Congenital diaphragmatic hernia: a genetic-environmental mismatch

Dick Tibboel,
on behalf of the pulmonary development research group

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

Erasmus MC – Sophia Children’s Hospital
Rotterdam the Netherlands
“Without the knowledge of the causes of diseases a man cannot be a surgeon / pediatrician. Surgeons / pediatricians have been too much satisfied with considering and treating the effects of diseases only”.

Modified after John Hunter
Wonder is the base of all knowledge

Aristoteles
Congenital anomalies

DISTURBANCES IN DEVELOPMENTAL PATHWAYS

GENES

ENVIRONMENT

MATERNAL METABOLISM
CONGENITAL ANOMALIES AT BIRTH

NO

YES

Genes

Environment

Maternal metabolism
THE “SOLUTION” OF CONGENITAL ANOMALIES DEPENDS ON

- Increased knowledge of risk factors (nutrition?; environmental exposure)
- Integrating knowledge of cell biological regulatory mechanisms and human DNA/gene data
- Structured interdisciplinary follow-up into adolescence and “targeted” genetic counseling (second generation)
Lung development

Primitive gut towards complex lung?
Branching morphogenesis

Early lung development, bronchial tree formation

Repetitive branching

Branching morphogenesis
Branching morphogenesis

Trachea development in Drosophila serves as model for lung development.
Individual morphogen gradients

Sum of all signals decides action!
Lung primordium specification

A  Liver Induction  B  Lung Induction  C  Refinement

6-7 somites  7-8 somites  9.5 days

FGF2  FGF2  FGF1

liver bud  cardiac

Erasmus MC
Lung primordium specification

Desai et al., 2006

Erasmus MC
Vascular development in fetal lungs

Canis Parera et al., 2005
CLASSICAL CONCEPTS IN CDH AND LUNG DEVELOPMENT

The diaphragmatic defect is the primary anomaly (Bochdalek 1848)

- Mesenchyme is essential for branching morphogenesis (Wessels 1976)

- Airway branches are established at 16 weeks in humans. Bloodvessels follow the airways (Reid 1979)
Carnegie Collection; Washington D.C.

Serial sections (6-10 micron) stained with H.E.

- Streeter Horizons stage 9-23 were investigated

CRL

<table>
<thead>
<tr>
<th>3-4 mm to</th>
<th>23-32 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 - 24</td>
<td>56-57</td>
</tr>
</tbody>
</table>
**Congenital Diaphragmatic Hernia (CDH)**

- **Incidence:** 1 in 3000
- **Diaphragm defect, lung hypoplasia, pulmonary hypertension**
- **Mortality:**
  - isolated CDH $\rightarrow$ 10 – 40% (case selection !)
  - non-isolated $\rightarrow$ up to 70%
- **Etiology:** unknown
  - environmental
  - genetic $\xrightarrow{\text{multifactorial}}$
Erasmus MC – Sophia Children’s Hospital
Level 3 University Children’s Hospital
Referral area 4 million; 35000 newborns
One of two designated ECMO centers

Number of CDH patients: 20 – 30 / year
Actual survival: 85% (last 3 years)
Strategy

CHD+ patients

abnormal karyotype? yes no

Search for smaller deletions / duplications

no

refine breakpoints

candidate region

candidate genes

mutation-analysis larger group of CDH patients
Techniques available

- Standard G-banding
- Fluorescent *In Situ* Hybridization (FISH)
- Array-based Comparative Genome Hybridization (Array-CGH): BAC, oligo, …
CDH and chromosome 15q anomalies

- 7 patients with CDH and deletion 15q (★)
- 3 patients with deletion 15q, but without CDH (#)

3D View CDH critical region

- genes
- BAC clones array
- BAC clones FISH

COUP-TF2
> 450 reported chromosomal anomalies

Rotterdam Cohort
Common pathway CDH candidate genes?
Common pathway CDH candidate genes?

Dietary sources of vit. A (β-carotene & retinol)

Raldh2

RA

Retinal

Retinol

Bound to RBP1 & RBP2

Bound to CRBP1 & CRBP2

Nitrofen

RA

RXR

RAR

COUP-TFI

FOG2

GATA4

GATA5

GATA6

RA

RAR

RARE

gene

transcription

promoter

Erasmus MC
Role for COUP-TF2 in the etiology of CDH?

- Transcription factor
- Involved in retinoic acid metabolism (sequesters RXR)
- Interacts with FOG2

- Essential for limb- and skeletal muscle development

- COUP-TF2 \(-/-\): \(\uparrow\) E9 (arrest of cardiac development)
- COUP-TF2 \(+/-\): 75% \(\uparrow\) neonatally

(Perreira, Tsai et al., 1999)
Yes: COUP-TF2 mouse model of CDH

- Tissue specific ablation
- Ablation in foregut mesoderm
  (incl. posthepatic mesenchymal plate)
  → left-sided CDH

(You et al., PNAS, 2005)
Where did this approach bring us???

>450 chromosomal aberrations

- Involved in RA pathway
- Candidate genes??

Mutation analysis

**COUP TF II** (Tsai et al; KO mouse model)
- 150 CDH pt for 15q gene COUP TF 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!
Proteins involved in CDH related phenotypes in mice or humans are yellow

Donahoe PK 2009 Birth Defects Res
mRNA expression of retinoid receptors in human lungs

RAR-alpha

RXR alpha

RAR-beta

RXR beta

RAR-gamma

RXR gamma

Distribution of retinoid receptors in human lungs

RXR beta 15 wk GA x 10

RXR beta 15 wk GA x 40

15 wk GA x 20

Adult x 40
**GENES OF INTEREST FROM CDH-CRITICAL REGIONS**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Genomic Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST8SIA2</td>
<td>15q26.1</td>
<td>Enzyme catalyzing polysialic acid synthesis</td>
</tr>
<tr>
<td>CHD2</td>
<td>15q26.1</td>
<td>Chromatin remodeling</td>
</tr>
<tr>
<td>RGMA</td>
<td>15q26.1</td>
<td>Repulsive guidance molecule</td>
</tr>
<tr>
<td>MCTP2</td>
<td>15q26.2</td>
<td>Calcium-mediated signaling</td>
</tr>
<tr>
<td>COUP-TFII</td>
<td>15q26.2</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>ARRDC4</td>
<td>15q26.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>IGFIR</td>
<td>15q26.3</td>
<td>Signaling receptor</td>
</tr>
<tr>
<td>DMN</td>
<td>15q26.3</td>
<td>Structural protein</td>
</tr>
<tr>
<td>TTC23</td>
<td>15q26.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>LRRC28</td>
<td>15q26.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>FOG2</td>
<td>8q23.1</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>GATA4</td>
<td>8p23.1</td>
<td>Transcription factor</td>
</tr>
</tbody>
</table>

AJP-Lung Cell Mol Physiol 2008;294:L666-
Congenital Diaphragmatic Hernia

Adapted from Beurskens et al., 2009
Nutrition Reviews Erasmus MC
Embryogenesis of diaphragm defects in congenital diaphragmatic hernia (CDH)  Robin D. Clugston, Wei Zhang and John J. Greer
Birth Defects Research 2010 (Part A) 88:15-24
Three-dimensional reconstruction of PPF’s recreated from control (upper left) nitrofen-created (upper right) VAD (lower left) and wt1 null-mutant (lower right) tissue sections. PPF defects are highlighted by an asterik.
Robin D. Clugston, Wei Zhang and John J. Greer
Congenital Diaphragmatic Hernia

Defects

A1
Posterolateral without rim (Bochdalek)

Defect: 
\( RARa/RARb2 \)
\( Wt1 \)

A2
Posterolateral with rim (Bochdalek)

Muscularisation defect: 
\( SF/HGF \)

Central

B
Rupture: 
\( Lox \)
Muscularisation defect: 
\( Gata4 \)
\( Slit3 \)

Eventration

C
Muscularisation defect: 
\( Pax3 \)  \( Cnot \)  \( Fog2 \)
\( Gab1 \)  \( MyoD \)
\( Myogenin \)

Anterior

D

Morgagni

E

Adapted from Beurskens et al., 2009 Nutrition Reviews
Embryogenesis of diaphragm defects in congenital diaphragmatic hernia (CDH)  Robin D. Clugston, Wei Zhang and John J. Greer
Birth Defects Research 2010 (Part A) 88:15-24
Integrity of the pleural mesothelium in Slit3 -/- diaphragm (a and b).
Wenlin Yuan, Yi Rao, Randal P Babiuk et al.
PNAS 2003;100:5217-5222
Model showing the function of Slit3 in diaphragm development
Wenlin Yuan, Yi Rao, Randal P Babiuk et al.
PNAS 2003;100:5217-5222
Abnormal pleuroperitoneal fold (PPF) development in nitrofen-exposed rat embryos.

Robin D. Clugston, Wei Zhang and John J. Greer

Birth Defects Research 2010 (Part A) 88:15-24
Retinal dehydrogenase (Raldh) 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
Retinoid receptor expression in the developing diaphragm at E13.5 (A)
RA receptor (RAR)-α is expressed in the PPF
Robin D. Clugston, Wei Zhang, Susana Alvarez.
Am J Respir Cell Mol Biol 2010;42:276-285
Chicken ovalbumin upstream promoter-transcription factor II (Coup tfII), IGF-I receptor (Igf1r) and repulsive guidance molecule A (Rgma) expression in the PPF.
Robin D. Clugston, Wei Zhang, Susana Alvarez, Angel R. de Lera and John J. Greer.
Am J Respir Cell Mol Biol 2010;42:276-285
Crabp expression in the developing diaphragm at E1 3.5 (A)
Crabpl expression is restricted in the pleuroperitoneal fold (PPF)
(dotted line represents the boundary of the PPF)
Friend of GATA 2 (Fog2) and GATA-binding protein 4 (Gata4) expression in the PPF.
Robin D. Clugston, Wei Zhang, Susana Alvarez, Angel R. de Lera and John J. Greer.
Am J Respir Cell Mol Biol 2010;42:276-285
Retinol in pregnancy

Cikot et al., Br J Nutr. 2001
Retinoic acid in human CDH; is it really relevant

A proof of principle study
Study design

- **T = 0**
  - 8th week pregnancy
  - **Diagnosis CDH**

- **T = 1**
  - During Pregnancy

- **T = 2**
  - Delivery

- **T = 3**
  - 15 months after delivery
The Rotterdam protocol: HERNIA-study

**T = 0**
- *Diagnosis CDH*
- Week 8
- Food frequency questionnaire
  - Questionnaire mother + child
  - Questionnaire father

**T = 1**
- *Delivery*
- Week 20
- Food frequency questionnaire
  - Questionnaire mother + child

**T = 2**
- *Week 38*
- Food frequency questionnaire
  - Questionnaire mother + child
  - Questionnaire father

**T = 3**
- *15 months pp*
- Food frequency questionnaire
  - Questionnaire mother + child
  - Questionnaire father

**Blood mother**
- Retinol, RBP, β-ctn
- DNA

**Amniotic fluid**
- Retinol, RBP, β-ctn

**Blood mother**
- Cord blood
  - Retinol, RBP, β-ctn

**Blood child**
- DNA

**Blood father**
- DNA
## HERNIA – study

**Congenital Diaphragmatic Hernia, Environment, Retinoids, Nutrition, Inheritance, other Associations**

<table>
<thead>
<tr>
<th>T = 0</th>
<th>T = 1</th>
<th>T = 2</th>
<th>T = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 8</td>
<td>Diagnosis CDH</td>
<td>Delivery</td>
<td>15 months pp</td>
</tr>
<tr>
<td><strong>cases</strong></td>
<td><strong>controls</strong></td>
<td><strong>cases</strong></td>
<td><strong>controls</strong></td>
</tr>
<tr>
<td>Blood mother</td>
<td>Blood mother</td>
<td>Blood mother</td>
<td>Blood mother</td>
</tr>
<tr>
<td>33</td>
<td>43</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>Cord blood / serum child</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>50</td>
<td>50</td>
<td>32</td>
</tr>
<tr>
<td>25</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>
## Levels of Retinol and RBP

Mean levels of Retinol and RBP in maternal serum and newborn cord blood

<table>
<thead>
<tr>
<th></th>
<th>CDH</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol (SD) μmol/l</td>
<td>n=22 1.21 (0.33)</td>
<td>n=32 1.24 (0.31)</td>
<td>0.74</td>
</tr>
<tr>
<td>RBP (SD) mg/l</td>
<td>n=22 11.07 (4.11)</td>
<td>n=34 11.85 (3.90)</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>Newborn</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol (SD) μmol/l</td>
<td>n=21 0.60 (0.19)</td>
<td>n=28 0.76 (0.18)</td>
<td>0.003</td>
</tr>
<tr>
<td>RBP (SD) mg/l</td>
<td>n=20* 5.42 (2.04)</td>
<td>n=28 7.11 (2.62)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*RBP 1 sample missing

*Accepted for publication, Pediatrics 2010*
Lung- and diaphragm development: common pathways?

Lung development

NCC development

Diaphragm development

Lung- and diaphragm development: common pathways?
In Conclusion:

- Animal data suggest disturbances in retinoic acid metabolism as an etiological factor in CDH
- The supplementation of retinoic acid diminishes the incidence of CDH in animal models
- A number of candidate genes in humans have been identified and are known to be involved in retinoic acid metabolism
- Patient recruitment of “the proof of principle study” is finalized, and revealed significant lower levels of retinol and RBP in neonatal cord blood
LET US DISCOVER THE SIGNIFICANCE OF BIRTH AND THE JOY OF LIVING