Pediatric acute respiratory distress syndrome: Host factors in Down syndrome and the general population
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The insertion/deletion polymorphism of the angiotensin-converting enzyme gene is not associated with respiratory syncytial virus-induced acute lung injury in children.
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Objective: Evidence indicates that the renin-angiotensin system, with its key enzyme angiotensin converting enzyme (ACE), plays an important role in the pathogenesis of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). The D allele of the I/D polymorphism in the ACE gene has been associated with an enhanced susceptibility to and worse outcome of ARDS in adults. We hypothesized that the development and the course of ALI due to respiratory syncytial virus (RSV) infection in children is associated with the D allele.

Design: Prospective, observational study.

Setting: Pediatric intensive care unit of a university hospital.

Patients: The RSV group consisted of 51 children who required mechanical ventilation due to RSV-lower respiratory tract infection. The control group consisted of 465 randomly selected, unrelated individuals drawn from the Amsterdam population register.

Measurements and main results: There was no significant difference in the I/D allele frequencies between the RSV and control group. Nor was there an association between the D allele and disease severity. Moreover, ACE activity and levels of inflammatory mediators in bronchoalveolar lavage fluid were similar for the different genotypes in the RSV patients.

Conclusions: The ACE I/D polymorphism does not influence disease severity of RSV infection in children.
CHAPTER 6

ACE gene polymorphism in RSV-induced ALI

Introduction

Respiratory syncytial virus (RSV) is the most important viral cause of lower respiratory tract infection in infants and young children. The clinical symptoms of a RSV infection vary from mild coryza to severe lower respiratory tract infection (LRTI). Severe RSV-LRTI is associated with significant morbidity and mortality and many children fulfill the criteria of acute lung injury (ALI) or even acute respiratory distress syndrome (ARDS). Since there is no specific treatment for RSV-LRTI other than supportive measures, more insight into the mechanisms by which RSV infection progresses to ALI/ARDS is needed.

Recently, the importance of genetic factors in the development and the course of ARDS have gained attention. In 2002, an association was described between a polymorphism of the angiotensin converting enzyme (ACE) gene and susceptibility and outcome of ARDS in adults. This polymorphism consists of the presence (insertion, I) or absence (deletion, D) of a 287-basepair marker and accounts for approximately half of the variance in plasma ACE activity, being highest in individuals with the DD genotype. The DD genotype frequency was found to be increased in adult patients with ARDS and significantly associated with mortality. Since then, several studies on the role of the ACE-polymorphism in ARDS have been performed, showing conflicting results.

ACE converts angiotensin (Ang) I into Ang II. Ang II predominantly exerts its activity via a type 1 (AT1) receptor maintaining blood pressure homeostasis, as well as fluid and salt balance. In addition, this peptide is involved in key events of inflammation, fibrosis and apoptosis via the same receptor. Inhibition of ACE or blocking the Ang II receptor reduces the inflammatory response and lung injury in experimental animal models of ARDS. This and the higher serum ACE activity in the DD genotype suggest that this genotype is associated with an increased inflammatory response accounting for a severe course of ARDS in this group.

In the present study, we hypothesized that the presence of the D allele of the ACE gene is associated with the predisposition to and outcome of RSV-induced ALI in children. In addition, we hypothesized that this D allele is associated with higher (pulmonary) ACE activity in bronchoalveolar lavage fluid (BALF) and functional contributes to the inflammatory mediator response within the lung.

Materials and Methods

Patient selection

Patients requiring mechanical ventilation due to severe RSV-LRTI who were admitted to the pediatric intensive care unit (PICU) of the Emma Children’s Hospital in Amsterdam, the Netherlands, in the period 2006-2009 were enrolled...
in the study. RSV infection was confirmed by direct immunofluorescence assay (Imagen, DakoCyto-mation, UK). Hospital ethics committee approval was granted. Informed consent was obtained from the parents or legal caretakers. Population-based controls consisted of 465 randomly selected, unrelated individuals from the Amsterdam population register. The study population of the controls is based on a sample of 35-60-yr-old, non-institutionalized people, in Amsterdam South East, the Netherlands.

**Illness severity and outcome**
The Pediatric Index of Mortality (PIM), which calculates likelihood of death for children on intensive care, was scored for each patient according to the published algorithms. The duration of mechanical ventilation and length of stay in the PICU were recorded. In addition, mean $\text{PaO}_2/\text{FiO}_2$ and oxygenation index ($\text{OI} = \text{Mean Airway Pressure} \times [\text{FiO}_2/\text{PaO}_2]$) values on days 1, 3 and 5 were collected from the medical record for each infant.

**Collection of bronchoalveolar lavage samples**
Bronchoalveolar lavage (BAL) was performed according to the European Respiratory Society (ERS) guidelines. Briefly, a suction catheter was passed down the endotracheal tube. Three aliquots of sterile isotonic saline (1 ml/kg; with a maximum of 10 ml) were instilled down the suction catheter. The first aliquot was discarded and the remaining two aliquots were pooled. The retrieved BALF was centrifuged (450 g at 4°C for 10 min) and the supernatant was stored in aliquots at -80°C. BAL was performed on the day of intubation (day 1) and subsequently on day 3 and 5 of mechanical ventilation.

**ACE genotyping**
DNA was extracted from blood samples using standard salting out procedures. The II, ID, and DD genotypes were detected by PCR performed by staff blinded to all subject data. The PCR yields amplification products of 319 bp for the D allele and 597 bp for the I allele. Because the D allele in heterozygous samples is preferentially amplified, there is a tendency towards misclassification of about 4-5% of ID genotypes to DD. In order to avoid this, a second independent PCR was performed of each sample found to have the DD genotype with a primer pair that recognizes an insertion specific sequence. The reaction yields a 335 bp amplicon in the presence of an I allele and no product in samples homozygous for DD.

**ACE-activity**
ACE-activity was measured in BALF monitoring the degradation of the fluorogenic peptide substrate Mca-R-P-P-G-F-S-A-F-K(Dnp)-OH (R&D Systems, Uithoorn, the
Netherlands) over time in a spectrofluorometer (FLUOstar Galaxy, BMG LABTECH GmbH, Ortenberg, Germany) at 320 nm excitation and 405 nm emission. As endothelin-converting enzyme also converts this substrate, ACE activity was distinguished as captopril inhibitible.

**Inflammatory mediators**
BALF levels of IL-6, IL-8, IL-1β, IL-10, TNF-α, VEGF, MIP-1α, MIP-1β and MCP-1 were measured with commercially available microspheres in a multiplex fluorescent bead assay (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescence was quantified with a Bio-Rad Bio-Plex 100.

**Statistical analysis**
The chi-square tables were used to compare the number of each genotype with those expected for a population in Hardy-Weinberg equilibrium and to compare genotype frequencies between the RSV population and the control group. The power of this study was retrospectively calculated to show an 18% difference in D allele frequency between the RSV population and the control group with a power of 80%, at a two-tailed α of 0.05. We used one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls, to compare group means for the different parameters studied. Log transformations of the variables were analyzed in case of lack of normality and/or homoscedasticity. For comparison of the changes of the inflammatory mediator levels in time, repeated measures ANOVA was used. Finally, Spearman’s correlation analysis was performed to assess the degree of association between BALF ACE activity and severity of illness measures. For all tests, a p-value of <0.05 was considered significant.

**Results**

**Genotypes and allele frequencies of ACE polymorphism and clinical data**
In total, 51 patients (age 70 ± 12 days; 32 male and 19 female) were enrolled in the study. All patients fulfilled the 1994 criteria of the American-European Consensus Conference for ALI/ARDS. Table 6.1 summarizes the ACE genotype distribution (II, ID and DD) and allele (I and D) frequencies of the RSV patients and the control group. The allele frequencies of both groups were in Hardy-Weinberg equilibrium. There was no significant effect of the genotype and allele frequencies in the predisposition to RSV-induced ALI. In addition, there was no association between the ACE genotype and disease severity and outcome (Table 6.2). No statistically significant differences were found in PIM scores, duration of mechanical ventilation, length of PICU stay, PaO$_2$/FiO$_2$ ratios and OI.
TABLE 6.1  Genotype and allele frequencies of the angiotensin-converting enzyme polymorphism.

a Patients compared to control population by Chi-squared analysis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n = 51)</th>
<th>Control population (n = 465)</th>
<th>p-valuea (Patients vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>7 (13.7)</td>
<td>99 (21.3)</td>
<td>.36</td>
</tr>
<tr>
<td>ID</td>
<td>27 (52.9)</td>
<td>241 (51.8)</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>17 (33.3)</td>
<td>125 (26.9)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (n = 51)</th>
<th>Control population (n = 465)</th>
<th>p-valuea (Patients vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>41 (40.2)</td>
<td>439 (47.2)</td>
<td>.21</td>
</tr>
<tr>
<td>D</td>
<td>61 (59.8)</td>
<td>491 (52.8)</td>
<td></td>
</tr>
</tbody>
</table>

Hardy-Weinberg equilibrium

| Chi-square | 0.52 | 0.74 |
| p-value    | .47  | .39  |

TABLE 6.2  Markers of disease severity and outcome among 51 children with respiratory syncytial virus infection. Data are means ± standard deviation.

b Comparison between the three genotypes by ANOVA

Abbreviations: PIM = pediatric index of mortality; MV = mechanical ventilation; PICU LOS = pediatric intensive care unit length of stay; OI = oxygenation index (Mean Airway Pressure x [FiO₂/PaO₂])

<table>
<thead>
<tr>
<th></th>
<th>DD (n = 17)</th>
<th>ID (n = 27)</th>
<th>II (n = 7)</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIM (%)</td>
<td>3.1 (1.4)</td>
<td>(n = 17)</td>
<td>3.0 (0.9)</td>
<td>0.24</td>
</tr>
<tr>
<td>MV, days</td>
<td>8.2 (5.2)</td>
<td>6.8 (3.1)</td>
<td>6.1 (2.1)</td>
<td>0.44</td>
</tr>
<tr>
<td>PICU LOS, days</td>
<td>9.8 (5.3)</td>
<td>7.9 (3.6)</td>
<td>7.1 (2.4)</td>
<td>0.26</td>
</tr>
<tr>
<td>PaO₂/FiO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>163 (55)</td>
<td>176 (49)</td>
<td>154 (53)</td>
<td>0.64</td>
</tr>
<tr>
<td>Day 3</td>
<td>166 (68)</td>
<td>190 (92)</td>
<td>198 (51)</td>
<td>0.58</td>
</tr>
<tr>
<td>Day 5</td>
<td>169 (63)</td>
<td>187 (51)</td>
<td>172 (55)</td>
<td>0.74</td>
</tr>
<tr>
<td>OI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>6.8 (2.9)</td>
<td>6.1 (2.5)</td>
<td>7.1 (3.1)</td>
<td>0.63</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.2 (4.0)</td>
<td>5.4 (2.2)</td>
<td>4.9 (2.3)</td>
<td>0.16</td>
</tr>
<tr>
<td>Day 5</td>
<td>7.2 (5.9)</td>
<td>5.0 (2.4)</td>
<td>5.7 (2.8)</td>
<td>0.41</td>
</tr>
<tr>
<td>Observed mortality</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
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</table>
Relation between the ACE genotype and BALF ACE activity and levels of inflammatory mediators

BALF ACE activity and levels of inflammatory mediators were monitored during five days of mechanical ventilation (Figure 6.1 and 6.2). ACE activity did not change during the course of the mechanical ventilation period (Figure 6.1). In contrast, there was a moderate to marked pulmonary inflammatory mediator response on the first day of admission as reflected by BALF levels of nine inflammatory mediators (Figure 6.2). Levels decreased sharply for eight of the mediators ($p<0.05$, except for IL-6 $p=0.07$). Only MCP-1 remained at a high level throughout the mechanical ventilation period.

Mean BALF ACE activities and mean levels of inflammatory mediators were compared among the three genotypes (Figure 6.1 and 6.2). There was no significant relationship between the polymorphism and the BALF ACE activity. In addition, no difference was observed between the genotypes with respect to BALF levels of the inflammatory mediators (Figure 6.2). Finally, no correlation was found between BALF ACE activity and severity of illness measures (data not shown).

**FIGURE 6.1** Box-whisker plots of angiotensin converting enzyme (ACE) activity in bronchoalveolar lavage fluid (BALF) of mechanically ventilated patients (total n=51) infected with respiratory syncytial virus on day of admission (day 1), day 3 and day 5. Patients were divided into three groups according to their ACE genotype.
We did not find an association between the I/D polymorphism in the ACE gene and predisposition to and severity of RSV-induced ALI in children. For the first time, the ACE genotype was linked to measured BALF ACE activity and levels of inflammatory mediators. In line with the absence of a correlation between ACE genotype and disease severity, we could not detect significant differences in BALF ACE activity nor in BALF levels of inflammatory mediators.

The findings of this study are similar to those of Plunkett et al. who described the absence of an association between the D allele and the susceptibility of children to acute hypoxic respiratory failure. However, our findings are in contrast to the reported association between genotypes of the ACE I/D polymorphism and worse outcome in ARDS in adults. The differences in
association between the ACE I/D polymorphism and susceptibility and course of ARDS between children and adults may be affected by age related differences in epidemiology and pathophysiology of ARDS. There is a striking difference in the reported incidence of ARDS between children and adults (12.8 versus 78.9 cases per 100000 person-years). Furthermore, mortality in adults with ARDS is substantially higher compared to children (40% versus 18%). Animal studies have shown less cytokine production during mechanical ventilation of pre-injured lungs in juvenile compared to adult mice. In our patient population, we found a marked pulmonary inflammatory mediator response on the first day of admission, which is a hallmark of ARDS. However, already after 48 hours there was a prompt resolution of this response. In contrast, BALF levels of inflammatory mediators of adult ARDS patients peaked during the first 3 days of admission and returned to near normal values only after 14 days. This difference in inflammatory mediator response may be a clue for the observed difference in disease severity and mortality between children and adults. Further studies are required to confirm this hypothesis.

It can not be excluded that the difference in ACE I/D polymorphism and development and outcome of ARDS is caused by the etiology of the syndrome. In the present study, viral lower respiratory tract infection was the underlying cause of respiratory failure, while pneumonia was diagnosed in only 27% of the cases in the study of Marshall et al. In addition, further studies are required to assess the correlation between ACE I/D polymorphism and other causes of direct (pulmonary) ARDS (e.g. bacterial pneumonia, aspiration) in children. Moreover, it may be that the association between the D allele and disease severity of ARDS exists only in secondary (indirect) ARDS. This is supported by data of the Asian epidemic of Severe Acute Respiratory Syndrome (SARS). Also in this cohort no association was found between the D allele of the ACE polymorphism and susceptibility for ARDS.

In this study, only patients were included that exhibited severe symptoms and required mechanical ventilation. Possibly, there is a correlation between ACE I/D polymorphism and less severe RSV infection, which represents the majority of the cases. However, a possible admission bias seems unlikely given the fact that genotype distribution of the patients and of the control group were in Hardy-Weinberg equilibrium.

The I/D polymorphism of the ACE gene has been found to associate with systemic ACE activity. There is however, considerable evidence to support the existence of local renin-angiotensin systems in human tissues. Whether this polymorphism also affects local tissue ACE activity is not clear yet. It was found that cardiac ACE activity is highest in subjects with the DD genotype. In the present study, pulmonary (BALF) ACE activity did not associate with the polymorphism. It can not be excluded that tissue factors oppose the local ACE
activity, obscuring linkage with the polymorphism.

In conclusion, we have not found an association between the ACE I/D polymorphism and RSV-induced ALI in children. It may thus well be that it is not appropriate to apply adult-based phenotype criteria for ARDS in a pediatric context. In addition, as ARDS is a heterogeneous syndrome, it may be necessary to make a distinction between direct and indirect lung injury to analyze the impact of the ACE genotype on the different causes of ARDS.