten Brandt

Heleen
Petra
Donahoe PK, Longoni M, High FA. 
*Am J Pathol* 2016, 186: 2532–2543
Epithelial differentiation - application