The role of the renin-angiotensin system in acute lung injury
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The insertion/deletion polymorphism of the angiotensin-converting enzyme gene is not associated with severity of RSV infection in children

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Chapter 5

Abstract
The clinical spectrum of respiratory syncytial virus (RSV) infection in children varies from coryza to lower respiratory tract infection (LTRI) of whom many even develop Acute Lung Injury (ALI) or its severe form, Acute Respiratory Distress Syndrome (ARDS). In adults, a polymorphism of the angiotensin-converting enzyme (ACE) gene is associated with an increased risk of developing ARDS. We found no such association with the development of ALI/ARDS in children with RSV-LTRI. Moreover, ACE activity and levels of inflammatory mediators in bronchoalveolar lavage fluid were similar for the different genotypes. Our results suggest that the ACE polymorphism does not influence disease severity of RSV infection in children.
Introduction

Respiratory syncytial virus (RSV) is a common cause of viral lower respiratory infection. The clinical symptoms of a RSV infection vary from mild coryza to severe lower respiratory tract infection (LRTI). Traditionally, it has been described in young children. However, there is growing awareness that RSV is also an important pathogen in adults (Falsey et al., 2005). RSV-LRTI is associated with significant morbidity and many children even fulfill the criteria of Acute Lung Injury (ALI) or its severe form, known as Acute Respiratory Distress syndrome (ARDS) (Nair et al., 2010). Since treatment of severe RSV-LRTI is largely supportive, more insight into the pathophysiological mechanisms as well as the significance of underlying host factors is warranted.

Recently, the importance of genetic factors in the susceptibility and course of severe RSV-LRTI, have gained attention. In this context, the polymorphism of the angiotensin-converting enzyme (ACE) gene is of interest. This insertion (I)/deletion (D) polymorphism has been associated with several adverse pulmonary phenotypes, including ARDS in critically ill adults (Marshall et al., 2002). The I/D polymorphism accounts for approximately half of the variance in plasma ACE activity, being highest in individuals with the DD genotype (Rigat et al., 1990). The DD genotype frequency was increased in patients with ARDS compared to non-ARDS patients and was significantly associated with mortality in the ARDS group (Marshall et al., 2002).

ACE converts angiotensin (Ang) I into Ang II. Ang II predominantly exerts its activity via a type 1 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 (Li et al., 2003; Wösten-van Asperen et al., 2010). Inhibition of ACE or blocking the Ang II receptor reduces the inflammatory response and lung injury in experimental animal models of ARDS (Imai et al., 2005; Hagiwara et al., 2009; Wösten-van Asperen et al., 2010). 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.

As severe RSV-LRTI is characterized by an acute inflammatory reaction as reflected by recruitment of neutrophils and enhanced amounts of inflammatory mediators similar to that found in ARDS, a role of ACE in the susceptibility and severity of RSV-LRTI might be expected. 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 severe RSV-LRTI in children. In addition, we hypothesized that this D allele is associated with higher (pulmonary) ACE activity in bronchoalveolar lavage fluid (BALF) and functionally contributes to the inflammatory mediator response within the lung.


Chapter 5

**Subjects, materials and methods**

**Patient selection**
Children admitted to our pediatric intensive care unit and requiring intubation and mechanical ventilation due to RSV infection were studied in the period 2006-2009. RSV infection was confirmed by direct immunofluorescence assay (Imagen, DakoCytomation, UK). Hospital ethics committee approval was granted. Informed consent was obtained from the parents or legal caretakers. In line with previous studies, we excluded non-Caucasians from our study because the association of the ACE I/D polymorphism with ACE activity varies according to ethnic background (Marshall et al., 2002; Bloem et al., 1996). The ACE I/D polymorphism has only been demonstrated to be functional in Caucasians (Rigat et al., 1990). Population-based controls consisted of 465 white, randomly selected, unrelated individuals from the Amsterdam population register (van Valkengoed et al., 2008). 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 (van Valkengoed et al., 2008).

**Illness severity and outcome**
The Pediatric Index of Mortality (PIM) score, from which the likelihood of death for children admitted to the intensive care can be calculated, was scored for each patient according to the published algorithms (Shann et al., 1997; Slater et al., 2003). The duration of mechanical ventilation and the length of stay in the PICU were recorded. In addition, the mean PaO$_2$/FiO$_2$ and oxygenation index (OI = Mean Airway Pressure x FiO$_2$/PaO$_2$) on day 1, 3 and 5 after admission were calculated.

**Collection of bronchoalveolar lavage samples**
Bronchoalveolar lavage (BAL) was performed according to European Respiratory Society guidelines (de Blic et al., 2000). 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 (Meduri et al., 1995; Meduri et al., 1995; Meduri et al., 1998). 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 start of the mechanical ventilation (day 1) and subsequently on day 3 and 5.

**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 (Lindpainter et al., 1995). 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\textsuperscript{\textregistered} Galaxy: BMG Labtechnologies) at 320 nm excitation and 405 nm emission. As endothelin-converting enzyme also converts this substrate, ACE activity was distinguished as captopril inhibitable.

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 reagents in a multiplex fluorescent bead assay (Bioplex; BioRad). Fluorescence was quantified with a Bioplex 100 system (BioRad).

Statistical Analysis
The chi-square 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 a 15% 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
A total of 46 patients (mean (SD) age 70 (12) days; 28 males and 18 females) was enrolled in the study. On admission, all patients fulfilled the criteria of the American-European
Consensus Conference for ALI/ARDS. Table 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 ACE genotype distribution demonstrated a Hardy-Weinberg equilibrium and did not differ from controls (Table 1). In addition, there was no association between the ACE genotype and course of the disease (Table 2). No statistically significant differences were found in PIM scores, duration of mechanical ventilation and length of PICU stay, PaO2/FiO2 ratios and OI.

Table 1. Genotype and allele frequencies of the angiotensin-converting enzyme polymorphism.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n = 46)</th>
<th>Control population (n = 465)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>6 (13.0)</td>
<td>99 (21.3)</td>
<td>.31</td>
</tr>
<tr>
<td>ID</td>
<td>24 (52.2)</td>
<td>241 (51.8)</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>16 (34.8)</td>
<td>125 (26.9)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>36 (78.3)</td>
<td>439 (47.2)</td>
<td>.15</td>
</tr>
<tr>
<td>D</td>
<td>56 (21.7)</td>
<td>491 (52.8)</td>
<td></td>
</tr>
</tbody>
</table>

Hardy-Weinberg equilibrium

| Chi-square | 0.42 | 0.74 |
| p value    | .52  | .39  |

* Patients compared to control population by Chi-squared analysis

Relationship between the ACE genotype and BALF ACE activity and levels of inflammatory mediators.

BALF ACE activity and levels of inflammatory mediators were monitored on day 1, 3 and 5 of mechanical ventilation (Figure 1 & 2). ACE activity did not change during the course of the mechanical ventilation period (Figure 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 2). Except for MCP-1, all levels decreased sharply within 2 days (p<0.05, except for IL-6 p = 0.07).

Mean BALF ACE activities and mean levels of inflammatory mediators were compared among the three genotypes (Figure 1 & 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 2). Finally, no correlation was found between BALF ACE activity and severity of illness measures (data not shown).
Figure 1. Box-whisker plots of ACE activity in bronchoalveolar lavage fluid of mechanically ventilated patients (total n=46) 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.

Figure 2. Box-whisker plots of levels of IL-6, IL-8, IL-1β, IL-10, TNF-α, VEGF, MIP-1α, MIP-1β and MCP-1 in bronchoalveolar lavage fluid of mechanically ventilated patients (total n=46) 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. *Statistical significant (p<0.05) day 3 vs. day 1, as determined by repeated measures ANOVA.
Discussion

Only a small proportion (10%) of children with an RSV infection develops severe LRTI necessitating hospital admission (Nair et al., 2010). This suggests that the host response is important in determining the severity of the disease. Indeed, it has been shown that genetic factors play a role in the course of a severe RSV-LRTI. In this study, we examined the association between the I/D polymorphism of the ACE gene and predisposition to and severity of RSV-LRTI in children. The main finding of this study is the lack of such an association. In addition to the ACE genotype, we measured BALF ACE activity and levels of inflammatory mediators over time. In line with the absence of a correlation between ACE genotype and disease severity, we could neither detect significant differences in BALF ACE activity nor 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 (Plunkett et al., 2008). Although in this latter study, other causes of acute hypoxic respiratory failure besides RSV infection were included, a large portion of their patients suffered from RSV-LRTI. Our findings are, however, in contrast to the reported association between genotypes of the ACE I/D polymorphism and worse outcome in ARDS in adults (Marshall et al., 2002). This difference in ACE I/D polymorphism and development and outcome of ARDS may be caused by the etiology of the syndrome. In the present study, RSV-LRTI was the underlying cause of

Table 2. Markers of disease severity and outcome among 46 children with Respiratory Syncytial Virus infection.

<table>
<thead>
<tr>
<th></th>
<th>IU (n = 16)</th>
<th>II (n = 24)</th>
<th>III (n = 6)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIM (%)</td>
<td>3.1 (1.4)</td>
<td>2.5 (0.9)</td>
<td>2.6 (0.3)</td>
<td>0.24</td>
</tr>
<tr>
<td>MV, days</td>
<td>8.4 (5.3)</td>
<td>6.9 (3.0)</td>
<td>6.2 (2.1)</td>
<td>0.55</td>
</tr>
<tr>
<td>PICU LOS, days</td>
<td>9.9 (5.4)</td>
<td>8.0 (3.5)</td>
<td>7.3 (2.5)</td>
<td>0.29</td>
</tr>
<tr>
<td>PaO2/FiO2, mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>161 (67)</td>
<td>165 (49)</td>
<td>133 (46)</td>
<td>0.38</td>
</tr>
<tr>
<td>Day 3</td>
<td>144 (57)</td>
<td>160 (41)</td>
<td>172 (56)</td>
<td>0.72</td>
</tr>
<tr>
<td>Day 5</td>
<td>169 (62)</td>
<td>187 (51)</td>
<td>151 (43)</td>
<td>0.21</td>
</tr>
<tr>
<td>UI, mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>7.4 (3.5)</td>
<td>6.9 (3.0)</td>
<td>8.3 (4.1)</td>
<td>0.49</td>
</tr>
<tr>
<td>Day 3</td>
<td>8.2 (4.1)</td>
<td>6.3 (2.2)</td>
<td>6.2 (2.7)</td>
<td>0.25</td>
</tr>
<tr>
<td>Day 5</td>
<td>7.2 (5.9)</td>
<td>5.0 (2.4)</td>
<td>6.4 (2.8)</td>
<td>0.42</td>
</tr>
<tr>
<td>Observed mortality</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation; Definition of 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); *Comparison between the three genotypes by ANOVA
respiratory failure, while pneumonia was diagnosed in only 27% of the cases in the study of Marshall et al. (2002). Furthermore, there is evidence that RSV-induced respiratory failure represents a relatively benign course of ARDS in children (Hammer et al., 1997). In line herewith, we found a marked pulmonary inflammatory mediator response on the first day of admission followed by a prompt resolution within 48 hours. 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 (Park et al., 2001).

Another explanation for the lack of association of the ACE polymorphism and disease severity might be age-dependent differences for the renin-angiotensin system (RAS) (Xie et al., 2006). Experimental data indicate that aging is associated with a decreased production of the counter-regulatory enzyme of ACE, i.e. ACE2. This is based on epidemiologic data that show that there is a predominance of young adult patients in severe acute respiratory syndrome (SARS) attacks (Poutanen et al., 2003). The coronavirus that causes SARS uses ACE2 as its functional receptor. Reduction of ACE2 production in time may cause an imbalance in the pulmonary RAS towards the ACE/Ang II pathway, thereby stimulating the inflammatory response.

In this study, only patients were included that exhibited severe symptoms and required mechanical ventilation. We can not exclude that there is a correlation between ACE I/D polymorphism and less severe RSV infection, which represents the majority of the cases (Nair et al., 2010). 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.

In conclusion, it is unlikely that the ACE polymorphism is causally related to severe RSV-LRTI and the concurrent local inflammation. Moreover, the ACE polymorphism does not relate to local ACE activity, at least in our cohort of children with RSV-LRTI. Given the recently recognized ACE-counteracting role of ACE2 in RAS, it may be of interest to determine whether there is an ACE2 polymorphism associated with severe RSV-LRTI. So far, the presence of several gene polymorphisms, related to the innate response, appear useful markers to distinguish those patients with an enhanced risk to develop severe RSV-LRTI. The underlying mechanism for developing severe RSV-LRTI, however, remains unknown.