Pediatric acute respiratory distress syndrome: Host factors in Down syndrome and the general population
Bruijn, M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3
Pulmonary epithelial apoptosis in fetal Down syndrome: not higher than normal
Children with Down syndrome (DS) are at high risk for acute lung injury (ALI). Pulmonary epithelial apoptosis is an important factor in the pathophysiology of ALI. Whether the risk of ALI in DS is associated with a high level of pulmonary epithelial apoptosis is not known. We hypothesized that the percentage of apoptotic epithelial cells is higher in DS than in control lungs. Lung tissue sections from autopsies of 21 fetuses with DS and 12 controls were stained with antibodies against the epithelial marker pan-cytokeratin (CK) and apoptosis marker activated caspase-3 (aC3). Spectral imaging software was used to quantify the mean percentage of pixels that showed colocalization of CK and aC3. Mean (standard deviation[SD]) gestational age in weeks was 18.7 (1.4) in DS and 18.9 (2.0) in controls (p=0.67). The mean (SD) percentage of CK-positive pixels was 27.2% (4.7%) in DS compared to 27.1% (6.2%) in controls (p=0.97). The median (interquartile range [IQR]) percentage of CK-positive pixels that showed colocalization of aC3 was 0.16% (0.18%) in DS compared to 0.27% (0.24%) in controls (p=0.45). The mean (SD) number of CK-positive pixels increased from 22.5% (5.2%) to 30.4% (4.6%) with the appearance of saccular morphology in controls but not in DS (p=0.01). The percentage of apoptotic epithelial cells in DS fetal lungs does not differ from that in controls. However, we did find a difference in the development of epithelial structures between DS and controls that may be associated with anomalies in alveolar development found at birth in DS.
Introduction

Down syndrome (DS) is the most common chromosomal disorder occurring in approximately 1 in 1000 live births.\textsuperscript{1} Down syndrome is associated with a high incidence of pulmonary diseases, such as pulmonary arterial hypertension, parenchymal lung disease and airway abnormalities.\textsuperscript{2} Other examples of diseases found frequently in DS are structural heart disease, leukemia and Alzheimer’s disease.\textsuperscript{1} The high incidence of several of these conditions found frequently in DS, has been associated with a higher than normal level of apoptosis, or regulated cell death, in DS.\textsuperscript{3-4}

We previously showed that children with DS are at risk to develop yet another condition associated with increased apoptosis. Children with DS who are admitted to the pediatric intensive care unit have a ten-fold higher risk to develop acute lung injury (ALI) compared to controls.\textsuperscript{5} Acute lung injury is an acute and life-threatening inflammatory condition of the lungs associated with severe impairment of oxygenation.\textsuperscript{6} Apoptosis of the pulmonary epithelium is a key process in the development of ALI.\textsuperscript{7} However, whether a higher-than-normal level of apoptosis in the pulmonary epithelium in DS contributes to the higher risk of ALI in DS is unknown.

Interestingly, apoptosis is also an important physiological process in normal fetal lung development, but maintaining a delicate balance is important.\textsuperscript{8} Induction or inhibition of apoptosis can dramatically change lung development.\textsuperscript{9-10} In DS, normal fetal lung development is disrupted, although there is controversy: some studies showed alveolar hyperplasia, while others showed hypoplasia at birth.\textsuperscript{11-13} Whether a dysregulation of apoptosis in the fetal pulmonary system plays a role in this early disruption of lung development in DS is unclear.

If we could show a high level of apoptosis in the lungs in DS patients compared to controls this might explain both the high risk for ALI as well as the abnormal fetal lung development found in DS and could provide a possible target for drug treatment to prevent or treat ALI in DS. We hypothesized that the expression of activated caspase-3 (aC3) in pulmonary epithelial and mesenchymal cells is different in DS fetuses compared to controls.

Materials and methods

Patients

Formalin-fixed, paraffin-embedded lung tissue samples from autopsies of fetuses with DS and without DS who were stillborn or born after chemical induction of labor in the period ranging from 1994 to 2009 were obtained from the Department of Pathology of the Erasmus MC (Rotterdam, the Netherlands). The lung specimens
of non-macerated fetuses were obtained within 24 hours of delivery by standard hospital autopsy procedures and fixed in buffered formalin for 24 hours prior to embedding in paraffin blocks. Informed consent was given when the parents agreed to perform an autopsy, and tissues were used anonymously according to the guidelines of the Dutch code for proper use of human tissues.\textsuperscript{14}

**Immunohistochemistry**

Of each lung tissue sample, a 5 µm thick section was double-stained with antibodies against a pan-cytokeratin (CK) specific for epithelium, and a marker for apoptosis, aC3. Caspase-3 is one of the cysteine aspartyl-specific proteases that, when activated, cleave cellular substrates, resulting in the characteristic morphological and biochemical alterations of apoptosis.\textsuperscript{15} More specifically, caspase-3 is one of the executioner caspases, which can be activated by multiple pathways, but always results in apoptosis. Activated caspase-3 detection is a well known immunohistochemical method to detect apoptosis in histological sections.\textsuperscript{16}

Sections were xylene deparaffinized, rehydrated in an alcohol series, and treated for 20 min with 0.3% H\textsubscript{2}O\textsubscript{2} in methanol to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed in tris-ethylenediaminetetraacetic acid (pH 9.0) at 98°C. After rinsing with tap water, the sections were treated with Ultra V Block (Immunologic, Duiven, the Netherlands) to block nonspecific binding sites. Primary antibody labeling was performed with a cocktail of a 1:5000 dilution of rabbit anti-human aC3 (Asp175) mAb (Cell Signaling, Danvers, MA, USA) and a 1:2000 dilution of mouse anti-human cytokeratin 1–8, 10, 13–16, and 19 mAb (AE1/AE3 clone, Thermo Fisher Scientific, Waltham, MA, USA), followed by secondary labeling with anti-rabbit immunoglobulin G horseradish peroxidase (HRP) polymer and anti-mouse alkaline phosphatase (AP) polymer (Immunologic). Visualization of AP activity was performed with an AP Substrate Kit (Vector Blue, Vector Laboratories, Burlingame, CA), and HRP activity was visualized with a Peroxidase Substrate Kit (Vector NovaRED, Vector Laboratories). All sections were stained in the same experiment. Positive and negative controls are shown in Figure 3.1.

In addition, sections of all tissue samples were stained with hematoxylin and eosin (H&E) to determine the developmental stage of the lung. The presence of canalicular and/or saccular developmental stage characteristics were evaluated by two authors (M.B. and J.vdT.). Canalicular characteristics were blind-ending primitive bronchioles and alveolar ducts lined by cuboidal epithelium. Saccular characteristics were defined as enlargement of distal airspace with presence of secondary crests, decrease in interstitial tissue and increase of the capillary bed.
Quantification

Both the CK and aC3 staining have cytoplasmic localization. Detection of colocalization of the Vector NovaRED and Vector Blue signals was performed by spectral imaging, as described previously.\(^\text{17}\) Per section, seven random digital images at moderate (x200) magnification were taken by a Leica microscope using Nuance 2.10.0 software (Caliper Life Sciences/PerkinElmer, Hopkinton, MA). Images containing large airways or large blood vessels were discarded and replaced by images containing solely small airways. The Nuance software was used to unmix these images, using the spectral characteristics of Vector NovaRED and Vector Blue (Figure 3.2). For quantification, the colocalization tool of the Nuance software was used to calculate the percentage of pixels with positive CK signal colocalizing with positive aC3 signal. Pixels representing mesenchymal cells were defined as CK negative. Pixels representing intra-luminal space, being CK negative, were therefore included in the mesenchymal pixel count, in both groups. The same threshold and spectral settings were used for analysis of all images. For each section, the pixel counts from all seven images were used to calculate means per section.
In twenty random x200 images we visually counted the number of double-positive cells to assess the degree of correlation with the spectral imaging quantification method.

**Statistical Analysis**

Statistical analysis was performed using a statistical software program (PASW Statistics 18, Release 18.0.2 2010. Chicago: SPSS Inc.). The comparability of pixel counts between groups was tested by means of a Mann-Whitney U test for data that were not normally distributed and a Student’s t-test for normally distributed data.

Not-normally distributed data were log-transformed to obtain normality and allow for a two-way analysis of variance (ANOVA) to test for differences in pixel counts depending on both the presence of Down syndrome and developmental stage. Spearman’s rho was calculated to assess the correlation between visual cell counts and computerized pixel counts and to assess the correlation between gestational age (GA) and developmental stage.

**FIGURE 3.2** Panel showing lung sections of Down syndrome (A,B) and controls (C,D) in both canalicular (A,C) and saccular (B,D) stages of lung development. The sections are double-stained with antibodies against pan-cytokeratin (blue) and activated-caspase-3 (red/brown). An example of cytoplasmic colocalization is marked with an arrow.
Results

We obtained tissue samples from 21 fetuses (16-24 weeks GA) with DS (mean [standard deviation, SD] age 18.7 weeks [1.4 weeks]) and samples from 12 control fetuses (mean [SD] age 18.9 weeks [2.0 weeks], p=0.67). In 2 DS fetuses, the heart was structurally abnormal. A strong correlation was found between the visually counted cells showing colocalization of CK and aC3, and the results of the pixel count using the Nuance software (Spearman’s rho 0.801, p<0.001).

The mean (SD) percentage of CK-positive pixels was 27.2% (4.7%) in DS compared to 27.1% (6.2%) in control sections (p=0.97). The median (interquartile range, IQR) percentage of CK-positive pixels (epithelial cells) that showed colocalization of aC3 was 0.16% (0.18%) in DS compared to 0.27% (0.24%) in controls (p=0.45). The median (IQR) percentage of CK-negative pixels (mesenchymal cells) that were aC3 positive was 0.09% (0.13%) in DS compared to 0.10% (0.13%) in controls (p=0.45).

Of the total of 33 samples, 19 showed (early) saccular morphology. The GA of the samples correlated strongly with the presence of saccular characteristics (Spearman’s rho 0.728, p<0.001). There was no correlation between the developmental stage of the tissue and the presence of DS (Spearman’s rho -0.081, p=0.95).

The data were divided in four groups according to the presence of DS and saccular morphology. As expected, in the control group, the mean (SD) percentage of CK-positive pixels was lower in samples with canalicular morphology compared to sections with saccular characteristics (22.5% [5.2%] versus 30.4% [4.6%], respectively, p=0.02, Figure 3.3). However, in the samples from fetuses with DS, we did not find a statistically significant difference in mean (SD) percentage of CK-positive pixels between the canalicular group and the saccular group (28.5% [4.3%] versus 26.4% [5.1%], p=0.42; Figure 3.3). This finding was supported by two-way ANOVA analysis: the increase in cytokeratin-positive pixels together with the appearance of saccular morphology is dependent on the absence of DS (p=0.01).

In addition, differences in aC3 expression between these four groups were analyzed. In epithelial cells we did not find statistically significant differences in the percentage of aC3-positive pixels between samples with canalicular and samples with saccular morphology, neither in DS nor in controls (Table 3.1).

Thus, in controls, but not in DS, the number of epithelial cells increases with the appearance of saccular morphology, and this coincides with a (statistically non significant) increase in apoptosis in mesenchymal cells.
**TABLE 3.1** Distribution of activated caspase-3–positive pixels (% [IQR]) in epithelial cells across different stages of fetal lung development in Down syndrome and controls

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Controls</th>
<th>Down syndrome</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canalicular stage</td>
<td>0.29% (0.28), n = 5</td>
<td>0.11% (0.32), n = 9</td>
<td>0.95</td>
</tr>
<tr>
<td>Saccular stage</td>
<td>0.18% (0.23), n = 7</td>
<td>0.20% (0.15), n = 12</td>
<td>0.67</td>
</tr>
</tbody>
</table>

*<sup>a</sup>p-value stated for Mann-Whitney U-test.

**TABLE 3.2** Distribution of activated caspase-3–positive pixels (% [IQR]) in mesenchymal cells across different stages of fetal lung development in Down syndrome and controls

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Controls</th>
<th>Down syndrome</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canalicular stage</td>
<td>0.07 [0.07]</td>
<td>0.09 [0.17]</td>
<td>0.84</td>
</tr>
<tr>
<td>Saccular stage</td>
<td>0.16 [0.15]</td>
<td>0.09 [0.13]</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*<sup>a</sup>p-value stated for Mann-Whitney U-test.

**FIGURE 3.3** Comparison of the percentage of cytokeratin-positive pixels in sections of fetal lungs in Down syndrome and controls.

![Box plot comparing cytokeratin positivity between controls and Down syndrome](image-url)
Discussion

We showed that the expression of activated caspase-3 in the pulmonary epithelial and mesenchymal cells of DS fetuses does not significantly differ from that in control fetuses. We did find a difference in the development of epithelial layers between DS and controls. In controls there is an increase in epithelial cells together with the appearance of saccular morphology. This increase is not present in DS.

Although children with DS are at high risk for ALI, a condition in which dysregulation of pulmonary apoptosis is important, we did not find a difference in levels of pulmonary apoptosis between DS fetuses and controls. This might indicate that, indeed, apoptosis is not different in DS fetal lungs compared to controls, and, thus, there is no higher intrinsic level of pulmonary apoptosis in DS that could explain the high incidence of ALI.

However, the absence of a difference in epithelial apoptotic activity between DS fetuses and control fetuses does not preclude a different apoptotic profile at older ages. This is illustrated by research in DS brains. High levels of apoptosis and of pro-apoptotic proteins have been found in adult but not in fetal DS brains.19-22 We would have preferred to look for differences in the level of apoptosis in histological sections of older children with Down syndrome and ALI, but that material is not readily available. The rationale for performing this study was therefore based on the assumption that fetuses are challenged by abortion, as compared to older children that are challenged by conditions that can induce ALI. Although this rationale might be audacious, it does not make our primary findings less valid.

Our study is one of a few studies in fetal lung development in DS. In 1982, Cooney et al. found a decreased number of alveoli in DS, using radial alveolar counts.11 It was later shown that this becomes apparent only after birth.12 Schloo et al. also found few patients with normal numbers of alveoli.13 However, they found not only decreased but also increased numbers of alveoli in DS lungs.

In addition to normal levels of apoptosis in DS fetal lung, we found an increase in epithelial cells together with the appearance of saccular morphology in controls but not in DS lungs. In fact, in our samples of DS fetal lungs with only canalicular features, the number of epithelial cells was already high compared to controls, but this number did not increase further. The increase of epithelial cells in controls was accompanied by an increase in mesenchymal apoptosis.

Unfortunately, we are not informed with regard to the increase in epithelial cells in DS fetuses <16 weeks GA and whether this coincides with an increase in mesenchymal apoptosis. Samples from fetuses <16 weeks GA and >24 weeks GA were unavailable. One could hypothesize that the decreased airway branching found in DS at birth, might be caused by a disruption of normal apoptosis before
the 16th week of intrauterine development, when the bronchial tree is formed. In murine fetal lungs, inhibition of apoptosis results in diminished lung branching.\textsuperscript{10} Thus, we cannot exclude the possibility that there is a deregulation of pulmonary apoptosis in DS during fetal lung development before or after the GA group included in our study. Therefore, future studies into alveolarization in DS should also include material from these stages of lung development.

The spectral imaging technique used in this study counts pixels and thus volume, not cells. However, we showed a strong correlation between the computerized volume count and manually performed cell count. Visual comparison did not suggest differences in cell volume between control and DS samples. Therefore, we argue that the difference in CK-stained pixels reflects a real difference in the number of epithelial cells. This adds to the existing evidence that DS fetal lung development is different from normal development. In what way it is different, is far from elucidated.

We can only speculate on the pathophysiology of the alterations in epithelial cell development in DS. There might be a difference in the level of or sensitivity for epithelial growth factors. Thyroid hormones (especially T3) are known growth-inducing substances, and there is evidence of a relative hypothyroidism in Down syndrome that is already present prenatally.\textsuperscript{23} However, tissue levels of T3 are mainly dependent on local activation and deactivation regulation rather than on plasma levels of T3. In any case, it seems unlikely that a simple gene dosage effect caused by trisomy of chromosome 21 is sufficient to explain characteristics of the DS phenotype, such as abnormal lung development.\textsuperscript{24}

In conclusion, we did not find a higher level of pulmonary epithelial apoptosis in fetal Down syndrome. This makes it less likely that a higher intrinsic level of apoptosis contributes to the higher risk of acute lung injury in DS. Therefore, this study does not provide theoretical support for the use of pharmacological inhibition of apoptosis to prevent or treat ALI in DS. However, we cannot exclude that the level of apoptosis changes during postnatal life and further maturation. We did find alterations in epithelial proliferation in DS lungs compared to controls that suggest a different lung development in very early pregnancy in DS that supports further research to differences in alveolarization in DS.
Acknowledgments

The authors wish to thank Tamara Dekker, Department of Experimental Immunology and Respiratory Medicine, Academic Medical Center, University of Amsterdam, the Netherlands for laboratory assistance and professor Dick Tibboel, Department of Pediatric Surgery, Erasmus MC-Sophia Children’s Hospital, Rotterdam, the Netherlands, for his comments on the manuscript.
17. van der Loos CM. Multiple immunoenzyme staining: methods and visualizations for the observation with spectral imaging. J Histochem Cytochem 2008;56:313-328