RSV bronchiolitis in healthy term infants

Prediction and pathogenesis

Michiel Houben

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RSV bronchiolitis in healthy term infants:
Prediction and pathogenesis

RSV bronchiolitis bij gezonde aterm geboren zuigelingen:
Predictie en pathogenese

(met een samenvatting in het Nederlands)

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Contents

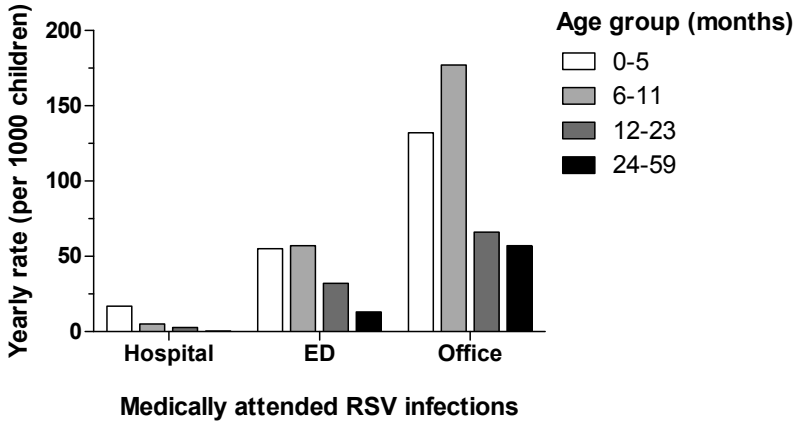
Chapter 1

General introduction

1. THE IMPACT OF RSV INFECTIONS

Respiratory syncytial virus (RSV) is the most important causative pathogen in bronchiolitis in children under the age of one year.[1] RSV is a single stranded RNA virus (member of the Paramyxoviridae) that has first been described in 1956.[2-4] The incidence of RSV bronchiolitis is high and has a substantial impact on children, their families, the health-care system and society. In 2005, globally 34 million children under the age of five years developed RSV bronchiolitis, and about 3.4 million children required hospitalization.[1] An estimated total of 66,000 to 199,000 children under the age of five years died from RSV bronchiolitis worldwide, 99% of which occurring in developing countries.[1] Annually, approximately 2 million children under the age of five years in the USA require medical attention for RSV infections.[5] More than 97% of these children are treated as outpatients in pediatric practices or at emergency departments. For The Netherlands, the estimated annual numbers of RSV associated hospitalizations and primary care visits are 2,400 and 48,000 for children below the age of two years and 300 and 35,000 for children aged two to four years, respectively.[6,7] ***Figure 1*** demonstrates that the majority of medically attended RSV infections occur in children above the age of six months who do not require hospital admission.[5] RSV infections in children under the age of 2 years are associated with a fourfold increased rate of emergency visits and a fivefold increased rate of primary care visits and of parents missing work, as compared to influenza virus.[8] It is known that children with severe (hospitalized) RSV bronchiolitis have a reduced health-related quality of life (HRQoL), and 50% suffer from recurrent wheezing symptoms.[9,10]

Figure 1. Annual rate of medically attended RSV infections in the USA



Incidence of RSV infection leading to hospital admission, emergency department (ED) visit and office visit in children under the age of 5 years in the USA. Numbers from Hall et al., NEJM 2009.[5]

2. PREDICTION OF RSV INFECTIONS IN HEALTHY TERM INFANTS

The majority of the children (~70%) admitted to hospital with RSV bronchiolitis are healthy term infants without any traditional risk factor, such as premature birth, congenital heart disease, chronic lung disease of prematurity, Down syndrome, neurological disease, or immunodeficiency.[11-13] Most probably, children that develop RSV bronchiolitis requiring medical attention without hospitalization have even less traditional risk factors. Establishing new predictive factors and prediction models specifically for these children is of major importance given the large socioeconomic impact of RSV bronchiolitis in the community. Simple prediction rules for RSV bronchiolitis could help general practitioners and pediatricians to target preventive strategies, such as intensified hand hygiene and reduction of viral exposure.[14-16] Prediction rules can be applied as stratification tools to distinguish between individuals at high and at low risk. Similar stratification strategies could be used to study and apply novel preventive and therapeutic interventions.[17-20]

3. PATHOGENESIS OF RSV INFECTIONS IN HEALTHY TERM INFANTS

Recently, nasal RSV viral loads were shown to determine disease severity during experimental RSV infections in healthy volunteers.[21] Although previous observational studies have demonstrated similar results in hospitalized infants with RSV infections, it is unknown whether RSV viral load is associated with disease severity during RSV infection in the community. If RSV viral load is indeed associated with RSV severity, early interventions aiming at rapid reduction of RSV viral load could diminish the disease severity and the duration of symptoms during RSV bronchiolitis.[18]

In 1988, Martinez and colleagues showed that healthy newborns with a high airway resistance at birth had a fourfold increased risk of bronchiolitis with signs of airflow limitation in the first year of life.[22] The underlying causes of impaired neonatal airway function remained unknown. In preterm infants, exposure to chorioamnionitis and/or funisitis has recently been shown to be associated with a reduced risk of respiratory distress syndrome and chronic lung disease of prematurity.[23-25] In addition, the suggested adverse effects of chorioamnionitis on respiratory outcomes in preterms was absent after adjustment for low gestational age.[26] Moreover, in term infants, an intra-uterine "acute inflammation gene expression signature" was demonstrated during spontaneous onset of labor deliveries without chorioamnionitis.[27] Exposure of the fetal lung to high levels of intra-amniotic pro-inflammatory signals might enhance maturation of the newborn respiratory mucosal immune system and airway function. Establishing and unraveling this mechanism would increase our insight in the physiologic development of the newborn airway mucosal immune system and its function. Ultimately, amniotic fluid

pro-inflammatory proteins could serve as biomarkers for early identification of, and preventive strategies for, healthy term newborns at high risk of early childhood respiratory diseases, such as RSV bronchiolitis.[28]

4. THE NETHERLANDS AMNIOTIC FLUID COHORT STUDY

A healthy unselected term birth cohort (the Netherlands Amniotic Fluid Cohort Study) was founded to study the prediction and the pathogenesis of RSV bronchiolitis in healthy term infants. From January 2006 to December 2010, approximately 500 healthy term infants were included for follow-up measurements. After collection of amniotic fluid samples, placentas and cord blood plasma, airway function was measured at the age of one month. Until the age of one year, daily respiratory symptoms and symptomatic nasopharyngeal virology samples were collected by the parents of participating infants. At the age of one year, detailed questionnaires on general health, respiratory symptoms and disorders and on HRQoL were filled out by the child's parents and the general practitioner. The parents of all participating children provided written informed consent and the study was approved by the ethical review board of the University Medical Center Utrecht and the Diakonessen Hospital (both Utrecht, The Netherlands).

5. OBJECTIVES OF THE THESIS

The general aim of this thesis is to gain insight in the etiology of RSV bronchiolitis and to contribute to its prediction in healthy term infants. The derived specific objectives are:

- To determine predictive factors and to develop a prediction model for RSV bronchiolitis in healthy term infants.
- To determine whether vitamin D deficiency at birth is associated with an increased risk of RSV bronchiolitis.
- To determine whether the severity of a RSV infection in the community is associated with RSV viral load.
- To determine the sensitivity of a nasal swab for the molecular detection of RSV infections.
- To determine whether intra-uterine inflammation at term is associated with spontaneous onset of labor.
- To determine whether fetal exposure to an intra-amniotic pro-inflammatory profile is associated with reduced risk of RSV bronchiolitis, and with normal newborn airway function.

6. OUTLINE OF THE THESIS

All studies that are reported in this thesis were carried out within children participating in the Netherlands Amniotic Fluid Cohort Study. In ***section one***, studies on risk factors and prediction of RSV bronchiolitis in healthy term infants are reported. In ***chapter 2***, new clinical predictive factors for RSV bronchiolitis in the first year of life are identified and a novel prediction rule is developed. In ***chapter 3***, we studied cord blood vitamin D deficiency as a risk factor for RSV bronchiolitis. The results of this study suggest that cord blood vitamin D deficiency is indeed a risk factor for RSV bronchiolitis.

In ***section two*** of this thesis, the results of our studies into the pathogenesis of RSV infections in healthy term infants are described. We showed that viral load is associated with disease severity in primary RSV infection, and that viral load also determines the sensitivity of nasal swabs for the detection of RSV infection (***chapters 4 & 5***). In ***chapter 6***, we describe that term spontaneous onset of labor vaginal deliveries are associated with marked intra-uterine inflammation. As compared with elective cesarean sections, spontaneous onset of labor deliveries have twofold higher amniotic fluid concentrations of pro-inflammatory cytokines interleukin-8 (IL-8), IL-6 and tumor necrosis factor-α (TNF-α), while the proportion of placentas with histological signs of chorioamnionitis is fourfold increased. In ***chapter 7***, we show that healthy term infants with high amniotic fluid concentrations of IL-8 and TNF-α have a reduced risk of medically attended RSV infection in the first year of life. Moreover, higher levels of amniotic fluid pro-inflammatory signals appear to be related to a reduced risk of recurrent wheezing. In ***chapter 8***, the concentrations of soluble leukocyte associated IgG-like receptor-1 (sLAIR-1) in amniotic fluid are studied. This soluble form of the inhibitory receptor LAIR-1 is considered a distinct marker of general immune activation. The amniotic fluid concentrations of sLAIR-1 during term delivery were found positively correlated with newborn compliance of the respiratory system.

Finally, ***chapter 9*** provides a general discussion on the results reported in this thesis in the context of what is known about RSV bronchiolitis from previous studies.

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Section I

Prediction of RSV bronchiolitis in healthy term infants

Chapter 2

Clinical prediction rule for RSV bronchiolitis in healthy newborns: prognostic birth cohort study

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ABSTRACT

Objective

Our goal was to determine predictors of respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) among healthy newborns.

Methods

In this prospective birth cohort study, 298 healthy term newborns born in two large hospitals in The Netherlands, were monitored throughout the first year of life. Parents kept daily logs and collected nose-throat swabs during respiratory tract infections. The primary outcome was RSV LRTI, which was defined on the basis of the combination of positive RSV PCR results and acute wheeze or moderate/severe cough.

Results

Of the 298 children, 42 (14%) developed RSV LRTI. Independent predictors for RSV LRTI were: day-care attendance and/or the presence of siblings, high parental education level, birthweight of >4 kg, and birth in April to September. The area under the receiver operating curve (ROC area) was 0.72 (95% CI 0.64 to 0.80). We derived a clinical prediction rule; possible scores ranged from 0 to 5 points. The absolute risk of RSV LRTI was 3% for children with scores ≤2 (20% of all children) and 32% for children with all 4 factors (scores of 5; 8% of all children). Furthermore, 62% of the children with RSV LRTI experienced wheezing during the first year of life, compared with 36% of the children without RSV LRTI.

Conclusions

A simple clinical prediction rule identifies healthy newborns at risk of RSV LRTI. Physicians can differentiate between children with high and low risks of RSV LRTI and subsequently can target preventive and monitoring strategies toward children at high risk.

INTRODUCTION

The annual number of all children in their first year of life in the USA with medically attended respiratory syncytial virus (RSV) infection is estimated to be 44%.[1] The majority of these children (95%) will be treated as outpatient by general practitioners (GP) or at the emergency department.[1] Therefore, from a socioeconomic point of view, outpatient treated RSV infection has a large impact, including emergency department and office visits, drug prescriptions, and missed work days by parents.[2] Moreover, RSV lower respiratory tract infection (LRTI) is associated with subsequent recurrent wheeze in approximately 40% of patients, leading to reduced health-related quality of life (HRQoL).[3-5]

Identifying those newborns that will develop RSV LRTI is important, since simple lifestyle changes, such as intensified hand hygiene, can prevent RSV infection.[6-9] In addition, current and future medical preventive measures may be used to target high-risk individuals.[10,11] Known risk factors for the occurrence of RSV LRTI are preterm birth, young age, male gender, heart and lung disease, Down's syndrome, absence or a short period of breastfeeding, presence of siblings, day-care attendance, and exposure to tobacco smoke.[12-20]

So far, clinical prediction models for RSV have only been developed regarding hospitalization in preterm infants.[21-23] A clinical prediction model for outpatient treated RSV LRTI in term children does not yet exist. The objective of this study therefore was to develop a clinical prediction rule to identify healthy term newborns at high risk of RSV LRTI in the first year of life.

METHODS

Population

Two large urban hospitals (University Medical Center, Utrecht; Diakonessenhuis, Utrecht) participated in this prospective birth cohort study. Children born after 37 weeks of gestation (term) after an uncomplicated pregnancy were eligible to participate. Newborns with major congenital anomalies and newborns whose parents had limited Dutch language skills were excluded. From January 2006 until December 2008, 1080 newborns were eligible and the parents of 341 (32%) agreed to participate and gave written informed consent. The most frequent reason for non-participation was reluctance of parents to perform daily follow-up measurements according to the study protocol. Baseline characteristics of non-participating children and their parents were similar to the characteristics of those participating (data not shown). Of these 341 included children, 298 (87%) had no missing values. The study protocol was approved by the institutional review boards of the two participating hospitals.

Predictive factors

The presence or absence of risk factors for RSV LRTI was assessed using data from the hospital delivery files (gender, gestational age, birth weight, and month of birth) or from standardized questionnaires filled out at age one month and one year. Gestational age was arbitrarily dichotomized using a cut-off of 40.0 weeks. Birth weight was dichotomized using an arbitrary cut-off of 4 kg.[24] Breastfeeding was defined as being given mother milk exclusively (without additional formula feeding) beyond the age of one month. Parental atopy was defined as the presence of any atopic diagnosis (asthma, eczema or hay fever) made by a physician in one or both parents. Exposure to maternal tobacco smoke was defined as maternal smoking of at least one cigarette per day at the age of one month. Day-care attendance was defined as attendance of any day-care during the first year of life. The presence of siblings in the household of the child was defined as one or more siblings under the age of 18 years living at least three days per week in the same house. A composite variable day-care and/or siblings was created to limit the number or potential predictive factors, because of the relatively small sample size. Parental educational level was dichotomized using the arbitrary cut-off of a bachelor's degree of at least one parent. Since maternal anti-RSV antibodies may protect infants against RSV disease in the community in their first months of life, being born within six months before the start of the RSV-season (April through September) was used as a potentially predictive variable.[25]

Outcomes

The primary outcome was RSV LRTI, which was defined as the presence of a LRTI and presence of RSV RNA. Parents were instructed to record daily respiratory symptoms in a log, including wheeze and cough.[26] Episodes in the log were defined to represent a LRTI using strict pre-defined criteria: moderate or severe cough or wheeze of any severity lasting for at least two days. A nose-throat swab sample was obtained by the parents at the start of every respiratory episode, and was subsequently sent to the researchers in a single vial containing 2 mL of viral transport medium. The samples were frozen at −80°C until polymerase chain reaction (PCR) was performed. The presence of RSV A or B RNA was determined with real-time PCR.[27]

A secondary outcome was 'GP attended RSV infection', which was defined as the occurrence of a respiratory episode for which the GP was attended and presence of RSV RNA. To study the burden of a RSV LRTI episode we also looked at wheezing during the first year of life and the HRQoL (measured with the TNO-AZL Preschool Quality of Life (TAPQOL) questionnaire) in both children with and without RSV LRTI (secondary outcomes).[28-30] Wheezing during the first year of life was derived from the logs.

Statistical analyses

The association between each prognostic factor and the presence or absence of RSV LRTI was examined with univariate logistic regression analyses. Predictors that were associated with the outcome in univariate analyses ($P < .15$) were included in multivariable logistic regression analyses. The model was reduced through exclusion of predictors with P-values of $> .10$. The predictive accuracy of the model was estimated by its reliability (goodness-of-fit) using Hosmer-Lemeshow tests.[31,32] The model's ability to discriminate between children with and without RSV LRTI was estimated as the area under the receiver operating characteristic (ROC) curve of the model. The ROC curve is a plot of the true positive rate (sensitivity) versus the false positive rate (1 – specificity) evaluated at consecutive cut-off points of the predicted probability. The area under the ROC curve (AUC) provides a quantitative summary of the discriminative ability of a predictive model. A useless predictive model, such as a coin flip, would yield an AUC of 0.5. When the AUC is 1.0, the model discriminates perfectly between those with and without developing a prognostic outcome.[33]

Prediction models derived with multivariable regression analysis are known for overestimated regression coefficients, which results in too extreme predictions when applied in new patients.[33] Therefore, we (internally) validated our models with bootstrapping techniques where in each bootstrap sample the entire modelling process was repeated. This yielded a shrinkage factor for the regression coefficients and the ROC AUC.[33]

To obtain a prediction rule that is easily applicable in clinical practice, the adjusted regression coefficients of the model were divided by the lowest coefficient and rounded to the nearest integer. Scores for each individual patient were obtained by assigning points for each variable and adding the results. Patients were classified according to their risk score and the number of children developing or not developing RSV LRTI, and corresponding positive and negative predictive values were calculated.

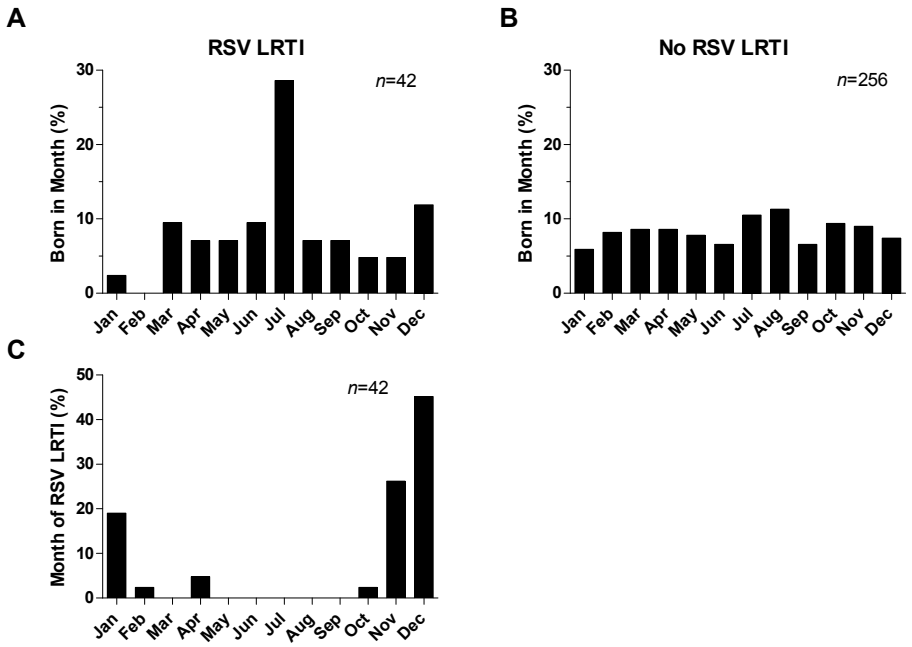
To test the robustness of the model, sensitivity analyses were carried out, using the alternative outcome GP attended RSV infection, and using alternative predictive factors (eg day-care and siblings as separate variables; duration of breastfeeding, intensity of maternal smoking, or duration of day-care attendance as continuous variables). The clinical relevance of the model was studied by comparing the proportion of children with wheeze per month, respiratory symptoms and HRQoL between children with and children without RSV LRTI in the first year of life. All analyses were performed in SPSS (version 15).

RESULTS

Of the 298 participating children, 42 (14%) developed RSV LRTI during their first year of life. One child developed two separate RSV LRTIs within the same season. The median age at the time of RSV LRTI was 6 months (interquartile range 4 to 8 months, **figure 1**). Twenty children (48%) were boys. Of the 42 children with RSV LRTI, 27 (64%) attended the GP and three (aged 2, 6 and 8 months) were hospitalized. Although RSV A (25/42) and B (17/42) were separately detected by PCR, clinical outcomes of children with RSV A and RSV B LRTI were comparable (data not shown).

Results of univariable and multivariable logistic regression analyses are presented in **table 1**. The final reduced regression model included four independent predictive variables: day-care-attendance and/or the presence of siblings (odds ratio (OR) 5.8), high parental education (OR 2.8), birthweight > 4 kg (OR 2.2), and month of birth between April and September (OR 2.2, table 1). The goodness-of-fit test indicated an acceptable fit of the final prognostic model ($P = .91$), and the AUC was 0.72 (95% confidence interval (CI) 0.64 to 0.80). The shrunk AUC was 0.70 (shrinkage factor 0.97). The sensitivity analyses with the alternative outcome GP attended RSV infection and with alternative

Figure 1. Distributions of month of birth of children with and without RSV LRTI and of month of RSV LRTI



Distributions of month of birth of children with (A) and without (B) RSV LRTI and of month of RSV LRTI (C).

Table 1. Univariable and multivariable analysis of predictors of RSV LRTI

Characteristic	RSV LRTI *n*= 42	No RSV LRTI *n*= 256	Univariable analyses			Multivariable analyses (Final model)			Points for rule
	n (%)	*n* (%)	OR	95% CI	*P*-value	OR	95% CI	*P*-value	
Child									
Breastfeeding	22 (52)	140 (55)	0.91	0.47-1.8	.78				
Male	20 (48)	138 (54)	0.78	0.40-1.5	.45				
Gestational age 40-42 wk	28 (67)	128 (50)	2.00	1.01-4.0	.05				
Birthweight > 4 kg	16 (38)	52 (20)	2.41	1.2-4.8	.01	2.24	1.1-4.6	.03	1
Environment									
Parental atopy	24 (57)	143 (56)	1.05	0.55-2.0	.88				
Maternal smoking	2 (5)	24 (9)	0.48	0.11-2.1	.33				
Born in April to September	28 (67)	132 (52)	1.88	0.95-3.7	.07	2.17	1.1-4.4	.03	1
Day-care or siblings	41 (98)	214 (84)	8.05	1.1-60.1	.02	5.80	0.76-44.4	.09	2
High parental education level	38 (91)	186 (73)	3.58	1.2-10.4	.01	2.79	0.94-8.3	.07	1
Hosmer-Lemeshow X^2						2.74		.91	
ROC AUC						0.72	0.64-0.80		Σ=5

CI indicates confidence interval. The prediction rule was as follows: score = (2 for day-care attendance and/or siblings) + (1 for high parental education level) + (1 for birthweight >4 kg) + (1 for birth in April to September). All variables were dichotomous (0 or 1), and scores ranged from 0 through 5.

predictive factors yielded a similar prognostic model with identical discriminating ability (ROC AUC = 0.72 and 0.71, respectively).

Using the regression coefficients of the final predictive model, the probability of developing RSV LRTI can be estimated for each child using the formula given in **table 1**. For example, a child born in July (1 point), who goes to day-care (2 points), with a birthweight of 4.2 kg (1 point), and whose parents are not highly educated (0 points), has a total score of 2 + 1 + 1 + 0 = 4 points, corresponding with a probability to develop RSV LRTI of 23%. **Table 2** shows the number of children in the cohort with and without RSV LRTI across different categories of the risk score. **Figure 2** shows that children with the lowest score (0 to 2 points, 20% of all children) had an absolute risk of 3% to develop RSV LRTI, while children with all risk factors (8% of all children) had an absolute risk of 32% (risk ratio (RR) 9.6).

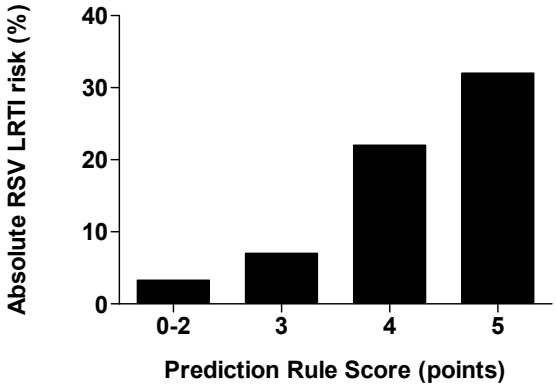
Furthermore, 62% of the children with RSV LRTI suffered from wheezing during the first year of life versus 36% of the children without RSV LRTI (RR 1.72, *P* = .003, **figure 3**). Exclusion of the episodes that defined the RSV LRTI group gave similar results (59 vs 36%, RR 1.65, *P* = .005). Children with RSV LRTI used more respiratory drugs at the age of one year, although not significant (15% vs 8%, NS) and more often visited a physician for respiratory problems than children without RSV LRTI (48% vs 30%, *P* = .03).

Table 2. Performance of different thresholds for prediction rule for RSV LRTI (*n*= 298)

Threshold	True-positive results *n*= 42 (%)	True-negative results *n*= 256 (%)	Positive predictive value %	Negative predictive value %
≥ 3	40 (95)	58 (23)	16.8	96.7
≥ 4	33 (79)	148 (58)	23.4	94.3
5	8 (19)	239 (93)	32.0	87.5

The prediction rule was as follows: score = (2 for day-care attendance and/or siblings) + (1 for high parental education level) + (1 for birthweight >4 kg) + (1 for birth in April to September). All variables were dichotomous (0 or 1), and scores ranged from 0 through 5.

Figure 2. Absolute risk to develop RSV LRTI for children stratified for different prediction rule scores



Absolute risk to develop RSV LRTI for children with different prediction rule scores. Scores of 0, 1 and 2 points (pooled), *n*=60; score of 3 points, *n*=97; score of 4 points, *n*=116; score of 5 points, *n*=25 were compared using X^2 test, *P*< .001.

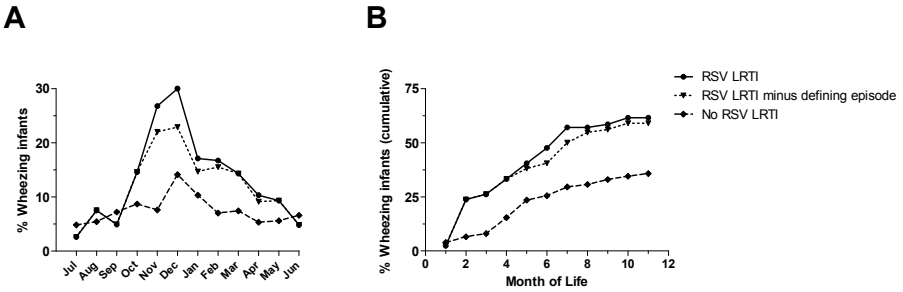
The HRQoL was lower in children with RSV LRTI regarding five of the ten domains (Lungs, Stomach, Appetite, Anxiety and Problem Behaviour) as compared to children without RSV LRTI (**figure 4**).

DISCUSSION

We developed a simple prediction rule that identifies healthy newborns at high risk of RSV LRTI in the first year of life. Independent predictors for RSV LRTI were day-care attendance and/or the presence of siblings, high parental education, birthweight > 4 kg, and month of birth between April and September.

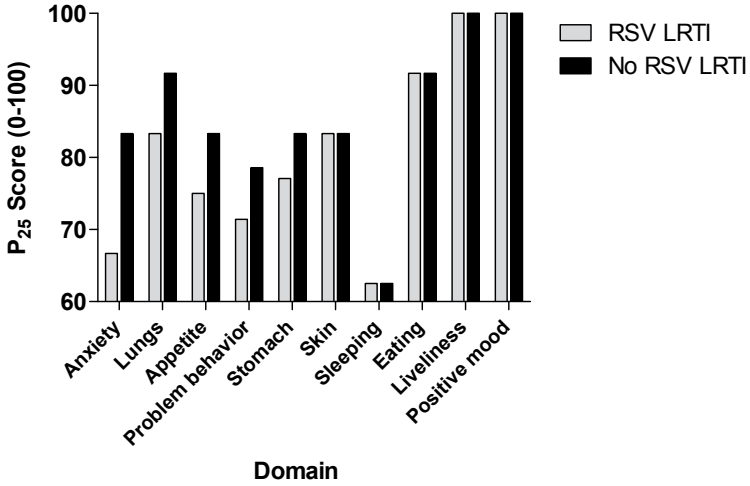
Our prognostic study differs from others with respect to the domain and outcome studied.[21;22] We focused on non-hospitalized RSV LRTI in term healthy infants, whereas others studied hospitalized RSV in premature infants. This may explain the small differences in predictive factors. The strongest predictor in our study (day-care attendance

Figure 3. Proportions of children with wheezing during the first year of life, for children with and without RSV LRTI in the first year of life



(A) Proportion of wheezing children in each calendar month, Fisher's exact test: November, $P = .001$; December, $P = .01$. Exclusion of the episodes that defined the RSV LRTI group yielded similar results (November, $P = .01$). (B) Cumulative proportion of wheezing in each month of life. Fisher's exact test or X^2 test: all $P < .01$, except month 1 (not significant) and month 5 ($P = .02$). Exclusion of the episodes that defined the RSV LRTI group yielded similar results: all $P < .01$, except month 1 (not significant), month 5 ($P = .05$), month 6 ($P = .05$), and month 7 ($P = .01$).

Figure 4 . Health-related quality of life (HRQoL) for children with and without RSV LRTI in the first year of life



HRQoL, measured at the age of one year using the TNO-AZL Preschool Quality of Life (TAPQOL) questionnaire, ranged from 100 (best) to 0 (worst). The 25th percentile (P_{25}) HRQoL scores for 10 different domains are shown (n=239).

and/or the presence of siblings) is in agreement with other studies.[21-23] High birth weight may be associated with delayed parturition and an altered immunologic phenotype.[21,34,35] Being born within six months before the start of the RSV season is a longer window than usually found.[16,21,23] However, it is consistent with the median age of six months during RSV LRTI in the community and/or at the GP in our cohort study and found by others.[1] Highly educated parents might be more careful or seek earlier medical advise in case

their child develops a respiratory infection.[36,37] However, parental education may also be associated with other environmental factors.

To our knowledge this is the first study that attempts to predict the risk of non-hospitalised RSV LRTI in healthy newborns using molecular detection of RSV. Some of our findings deserve additional discussion. First, only 341 of the 1080 eligible newborns participated in our study, which might have resulted in selection (bias). Comparison of the baseline clinical and demographical characteristic between participant and non-participants, however, showed no differences. We therefore believe that our results are generalisable to all healthy newborns. Second, due the design of our study, elective caesarean sections were overrepresented in this cohort (16% vs 6% in the Netherlands).[38] Mode of delivery, however, was not associated with RSV LRTI. We therefore assume that the results are generalisable to other modes of delivery. Third, misclassification due to parental incompliance with recording of respiratory symptoms and collection of nose-throat swabs cannot be completely ruled out. However, associations between parental compliance and any potential risk factor seem unlikely. Fourth, since missing values usually do not occur at random, exclusion of participants with missing values (complete case analysis) may have resulted in biased estimates.[39,40] We therefore used imputation to address the missing values, including missing values of the outcome, which showed similar results as the presented complete case analysis. Fifth, for a number of variables, we used arbitrary cut-offs / definitions, mostly in favour of a simple prediction rule or as a result of study design. Accessory analyses with alternative cut-offs yielded a similar prediction model. Likewise, using continuous variables (eg for duration of breastfeeding, number of cigarettes per day) did not change the final model. We therefore believe that our prediction rule is robust.

The clinical implications of our findings include the use of the prediction rule by primary care pediatricians, who manage the majority of children at risk of and/or with RSV LRTI.[1] The incidence of medically attended RSV infection in children below the age of one year is extremely high (approximately 44%), and highest for the group aged six to twelve months (24%).[1] Children classified as at high risk could be monitored more closely and lifestyle changes that reduce exposure could be applied.[6-9] When novel (preventive) treatment options become available, these could be used in targeted high risk populations. [41-43] Finally, the model may be used in randomized clinical trials when future RSV vaccines will become available for healthy term infants.[44]

In conclusion, the risk of RSV LRTI was ten times higher in children attending day-care also having older siblings, high parental educational level, birthweight > 4 kg, and who were born between April and September as compared to children without these factors. Clinicians can use these features to differentiate between children with high and low risk of RSV LRTI, and subsequently target preventive and monitoring strategies at high risk children.

Acknowledgements

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Chapter 3

Cord blood vitamin D deficiency is associated with RSV bronchiolitis

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ABSTRACT

Background

Respiratory syncytial virus (RSV) is the most important pathogen causing severe lower respiratory tract infection (LRTI) in infants. Epidemiologic and basic studies suggest that vitamin D may protect against RSV LRTI.

Objective

To determine the association between plasma concentrations of 25-hydroxyvitamin D (25-OHD) at birth and the subsequent risk of RSV LRTI.

Methods

We performed a prospective birth cohort study in healthy term neonates. Cord blood concentrations of 25-OHD were related to risk of RSV LRTI in the first year of life, defined as parent-reported LRTI symptoms in a daily log, and simultaneous presence of RSV RNA in a nose-throat swab.

Results

156 neonates were included. Eighteen (12%) developed RSV LRTI. The mean cord blood concentration of 25-OHD was 82 nmol/L. Overall, 4% of neonates had 25-OHD concentrations <25 nmol/L, 23% had 25-OHD 25-49 nmol/L, 27% had 25-OHD 50-74 nmol/L and 46% had 25-OHD ≥75 nmol/L. Maternal vitamin D3 supplementation during pregnancy was positively associated with cord blood 25-OHD ($P = 0.003$). Concentrations of 25-OHD were lower in neonates who developed RSV LRTI compared to those who did not (65 versus 84 nmol/L, $P = .009$). Neonates born with 25-OHD <50 nmol/L had 6.2-fold (95%-confidence interval 1.6-24.9, $P = .01$) increased risk of RSV LRTI in the first year of life compared to those with 25-OHD ≥75 nmol/L.

Conclusions

Vitamin D deficiency at birth is associated with increased risk of RSV LRTI in the first year of life. Intensified routine vitamin D supplementation during pregnancy may be a useful strategy to prevent RSV LRTI during infancy.

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important respiratory pathogen in young children, causing significant morbidity.[1,2] Although >90% of all infants encounter RSV before the age of 2, only 10% develop a severe lower respiratory tract infection (LRTI). Several risk factors for RSV LRTI have been described, but the majority of infections occur in infants without any known risk factors.[1,2] Insight into the factors predisposing to RSV LRTI may result in new strategies to prevent infection.

Vitamin D is an essential nutrient, with functions that extend beyond its classical role in bone metabolism. Vitamin D regulates >1000 human genes, with receptors present in most cells throughout the body.[3] In westernized countries, 40% of pregnant women and 50% of newborns and infants are vitamin D insufficient.[4-8] Fetal and newborn concentrations of vitamin D are dependent on and correlated with maternal serum 25-OHD concentrations.[9-12] Accordingly, maternal vitamin D insufficiency has been related to many diseases in the offspring, including type I diabetes,[13] multiple sclerosis,[14] schizophrenia,[15] infant wheeze,[16] and acute respiratory infections.[12,17]

Basal and epidemiologic evidence suggests that vitamin D may protect against severe RSV LRTI. *In vitro*, vitamin D decreases the inflammatory response of airway epithelial cells to RSV infection, without jeopardizing viral clearance.[18] In humans, RSV occurs in a seasonal pattern with peaks in winter, when serum concentrations of vitamin D are lowest.[19] Genetic polymorphisms in the vitamin D receptor are associated with hospitalization for acute lower respiratory tract infections, predominantly RSV bronchiolitis, in infancy.[20] Furthermore, several studies have demonstrated that plasma concentrations 25-OHD are lower in infants hospitalized for acute lower respiratory tract infection compared to healthy controls.[10-12,21,22] However, these studies were cross-sectional and included a limited number of subjects.

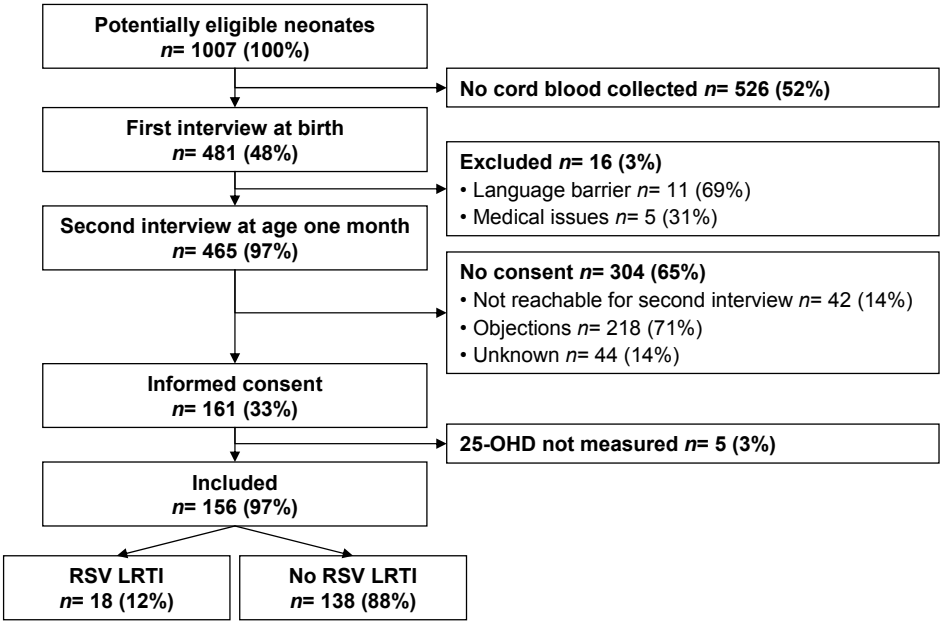
In this healthy birth cohort study, we aimed to determine the association between cord blood vitamin D status and the subsequent risk of RSV LRTI in the first year of life.

METHODS

Study design and recruitment criteria

This study is part of a prospective birth cohort study on early-life determinants of RSV LRTI in two medical centers in Utrecht, The Netherlands. Study design and recruitment criteria have been published previously.[23] Eligible were healthy newborns, born after uncomplicated gestation of ≥37 weeks. To avoid extensive counseling of parents just after delivery, a two-step approach was used (**Figure 1**). The first consent interview took place after delivery. During this interview, parents were informed that cord blood had been

Figure 1. Flow chart of study population



collected, oral and written information on the study was provided and a second interview was scheduled at the age of 1-3 weeks for further information and informed consent. The study was approved by the ethical review board of the University Medical Center Utrecht and the Diakonessen Hospital Utrecht, and parents of all participants provided written informed consent for study participation.

Clinical characteristics

Baseline characteristics and use of vitamin D supplements were collected from hospital charts and standardized parental questionnaires.[24] Maternal ethnicity was defined as Caucasian or non-Caucasian, based on country of birth. The following birth seasons were distinguished: winter (December, January, February), spring (March, April, May), summer (June, July, August) or fall (September, October, November). To assess the relation between cord blood 25-OHD levels and sun exposure, monthly hours of sunshine in The Netherlands during the study period were obtained from the archives of the Dutch Royal Meteorological Institute.[10]

Plasma vitamin D measurement

Cord blood was collected directly after delivery and anticoagulated using sodium heparin. Plasma was prepared by centrifugation (5 min, 500 *g*), and stored at −80°C. Plasma 25-OHD concentrations (nmol/L, to convert to ng/mL divide by 2.496) were measured with the Modular E170 analyzer (Roche). Inter assay variability for pooled serum analy-

ses was 19% at 33 nmol/L 25-OHD, 12% at 62 nmol/L and 10% at 99 nmol/L. Plasma concentrations of 25-OHD were analyzed both as a continuous variable, or divided into quartiles (<25 nmol/L, 25-49 nmol/L, 50-74 nmol/L and ≥75 nmol/L)(7). Because of the low number of neonates in the <25 nmol/L group (n = 7), for outcome analyses, the <25 and 25-49 nmol/L groups were pooled.

Primary and secondary outcome

The primary outcome was defined as parent-reported RSV LRTI, which was defined as (1) LRTI symptoms, and (2) simultaneous presence of RSV RNA in a nose-throat specimen. Parents were instructed to record presence and severity of respiratory symptoms during the first year of life in a daily log.[23] LRTI symptoms were defined by two independent researchers using strict criteria: moderate or severe cough or wheeze of any severity lasting for at least two days. At the second day of every respiratory episode, parents obtained a nose-throat swab specimen. Samples were sent to the researchers in viral transport medium and frozen at −80°C.[25] The presence of RSV RNA was determined by real-time polymerase chain reaction as described previously.[23]

The secondary outcome 'physician-attended RSV LRTI' was defined as (1) respiratory illness for which the general practitioner or paediatrician was visited, and (2) simultaneous presence of RSV RNA in a nose-throat swab.

Statistical analysis

Cord blood plasma 25-OHD concentrations were normally distributed, and means were compared using Student's T test. X^2 analysis was used to test associations between categorical variables. Seasonality of 25-OHD was tested by fitting the data to a sine function with a period of 12 months in a nonlinear regression model. Statistical significance of seasonal distribution was determined by comparing the resulting sinusoidal model with the best fitting linear model, using the F test. Logistic regression analysis was performed to determine the effect of cord blood 25-OHD concentrations on the risk of subsequent RSV LRTI while correcting for potential confounders. Due to the limited number of cases, only a restricted number of potential confounders could be analyzed. The variables birth month, birth weight and maternal ethnicity showed the highest association with both cord blood 25-OHD concentration and risk of RSV disease in single variable analyses, and were therefore included in regression models. To adjust for birth month, two approaches were used: (1) 'Deseasonalization' of 25-OHD concentrations **(Supplementary figure 1)**.[26] In this approach, the predicted 25-OHD concentrations for each subject, derived from the sinusoidal model, were subtracted from the actual observed value. Subsequently, the overall mean was added and the resulting deseasonalized 25-OHD concentrations were analyzed using logitistic regression analysis, also adjusting for maternal ethnicity and birth weight. (2) Regression analysis in which we adjusted

for birth ±10 weeks from the start of the RSV season (yes versus no), next to maternal ethnicity and birth weight. Analyses were performed in SPSS 15.0.

RESULTS

Population characteristics

From November 2006 to December 2009, 1007 neonates were eligible for study participation (**figure 1**). Of these, cord blood was collected in 481 (48%) neonates, and 161 (33%) of these agreed to participate in follow-up. Due to technical reasons, plasma 25-OHD concentrations were not measurable in 5 (3%) of participating neonates, resulting in a final cohort of 156 neonates. Baseline characteristics did not differ between participating subjects and non-participants (**supplementary table 1**). Of the participating neonates, 18 (12%) developed RSV LRTI in their first year of life, of which 10 had a physician-attended RSV LRTI. Neonates who subsequently developed RSV LRTI had a higher birth weight (3,903 versus 3,523 g, $P = .001$), and trended towards higher gestational age (40.4 versus 39.9 weeks, $P = .06$) compared to those who did not, as observed previously (**supplementary table 2**).[27] There were no differences in birth season, number of siblings, maternal ethnicity, mode of feeding and use of vitamin D supplements between neonates who did or did not develop RSV LRTI.

High prevalence of vitamin D deficiency in healthy newborns

The mean cord blood plasma 25-OHD concentration among healthy newborns was 82 nmol/L (standard deviation (SD) 44 nmol/L). Overall, 4% of neonates had 25-OHD levels <25 nmol/L and 23% had levels <50 nmol/L; 27% had 25-OHD levels of 50-74 nmol/L, and only 46% had 25-OHD levels of 75 nmol/L or higher.

Use of vitamin D supplements during pregnancy increases cord blood 25-OHD concentrations

Of participating women, 46% reported use of vitamin D-containing supplements during pregnancy. The majority of these (97%) were multivitamin preparations, containing a daily dose of 400 IU (10 μg) vitamin D3. Of these women, 74% used supplements during the first trimester, 86% during the second trimester, and 81% during the third trimester. In total, 54% of participating women used vitamin D supplements throughout pregnancy. Maternal use of vitamin D supplementation during pregnancy was associated with increased concentrations of 25-OHD in cord blood (73 versus 96 nmol/L, $P = .003$). After birth, 75% of all neonates received vitamin D supplements (daily recommended dose 400 IU vitamin D3) during the first month of life. Characteristics of participants according to vitamin D status at birth are shown in **table 1**.

Table 1. Characteristics of study population according to cord blood vitamin D status

	Cord blood 25-OHD				
	<25 nmol/L (*n*=7)	**25-49 nmol/L (*n*=29)**	**50-74 nmol/L (*n*=48)**	**>75 nmol/L (*n*=72)**	***P*-value**
Birth weight, g (SE)	3237 (167)	3529 (90)	3604 (74)	3574 (50)	.25
Gestational age, wks (SE)	39.8 (0.41)	40.1 (0.21)	40.2 (0.14)	39.8 (0.13)	.35
Any siblings, *n* (%)	0 (0)	12 (41)	18 (38)	31 (43)	.17
Male gender, *n* (%)	4 (57)	13 (45)	24 (50)	30 (42)	.75
Birth season (%)					.012
Winter	3 (43)	11 (38)	16 (33)	13 (18)	
Spring	1 (14)	6 (21)	7 (15)	18 (25)	
Summer	1 (14)	3 (10)	7 (15)	28 (39)	
Fall	2 (29)	9 (31)	18 (38)	13 (18)	
Maternal ethnicity (%)					
Caucasian	2 (29)	15 (52)	33 (69)	63 (88)	<.001
Other	5 (71)	14 (48)	15 (31)	9 (13)	
Ever breastfed, *n* (%)	6 (100)	19 (79)	33 (87)	51 (81)	.56
Vitamin D supplement use during pregnancy, *n* (%)	6 (100)	13 (68)	23 (62)	24 (39)	.005
Neonatal vitamin D supplement use, *n* (%)	4 (67)	12 (63)	30 (81)	46 (75)	.50

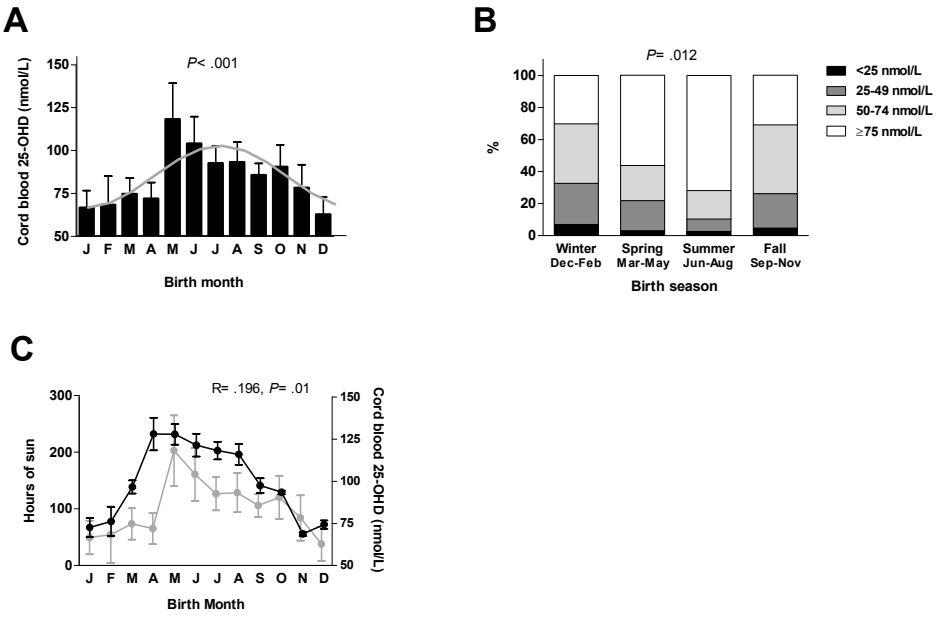
25-OHD concentrations show a seasonal pattern

To explore the seasonal variation in the concentrations of 25-OHD, cord blood 25-OHD concentrations and birth month were fitted to a sinusoidal model. Cord blood concentrations of 25-OHD showed a seasonal distribution with a baseline level of 84 nmol/L and an amplitude of 15 nmol/L ($P = .001$, **figure 2A**). Maximum fitted concentrations of cord blood 25-OHD were observed in newborns born in July, and concentrations reached their nadir in January. Of neonates born in winter, 33% had cord blood 25-OHD concentrations <50 nmol/L and 70% had concentrations <75 nmol/L, compared to 10% and 28% of neonates born in summer (**figure 2B**, X^2, $P = .012$). Seasonality of 25-OHD concentrations was present for all birth years in our cohort (data not shown). We also related cord blood 25-OHD levels to monthly hours of sunshine during the study period, according to the Royal Netherlands Meteorological Institute,[10] and found a strong correlation between cord blood 25-OHD levels and monthly sun hours (**figure 2C**, $\rho = 0.196$, $P = .01$).

Cord blood vitamin D concentrations are associated with RSV LRTI in the first year of life

Plasma concentrations of 25-OHD at birth were related to the risk of RSV LRTI in the first year of life (**figure 3**). Newborns who subsequently developed RSV LRTI had 1.3-fold

Figure 2. High prevalence of vitamin D deficiency in healthy newborns

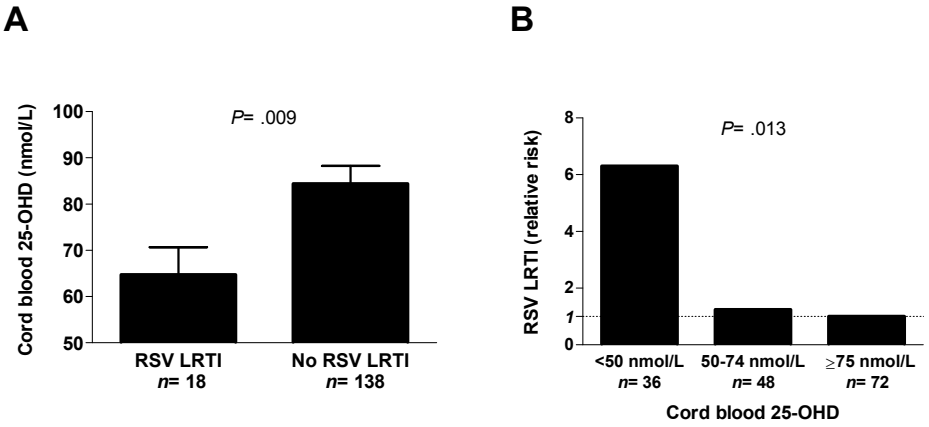


Concentrations of 25-OHD in cord blood plasma ($n = 156$) were measured with the Modular E170 analyzer, and related to birth month (A) or birth season (B). Seasonality of cord blood 25-OHD was assessed by fitting the data to the best fitting linear and sinusoidal model. For panel C, monthly hours of sun during the study period were obtained from the archives of the Dutch Royal Meteorological Institute[10], and related to cord blood 25-OHD using Pearson correlation. Bars represent mean+standard error of the mean (A and C), or % of subjects (B).

lower cord blood concentrations of 25-OHD compared to those who did not (65±7 versus 84±11 nmol/L, $P = .009$, **figure 3A**). Logistic regression analysis, correcting for birth month, birth weight and maternal ethnicity as potential confounders, demonstrated that cord blood 25-OHD concentrations were independently associated with subsequent risk of RSV LRTI (B = 0.978 (95% CI 0.959-0.997), $P = .024$). Sensitivity analyses, correcting for birth ±10 weeks of the start of the RSV season, maternal ethnicity and birth weight, also showed a significant negative association between cord blood 25-OHD and risk of RSV LRTI (B = 0.976 (0.957-0.996), $P = .018$). Using the secondary outcome, physician-attended RSV LRTI, a similar trend was observed (63 versus 83 nmol/L, B = 0.983 (0.962-1.004), $P = .117$).

We also analyzed the risk of RSV LRTI in neonates who were born with cord blood 25-OHD levels <50 nmol/L, 50-75 nmol/L and ≥75 nmol/L (**figure 3B**). Compared to neonates with cord blood 25-OHD levels ≥75 nmol/L, the adjusted relative risk (aRR) of RSV LRTI was 6.2 (95%-CI 1.6-24.9, $P = .01$) in neonates with 25-OHD levels <50 nmol/L.

Figure 3. Association between cord blood vitamin D concentrations and RSV LRTI in the first year of life



(A) Cord blood concentrations of 25-OHD in neonates who subsequently developed respiratory syncytial virus lower respiratory tract infection (RSV LRTI, $n = 18$) and those who did not ($n = 138$). (B) Risk of RSV LRTI per quartile of 25-OHD levels. Due to limited number of cases, the lower quartiles (<25 nmol/L, $n = 7$ and 25-49 nmol/L, $n = 29$) were pooled. Bars represent mean+standard error of the mean (A), or risk of RSV LRTI relative to neonates with 25-OHD ≥75 nmol/L (B).

DISCUSSION

In this prospective birth cohort study, we demonstrate that 54% of healthy newborns in the Netherlands are born with insufficient 25-OHD concentrations required for maximum health,[28,29] and that low plasma concentrations of 25-OHD at birth are associated with increased risk of RSV LRTI in the first year of life.

RSV is the most important respiratory pathogen in infancy, yet the mechanisms responsible for severe RSV disease are incompletely understood. Although antibody therapy is recommended for children at high risk of severe infection, the majority of infections occur in children without any known risk factors,[30] for whom no preventive strategies are currently available. Micronutrient supplementation to pregnant women and their newborns could be an easy and affordable strategy to prevent RSV LRTI.

The prevalence of vitamin D deficiency in our cohort is comparable to reported prevalence in other westernized countries.[6,28,29,31] Cord blood vitamin D concentrations demonstrated a seasonal pattern, with maximum concentrations in July and lowest concentrations in newborns born in December. This relatively early peak compared to previous cohort studies[11,32] might be due to the extraordinarily high sun exposure in spring months during the study period (**figure 1C**). In addition, the partial association between hours of sunshine and cord blood 25-OHD levels indicates that other factors, including time spent outdoors, use of sun protection,[33,34] and nutritional intake of vitamin D might contribute to cord blood vitamin D status.

To our knowledge, this is the first longitudinal study to relate plasma 25-OHD concentrations at birth to the subsequent risk of RSV LRTI. Previous cross-sectional studies have related low plasma concentrations of 25-OHD to increased severity of respiratory tract infection.[12,17,22] In Turkey and rural Bangladesh, plasma 25-OHD concentrations during infection were lower in children hospitalized with acute LRTI compared to age-matched healthy controls,[12,22] and subclinical vitamin D deficiency predisposed to acute RTI in Indian children.[17] In addition, a recent cohort study in 284 Finnish children hospitalized for acute wheezing demonstrated a significant association between plasma vitamin D levels and risk of viral co-infection, specifically co-infections with RSV, rhinovirus or both.[8] In contrast, studies in Canada failed to show a difference in plasma 25-OHD between children with and without RTI.[21,35]

There are several potential explanations for the protective effect of vitamin D at birth against subsequent RSV LRTI reported in our study. Severe RSV infection is thought to arise from a interplay between the host immune response, airway anatomy, and RSV viral load. All these factors may be affected by vitamin D.

First, vitamin D has immune modulatory properties that may influence the development of the fetal and neonatal immune system. Low vitamin D intake during pregnancy is associated with increased incidence of diseases related to immune dysfunction in the offspring, including type I diabetes, asthma and allergic rhinitis.[16,36,37] In vitro, vitamin D has many immune modulatory functions, including induction of tolerogenic dendritic cells,[38] development of $CD4^+CD25^+Foxp3^+$ regulatory T-cells,[39] activation of T-cell signalling,[40] and elaboration of tolerizing and anti-inflammatory cytokines, including IL-10.[39,41,42] Moreover, a recent study demonstrates that maternal vitamin D intake during pregnancy increased expression of tolerogenic genes in cord blood,[43] suggesting that the immune modulatory function of vitamin D may already occur prenatally.

Second, vitamin D may modulate early lung development. In animal models, vitamin D has been shown to promote lung development and surfactant production.[44-46] In humans, $1,25(OH)_2D$ also promotes surfactant production(47) and downstream effectors of vitamin D have been detected in fetal lungs as early as 14 weeks gestational age.[48] Vitamin D might thus accelerate fetal lung development, thereby potentially protecting against RSV disease.

Third, vitamin D has many antimicrobial properties that may result in decreased viral load during infection.[42] Neonates who are born vitamin D deficient may also have lower serum concentrations at the neonatal and infant age, thereby potentially confounding any association between cord blood vitamin D and RSV LRTI during infancy. We did not measure vitamin D concentrations during RSV infection. However, 75% of neonates and infants in our cohort received vitamin D supplements after birth (400 IU/day), and there was no association between postnatal vitamin D supplement use and cord blood vitamin D levels (data not shown) or risk of RSV LRTI (**supplementary table 2**).

Our results suggest that strategies aimed at improving maternal vitamin D status during pregnancy might decrease the risk of RSV disease in the offspring. In agreement with recommendations of the American Association for Pediatrics and the World Health Organization, the Dutch Health Council recommends daily supplementation of 400 IU (10 μg) vitamin D to all pregnant women and breastfed newborns.[49] However, the optimal dose of vitamin D supplementation is still under debate. Especially during pregnancy, doses up to 4000 IU/day may be needed to obtain optimal maternal and neonatal health.[29,42,50-52] In addition, adherence to the current guidelines is generally poor.[53] In our cohort, only 46% of women reported use of vitamin D-containing supplements during pregnancy. Although vitamin D supplementation during pregnancy resulted in increased cord blood 25-OHD concentrations, we did not find a significant association with risk of RSV LRTI. However, the current study was insufficiently powered to answer this question. The association between cord blood 25-OHD concentrations and subsequent RSV LRTI urges for larger clinical trials investigating the effect of vitamin D supplementation during pregnancy on the susceptibility to RSV LRTI in the offspring.

Potential limitations deserve discussion. First, the sample size and number of cases in our cohort was relatively low. Limited statistical power especially affected analyses using cord blood 25-OHD quartiles, resulting in wide confidence intervals. Nevertheless, despite the low number of cases, we were able to demonstrate significant differences in RSV risk. Second, lack of parental compliance may have caused misclassification of infants who did experience RSV LRTI, but whose parents forgot to take a nose-throat swab or fill out the diary. However, because of the low incidence of RSV LRTI, we do not think that this will significantly change our conclusions. Third, detailed information on sun exposure and dietary habits was not available for analysis. As a surrogate marker of sun exposure, birth month was included into our analysis. Similar results were found, indicating that cord blood 25-OHD is an independent predictor of RSV LRTI.

In conclusion, vitamin D deficiency was highly prevalent in Dutch newborns, and cord blood 25-OHD concentrations are associated with susceptibility to subsequent RSV LRTI. Increased awareness of vitamin D status of pregnant women and intensified routine vitamin D supplementation may help prevent RSV LRTI during infancy. Randomized trials are required to address this question.

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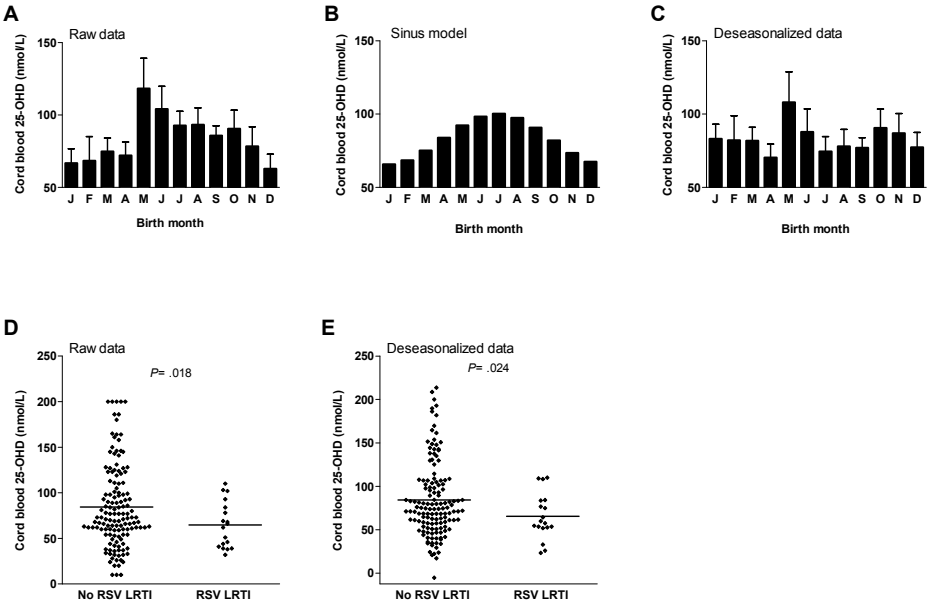
Supplementary figure 1. Strategy for deseasonalization of cord blood 25-OHD concentrations



A sinusoidal model was fitted to cord blood 25-OHD concentrations and birth month (t):

$$25\text{-OHD} = A + B^{*}\sin((C+2\pi t)/12)$$

The predicted values derived from this model (panel B) was subtracted from the actual 25-OHD concentration for each subject (panel A), and the overall mean was added to obtain deseasonalized 25-OHD concentrations (panel C). Regression analysis, using either uncorrected 25-OHD (panel D) or deseasonalized levels (panel E), showed similar results. Bars represent mean+standard error of the mean.

Supplementary table 1: Baseline characteristics of study population compared to all eligible

	Included in study population (*n*=156)	**Not included in study population (*n*=851)**	***P*-value**
Birth weight, g (SE)	3568 (37)	3583 (43)	.88
Gestational age, wks (SE)	40.0 (0.09)	40.0 (0.04)	.37
Siblings, *n* (%)	95 (61)	536 (64)	.59
Male gender, *n* (%)	71 (46)	425 (50)	.34
Birth season, *n* (%)			.77
Winter	43 (28)	239 (28)	
Spring	62 (21)	186 (22)	
Summer	39 (25)	181 (21)	
Fall	42 (27)	245 (29)	
Maternal ethnicity, *n* (%)			.32
Caucasian	106	541	
Other	50	310	

Supplementary table 2: Characteristics of study population according to RSV LRTI

	RSV LRTI (*n*= 18)	**No RSV LRTI (*n*= 138)**	***P*-value**
Birth weight, g (SE)	3903 (374)	3523 (457)	.001
Gestational age, wks (SE)	40.4 (0.25)	39.9 (0.09)	.06
Siblings, *n* (%)	13 (72)	82 (59)	.30
Male gender, *n* (%)	7 (39)	64 (46)	.55
Birth season, *n* (%)			.54
Winter	3 (17)	40 (29)	
Spring	3 (17)	29 (21)	
Summer	5 (28)	34 (25)	
Fall	7 (39)	35 (25)	
Maternal ethnicity, *n* (%)			.15
Caucasian	15 (83)	92 (67)	
Other	3 (17)	46 (33)	
Ever breastfed, *n* (%)	15 (83)	94 (83)	>.99
Vitamin D supplement use during pregnancy, *n* (%)	6 (43)	51 (47)	.78
Neonatal vitamin D supplement use, *n* (%)	10 (71)	82 (75)	.76

Section II

Pathogenesis of RSV bronchiolitis in healthy term infants

Chapter 4

Disease severity and viral load are correlated in infants with primary RSV infection in the community

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RW Hofland, JLL Kimpen, L Bont

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ABSTRACT

Background

Respiratory syncytial virus (RSV) is a major cause of respiratory tract infections in infants, with remarkable variability in disease severity. Factors determining severity of disease in previously healthy infants are still unclear. It was hypothesized that disease severity is correlated with viral load in primary RSV infection. Infants of a healthy birth cohort were included at signs of their first respiratory tract infection.

Methods

Nasopharyngeal aspirate was obtained within 48-96 hours and disease severity was assessed with a previously published severity scoring model. PCR was applied to test the aspirates in a semi-quantitative way for the presence of 10 respiratory pathogens. In case of multiple infection, the pathogen with the highest load was defined as the primary pathogen. The correlation between disease severity and viral load was analysed.

Results

A total of 82 infants were included over a period of 2 years. Median age at first respiratory tract infection was 3 months. Pathogens were detected in 77 (94%) infants; more than one pathogen was detected in 35 (43%) infants. RSV was present in aspirates of 30 infants; in 16 aspirates RSV was the primary pathogen. A negative correlation between RSV CT-value and disease severity was found in all RSV cases ($\rho = -0.52$, $P = .003$) and in cases with RSV as the primary pathogen ($\rho = -0.54$, $P = .03$).

Conclusions

In conclusion, this is the first report on viral loads in previously healthy infants with RSV infection in the community. Disease severity correlated positively with viral load during primary RSV infection.

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most common causes of acute respiratory tract infection in infants. Approximately two thirds of infants are infected during their first year of life.[1] There is large variability in the severity of RSV disease, ranging from insignificant clinical illness to severe respiratory distress. The overall hospital admission rate for RSV infection is estimated to be 0.5-2%.[1,2] Of infants admitted to hospital for RSV infection, about 10% require mechanical ventilation.[3,4]

The factors determining severity of RSV respiratory tract infection are still unclear and are likely to be determined by the host and viral factors.[1] Host derived risk factors for severe RSV infection include prematurity, low birth weight, young age (< 6 months), cardiopulmonary disease and Down's syndrome.[1,5,6] Despite these known risk factors, the majority of severe RSV infections occurs in healthy infants. The contribution to the pathogenesis of RSV infection of direct viral cytopathology versus the host immune response in these infants remains controversial.[1] Publications on viral factors that contribute to RSV disease severity are still sparse.[1] Published reports disagree as to whether or not there is a difference in the pathogenicity of the two RSV strains, A and B.[7-12]

Knowledge of the relationship between viral load and severity of disease will expand understanding of pathogenesis of RSV infection and is important for determining the potential benefit of antiviral treatment. Previous studies in infants treated in hospital disagree on the association between viral load in the nasopharynx and disease severity.[13-17] Since the populations in these studies were very heterogeneous, and none of these studies did take into account possible co-infections, the level of evidence is limited. Analysis of RSV infection in the community in a healthy birth cohort is relevant, because of the high incidence resulting in a large impact on public health, and because it reveals mechanisms of pathogenesis that potentially apply to infants admitted to hospital. The current study is the first to investigate the correlation between viral load and disease severity during primary RSV infections in the community in previously healthy infants. We hypothesize that higher viral load leads to more severe disease.

METHODS

Study population

This study is part of the Netherlands Amniotic Fluid (NAF) study (Wilhelmina Children's Hospital, Utrecht University Medical Center), a birth cohort focusing on the role of perinatal inflammation in the pathogenesis of RSV respiratory tract infection.[18] The included infants were born at term delivery after an uncomplicated pregnancy. For the present observational study, a subgroup of these infants were included at signs of their

first respiratory tract infection, in the period between April 2006 and February 2008. The Institutional Ethical Review Board approved the study protocol. Informed consent was obtained from parents of all participants.

Collection of data

Baseline characteristics (gestational age, birth weight, apgar score, gender, breastfeeding, presence of siblings, parental smoking, parental atopy, day-care attendance) and clinical characteristics (age during infection, duration of illness, wheeze, fever (≥38°C), severity score) were collected prospectively. Breastfeeding was defined as being given mother milk beyond the age of one month. The presence of siblings was defined as one or more siblings under the age of 18 years living at least three days per week in the same house. Parental smoking was defined as smoking by one or both parents of at least one cigarette per day at the age of one month. Parental atopy was defined as the history of any atopic diagnosis (asthma, eczema or hay fever) made by a physician in one or both parents. Day-care attendance was defined as attendance of any day-care during the first year of life.

Parents were instructed to notify the researchers on the second day of the (lifetime) first respiratory tract infection and a house visit was arranged within 36 hours. Disease severity was assessed by the researchers (MLH or RWH), using a previously published severity score by means of a standardized questionnaire and physical examination.[19,20] This scoring model is shown in **table 1**.

Nasopharyngeal aspirate was obtained using an infant mucus extractor (Vygon Pharmaceutiques, Ecouen France). The catheter was inserted in a nostril to a depth of 5 to 7 cm and drawn back while the researcher applied suction. Both nostrils were suctioned. The aspirate was directly and stored at −80°C until further work up.

Table 1 Scoring of disease severity (according to [19,20])

Item	Point score
Fever (≥38°C)	1
Cough	1-2-3
Rhinorrhea	1-2
Hoarseness	1
Duration of illness >4 days	1
Apnea	3
Wheezing	5
Cyanosis	5
Retractions	5
Tachypnea	5
Severity score (sum)[1]	0 through 31

[1]A higher severity score indicates more severe disease

Pathogen detection by real-time semi-quantitative polymerase chain reaction (PCR)

All samples were tested separately for influenzavirus, parainfluenzavirus, coronavirus, RSV, rhinovirus, human metapneumovirus, bocavirus and adenovirus. Because of the low incidence of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, DNA was pooled before testing for these bacteria. Positive pool samples were tested separately to identify the positive sample(s).

RNA extraction and cDNA synthesis. RNA extraction was performed on aspirates, diluted in 2 mL viral transport medium, using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). The isolated viral RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA, USA), followed by RT inactivation for 5 min at 95°C. Both kits were used according to the manufacturer's guidelines. Murine encephalomyocarditis virus (RNA) or Phocine herpes virus (DNA) was used as internal control.

Real-time TaqMan PCR. Type-specific primers and probes for both RSV A and B were based on the highly conserved genomic regions of the N gene. The following primers were used:

RSA-1: 5′ - AGATCAACTTCTGTCATCCAGCAA – 3′
RSA-2: 5′ - TTCTGCACATCATAATTAGGAGTATCAAT – 3′
RSB-1: 5′ - AAGATGCAAATCATAAATTCACAGGA – 3′
RSB-2: 5′ - TGATATCCAGCATCTTTAAGTATCTTTATAGTG – 3′
RSA probe: 5′ - CACCATCCAACGGAGCACAGGAGAT – 3′
RSB probe: 5′ - TTCCCTTCCTAACCTGGACATAGCATATAACATACCT – 3′

Primers and probes were tested for possible interactions to enable use in a multiplex assay. Samples were assayed in duplicate in a 25 µl reaction mixture containing 5 µl of cDNA, TaqMan universal PCR master mix (PE Applied Biosystems), primers (900 nM each), and fluorogenic probes (200 nM) labeled with the 5′ reporter dye 6-carboxy-fluorescein (FAM) and the 3′ quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an ABI Prism 7700 system for 2 min at 50°C to attain optimal AmpErase uracil-*N*-glycosylase activity, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C.

Viral load was determined by the number of amplification cycles needed for a positive PCR test (cycle threshold, CT). Previous studies have shown a highly significant inverse linear relationship between viral load and CT-values.[21] For reference, calibration assays in our laboratory showed that a CT-value of 20 equals $2.9*10^9$ particles/mL and a CT-value of 30 equals $6.9*10^6$ particles/mL. A CT-value of 45 was chosen as cut-off value for sample positivity. Samples were controlled for the presence of possible inhibitors of

the amplification reaction by the indicated internal controls – signals of which had to range within clear-cut intervals. In case of multiple pathogens the pathogen with the lowest CT-value was defined as the primary pathogen.

Statistical analysis

Non-parametric tests were used, because of non-normally distributed data. Severity score was not normally distributed after logarithmic transformation. Mann-Whitney U test, Kruskal-Wallis test, and Fisher's exact test were used to compare severity scores, CT-values and clinical variables between groups. Spearman's correlation was calculated to analyse the correlation between continuous variables. A *P*-value below 0.05 was considered statistically significant. Analyses were performed with the aid of the Statistical Package for the Social Sciences version 15.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

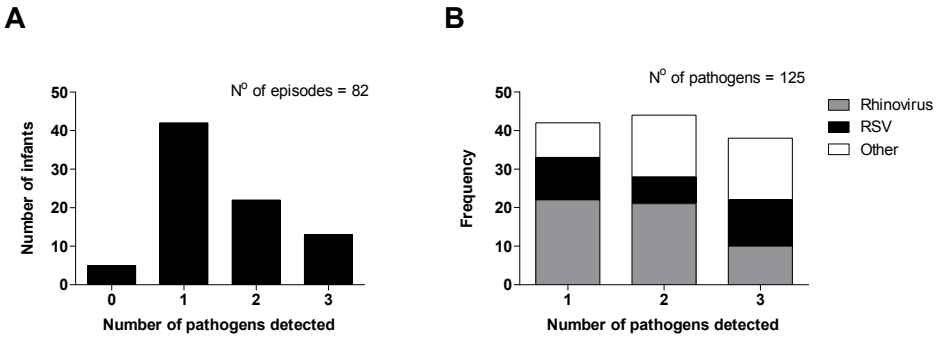
In this prospective study, 82 infants were enrolled during their first respiratory tract infection. All infants were born term, 61% was male, 61% had one or more siblings, and 70% attended day-care (**table 2**). The median age of the infants at their first respiratory tract infection was three months (IQR 2–5) and the median severity score was three (IQR 2–6) (**table 3**). For these 82 respiratory tract infection episodes, real-time PCR detected 125 pathogens: no pathogen was found in five infants, one pathogen in 42 infants and multiple pathogens in 35 infants (**figure 1**). RSV was detected in 30 infants (37%); in 11

Table 2. Baseline characteristics of infants with primary respiratory tract infection (*n* = 82)

Item	Value
Infant	
Gestational age, wk	39.9 (39.3–40.6)
Birth weight, kg	3.6 (3.3–4.0)
Apgar score, 5 min	10 (10-10)
Male gender	50/82 (61)
Breastfed	48/81 (59)
Parents / environment	
Siblings in household	50/82 (61)
Parental smoking	15/82 (18)
Parental atopy	37/80 (46)
Day-care attendance	57/81 (70)

Wk, weeks. Values represent median (interquartile range) or frequency (percentage). Data on breastfeeding, parental atopy, and day-care attendance were missing from 1, 2, and 1 infants, respectively.

Figure 1. Frequency of number of pathogens detected during primary respiratory tract infection



(A) Based on the total of 82 episodes. (B) Based on the total of 125 pathogens detected. Detection frequencies of the most prevalent viruses were: Rhinovirus 53 (65%), respiratory syncytial virus (RSV) 30 (37%), coronovirus 12 (15%), parainfluenzavirus 2/4 8 (10%), bocavirus 7 (9%), adenovirus 6 (7%).

Table 3. Clinical characteristics of infants with primary respiratory tract infection

Item	All infants *n*=82	No RSV infection *n*=52	RSV coinfection[a] *n*=14	RSV as the primary pathogen[a] *n*=16	*P*-value
Age, mo	3.3 (2.2-4.6)	3.0 (1.9-4.4)	3.8 (2.6-5.6)	3.9 (3.1-4.7)	.04
Duration of illness, d	3 (3-4)	3 (3-4)	3 (3-4)	3 (3-4)	.86
Wheeze	5 (6)	4 (8)	0 (0)	1 (6)	.57
Fever	16 (20)	8 (15)	1 (7)	7 (44)	.02
Severity score	3 (2-6)	3 (2-6)	3 (2-4)	5 (4-11)	.007

RSV, respiratory syncytial virus; mo, months; d, days. Values represent median (interquartile range) or frequency (percentage). Comparison between the three groups by Kruskal-Wallis test and Fisher's exact test, as appropriate. [a]In case of multiple infection the pathogen with the lowest CT-value was defined as the primary pathogen.

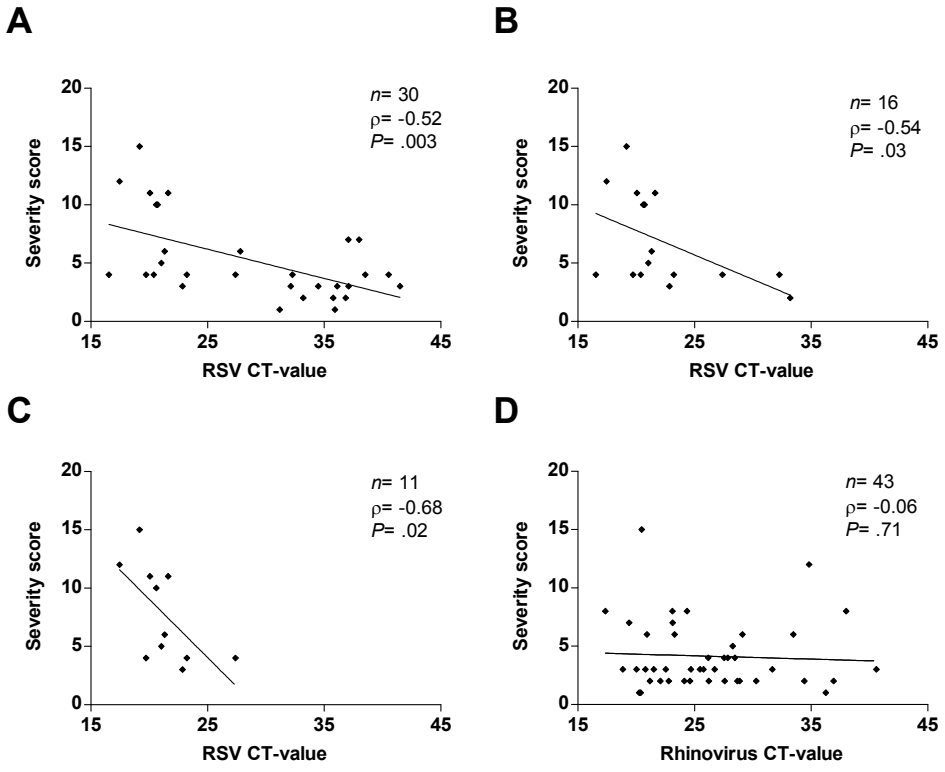
(13%) RSV was the single pathogen. The other 19 infants had aspirates that in addition to RSV contained Rhinovirus ($n=17$), Coronavirus (5), Bocavirus (3) or Parainfluenzavirus 2/4 (3), *Mycoplasma pneumoniae* (1), *Chlamydia pneumoniae* (1) or hMPV (1).

The median age of infants with RSV as the primary pathogen was four months (IQR 3–5); the median severity score was five (IQR 4–11, **table 3**). The severity score was higher in infants with RSV as the primary pathogen (median 5, IQR 4-11) than in infants with RSV co-infection in which another pathogen was the primary pathogen (median 3, IQR 2-4, $P=.007$). In addition, RSV CT-values were lower in infants with RSV as the primary pathogen (median 21, IQR 20-23) than in infants with RSV co-infection (median 36, IQR 34-38, $P<.001$). In infants with RSV being the primary pathogen, there was no association between the duration of illness and the RSV CT-value ($\rho=0.16$, $P=.56$), age and severity score ($\rho=-0.14$, $P=.61$), or any of the baseline characteristics and severity score (all $P>.05$, data not shown).

Correlation between viral load and disease severity

A positive correlation between RSV viral load and disease severity was observed in infants with RSV in their aspirate during first respiratory tract infection (CT-value and severity score ρ = –0.52, *n* = 30, *P* = .003, **figure 2A**). Similar results were found in the 16 cases in which RSV was the primary pathogen (**figure 2B**). In infants in which RSV was the only detected pathogen, the correlation between RSV CT-value and disease severity was even stronger (ρ = –0.68, *n* = 11, *P* = .02, **figure 2C**), while there was no correlation in the remaining 19 infants (ρ = –0.06, *P* = .81, not shown). No association was found between rhinovirus CT-value and disease severity in infants with rhinovirus as the primary pathogen (*n* = 43, ρ = –0.06, *P* = 0.71, **figure 2D**) or in infants in which rhinovirus was the only detected pathogen (*n* = 22, ρ = –0.23, *P* = .31, not shown).

Figure 2. Relation between cycle threshold value and severity score during primary respiratory tract infection



(A) Correlation of RSV cycle threshold (CT)-value and severity score during primary respiratory tract infection, Spearman's ρ. (B) Correlation of RSV CT-value and severity score in aspirates in children with RSV as the primary pathogen, Spearman's ρ. (C) Correlation of RSV CT-value and severity score in single RSV aspirates, Spearman's ρ. (D) Correlation of rhinovirus CT-value and severity score in aspirates in children with rhinovirus as the primary pathogen, Spearman's ρ.

DISCUSSION

In this prospective birth cohort study, it was found that disease severity during primary RSV infection in the community in previously healthy infants is associated positively with viral load. In infants with RSV as the primary pathogen, viral load was high and moderately related to disease severity.

RSV viral load was found to determine disease severity, which is consistent with conclusions drawn by others stating that RSV disease severity in children treated in hospital depends on viral load.[13-15] Studies that did not find this relation may have been confounded by the presence of risk factors such as cardiopulmonary disease, by RSV immunization, or by the occurrence of co-infections, since none of the before-mentioned studies considered co-pathogens during RSV infection.[16,17] In the current study, two or more pathogens were found in 43% of the first respiratory tract infections in previously healthy children, strongly suggesting asymptomatic acquisition of respiratory viruses in early infancy. However, the possibility that a previous mild respiratory tract infection was not noticed by the parents or not reported to the researchers cannot be excluded. The correlation between disease severity and viral load was highest in case of single RSV infection and was absent in RSV coinfections. Infants with RSV as the primary pathogen infection were more severely ill. Rhinovirus disease severity was not correlated to viral load or the presence of coinfections. Apparently, the contribution of viral load to disease severity cannot be extrapolated to other viruses, implying distinct pathophysiology of RSV and rhinovirus respiratory tract infection.

The high viral loads found in children with RSV as the primary pathogen in this cohort are in the range of viral loads in infants admitted to hospital with severe RSV lower respiratory tract infection. Similar CT-values (15-25) were found in infants with RSV infection that were ventilated mechanically, using a similar sampling technique (own unpublished data). Therefore, the conclusion must be that viral load is not a dominant or conditional factor in the pathogenesis of RSV infection. However, this study confirms that, even in relatively mild RSV infections, disease severity depends on viral load. Apparently, RSV viral load modifies the severity in all forms of the disease.

In this study, unique clinical and virological data were collected from otherwise healthy children during their first respiratory tract infection in life. Studying respiratory tract infections in the community in a healthy birth cohort has the advantage of a high incidence, homogeneity of participants, absence of comorbidity and a known and short interval between onset of symptoms and sampling. Home visits and a validated severity instrument made it possible to assess disease severity in a reliable manner.[19,20]. In order to prevent selection bias home visits were performed, rather than inviting infants and their parents to the hospital during illnesses. The broad panel of pathogens included in the quantitative PCR analysis enabled accurate determination of the primary pathogen.

Although strong correlations were observed, the study is limited by the relatively small sample size and the absence of data on the immunological response against the RSV infection.

In conclusion, this is the first study showing that disease severity in primary RSV respiratory tract infection in the community in previously healthy infants depends on RSV molecular viral load.

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Chapter 5

Detection of multiple respiratory pathogens during primary respiratory infection: nasal swab versus nasopharyngeal aspirate using real-time polymerase chain reaction

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ABSTRACT

Objectives

In this study, we present the multiple detection of respiratory viruses in infants during primary respiratory illness, investigate the sensitivity of nasal swabs and nasopharyngeal aspirates, and assess whether patient characteristics and viral load played a role in the sensitivity.

Methods

Healthy infants were included at signs of first respiratory tract infection. Paired nasopharyngeal aspirates and nasal swabs were collected. Real-time polymerase chain reaction was carried out for eleven respiratory pathogens.

Results

Paired nasopharyngeal aspirates and nasal swabs were collected in 98 infants. Rhinovirus ($n = 67$) and respiratory syncytial virus (RSV, $n = 39$) were most frequently detected and detection of more than one pathogen occurred in half of the infants. The sensitivity of the nasal swab was lower than the nasopharyngeal aspirate in particular for RSV (51% versus 100%) and rhinovirus (75% versus 97%). Sensitivity of the nasal swab was strongly determined by the cycle threshold value ($P < .001$). Sensitivity of the swab for RSV, but not rhinovirus, was 100% in children with severe symptoms (score ≥11).

Conclusions

It is concluded that for community based studies and surveillance purposes, the nasal swab can be used, though the sensitivity is lower than the aspirate, in particular for the detection of mild cases of RSV infection.

INTRODUCTION

Respiratory viruses are a common cause of illness in children, in particular during their first years of life and may lead to more severe morbidity and hospitalisation.[1-4] Different types of specimen are available for viral diagnosis. The nasopharyngeal aspirate (NPA) has been considered the best sampling technique, but is more invasive and results in significantly more distress of the infant than a nasal swab (NS).[5] A number of studies have compared the sensitivity of NPA with nasopharyngeal swabs,[6] nose-throat swabs[7] and NS.[8-11] Generally, conventional techniques such as viral culture and antigen detection methods were used. The use of real-time polymerase chain reaction (PCR) may overcome differences in sensitivity for respiratory viruses as a result of specimen type.[12]

Limited data is available on the comparison of these sampling methods combined with real-time PCR. It was observed that nose-throat swabs are a less invasive diagnostic technique with adequate sensitivity for use in outpatient and large community-based settings in children.[13] However, no tests were performed for rhinovirus, while this virus commonly infects infants.[14] The aim of this study was to present the detection of common respiratory pathogens in infants during primary respiratory illness, to investigate the sensitivity of NS and NPA, and assess the role of patient characteristics and viral load in the sensitivity of either sampling method.

METHODS

Study Cohort

The study is part of the Netherlands Amnion Fluid Study of the University Medical Center Utrecht (UMCU), The Netherlands. Healthy infants were included at birth, and were at risk for primary respiratory infection until the age of one year. The data collection and episode sampling stopped one year after birth. From April 2006 to April 2008, including two winter seasons, paired NPA and NS specimens were obtained from 98 infants. Parents were instructed to notify the clinical staff within 24 hours after onset of symptoms. Clinical staff visited the child within 36 hours and the history of illness was taken by a standardized questionnaire. Symptoms were scored, according to Gern,[15] with points presented in parentheses: fever (>38°C) (1); cough, mild (1), moderate (2), severe (3); rhinorrhea, mild (1), moderate to severe (2); hoarseness (1); duration of illness >4 days (1); apnoea (3); wheezing (5); retractions (5); tachypnea (5); cyanosis (5). Mild, moderate and severe infection were defined as sum scores 0 to 4, 5 to 10, and 11 and higher, respectively. Specially trained clinical staff obtained paired NS and NPA. The institutional review board of the UMCU approved the study protocol and written informed consent was obtained from the parents of all participating children.

Collection of specimens

The NPA was obtained by use of an infant mucus extractor (Vygon). Both nostrils were suctioned. In addition a NS was collected, samples were collected from one nostril and one from the hard palate using separate cotton-tipped swabs. The two swabs were then inserted in one vial containing 2 mL of virus transport medium (universal transport medium).

Real-time PCR

Semi-quantitative real-time PCR was conducted on both NS and NPA for RSV, rhinovirus, human metapneumovirus, adenovirus, coronavirus, influenza, parainfluenza virus type (type 1,3), parainfluenza (type 2,4), bocavirus, *Mycplasma pneumoniae*, and *Chlamydia pneumoniae*. Nucleic acids were extracted using the QIAamp DSP virus kit (QIAGEN, Valencia, CA). Each sample was eluted in 200 µl buffer. cDNA was synthesized by using MultiScribe reverse transcriptase (RT) and random hexamers (both from Applied Biosystems, Foster City, CA, USA).[16] Each 100 µl reaction mixture contained 60 µl cDNA mix and 40 µl of eluted RNA. After incubation for 10 minutes at 25°C, RT was carried out for 30 minutes at 48°C, followed by RT inactivation for 5 minutes at 95°C.[16] Samples were assayed in a 50 µl reaction mixture containing 20 µl (c)DNA, and 30 µl mix of the forward and reverse primers and probes. All samples had been spiked before extraction with an internal control virus (murine encephalomyocarditis virus [RNA] and phocine herpes virus [DNA]). The amplification and detection were performed by use of the ABI Prism 7700 sequence-detection system; 2 min at 50°C to acquire optimal AmpErase UNG activity and 10 min at 95°C to activate AmpliTaq Gold DNA polymerase, followed by 45 cycles of 15s at 95°C and 1 min at 60°C.[17] Primers and probes for real-time PCR detection of RSV, influenza virus, para-influenza virus and adenovirus are available by Van de Pol et al[18] and real-time PCR were performed as described previously.[19-21]

Statistical analysis

Similar to previous studies, a consensus standard was used to assess sensitivity of each testing method: a positive result in either NPA or NS was considered as gold standard for presence of a pathogen and used to calculate the sensitivity of the NPA and NS for detection of the respiratory pathogens. X^2 test was used and a logistic regression analysis was performed. The outcome variable was defined as the sample being positive. Variables inserted in the model were age, gender, symptom score, and multiple virus detection. Statistical significance was concluded if the *P*-value was < .05. The statistical analyses were performed in STATA 10.0 (StataCorp LP, TX USA).

RESULTS

A total of 163 respiratory pathogens were identified in 94 children. The majority of children (73.5%) were ill for less than 4 four days at the time of sampling. The median age at primary infection was 104 days (range 33-269) and the median score of symptoms was 3, indicating a mild illness (**table 1**). In all children with an illness of five days or longer, one or more pathogen was detected.

Table 1. Characteristics of infants during first airway infection

Characteristics		All infants (*n*= 98)	Rhinovirus detected (*n*= 67)	RSV detected (*n*= 39)
Median age in days (range)		104 (33-269)	99 (33-269)	115 (51-269)
Median days of illness at time sampling (range)		3.5 (2-31)[c]	4 (2-16)	4 (2-12)
Male		58%	60%	62%
Symptoms				
Rhinorrhea	None	15%	15%	18%
	Mild	55%	58%	46%
	Moderate-severe	30%	27%	36%
Cough	None	17%	18%	5%
	Mild	36%	46%	28%
	Moderate	37%	30%	46%
	Severe	10%	6%	21%
Wheezing[a]		8%	6%	10%
Fever >38°C		17%	12%	23%
Hoarseness		28%	22%	31%
Apnea[b]		3%	2%	5%
Cyanosis		1%	0%	3%
Retractions		6%	3%	5%
Tachypnea		20%	16%	28%
Median sum score (range)		3 (0-25)	3 (0-15)	4 (1-25)
	0-4	64%	72%	56%
	5-10	25%	22%	26%
	>10	11%	6%	18%

[a] reported by parents; [b] *n* = 97, [c] duration of illness: IQR = 3-5
Values represent percentages, unless indicated otherwise.

Multiple pathogens in half of the children

In 49 children (50%) one pathogen was detected: 29 rhinovirus, 11 RSV, 4 coronavirus, 2 hMPV, 2 parainfluenza virus type 2 and 4 and 1 bocavirus. In twenty-five children two pathogens were detected, of which 24 (96%) were rhinovirus with RSV. In 20 children more than two pathogens were detected during the first episode of respiratory symptoms (three pathogens: $n = 17$; four pathogens: $n = 2$; five pathogens: $n = 1$). Co-infection rates by pathogen were: rhinovirus (57%), hMPV (60%), RSV (72%), coronavirus (71%) and bocavirus (91%).

Sensitivity of nasal swab is lower than the aspirate for RSV and rhinovirus

Rhinovirus was found most frequently ($n = 67$), followed by RSV ($n = 39$) and coronavirus ($n = 14$, **table 2**). No influenza viruses and parainfluenza type 1 and 3 viruses were detected. Sensitivity for detecting any pathogen of the NPA was 92% ($CI_{95\%}$ 86.7-95.7), whereas the sensitivity of the NS was lower at 67% ($CI_{95\%}$ 59.1-74.0). For the detection of RSV and rhinovirus the sensitivity of the NS was lower than the NPA (**table 2**).

Table 2. Detection of respiratory pathogens and the sensitivity by sampling method

				NPA		NS	
Respiratory pathogen	**NPA**	**NPS**	**Total**	**Sensitivity**	**95%CI[a]**	**Sensitivity**	**95%CI[a]**
Rhinovirus	65	50	67	97%	89.6-99.6	75%	62.5-84.4
RSV	39	20	39	100%	91.0-100	51%	34.8-67.6
Coronavirus	13	10	14	93%	66.1-99.8	71%	41.9-91.6
Bocavirus	8	7	11	73%	39.0-94.0	64%	30.8-89.1
Adenovirus	9	6	11	82%	48.2-97.7	55%	23.4-83.3
Parainfluenza type 2 and 4	9	9	11	82%	48.2-97.7	82%	48.2-97.7
hMPV	4	3	5	80%	28.4-99.5	60%	14.7-94.7
Mycoplasma Pneumoniae	2	2	3	67%	9.4-99.2	67%	9.4-99.2
Chlamydia Pneumoniae	1	2	2	50%	1.2-98.7	100%	15.8-100
Total	**150**	**109**	**163**	**92%**	**86.7-95.7**	**67%**	**59.1-74.0**

RT-PCR: real-time polymerase chain reaction, NPA: nasopharyngeal aspirate, NS: nasal swab, RSV: respiratory syncytial virus, hMPV: human metapneumovirus, CI: Confidence interval. [a]One-sided 97.5% confidence interval was used in case sensitivity was 100%.

Sensitivity of nasal swab depends on viral load

Sensitivity values of the NPA and NS were investigated in more detail for rhinovirus and RSV (**table 3, figure 1**). For children with a low symptom score the sensitivity of the NS was lower than the NPA. The NS had a lower sensitivity than the NPA for the 30-40 CT values. To assess whether the sensitivity of the NS differed by age group, gender, multiple pathogens, symptom score and CT-value, X^2 tests were performed. The sensitivity of the NS for detection of RSV was related to the symptom score ($P = .001$), and the sensitivity of the NS was related to the CT values for both RSV and rhinovirus ($P < .001$).

Table 3. Sensitivity of the NPA and NS for the detection of rhinovirus and RSV presented by age group, gender, symptom score, presence of multiple pathogens and CT-value of the NPA

Respiratory pathogen	subgroups	NPA	NS	Total	NPA		NS	
		(*n*)	(*n*)		Sensitivity	95% CI[a]	Sensitivity	95% CI[a]
Rhinovirus								
Age	1-3 months	28	24	28	100%	87.7-100	86%	67.3-96.0
	3-6 months	30	22	32	94%	79.2-99.2	69%	50.0-83.9
	6-12 months	7	4	7	100%	59.0-100	57%	18.4-90.1
Gender	Boy	38	32	40	95%	83.1-99.4	80%	64.4-90.0
	Girl	27	18	27	100%	82.1-100	67%	46.0-83.5
Symptom score	0 - 4	46	36	48	96%	85.7-99.5	75%	60.4-86.4
	5 - 10	15	11	15	100%	78.2-100	73%	44.9-92.2
	≥ 11	4	3	4	100%	39.8-100	75%	19.4-99.4
Multiple pathogen	No	29	25	29	100%	88.1-100	86%	68.3-96.1
	Yes	36	25	38	95%	82.3-99.4	66%	48.7-80.4
CT NPA[b]	0-20	3	3	3	100%	29.2-100	100%	29.2-100
	20-25	22	22	22	100%	84.6-100	100%	84.6-100
	25-30	17	15	17	100%	80.4-100	88%	63.6-98.5
	30-35	10	6	10	100%	69.2-100	60%	26.2-87.8
	35-40	10	2	10	100%	69.2-100	20%	2.5-55.6
	40-45	3	0	3	100%	2.9-100	0%	0-70.8
RSV								
Age	1-3 months	12	7	12	100%	73.5-100	58%	27.7-84.8
	3-6 months	20	11	20	100%	82.3-100	55%	31.5-77.0
	6-12 months	7	2	7	100%	59.0-100	29%	7.6-64.8
Gender	Boy	24	11	24	100%	85.8-100	46%	25.6-67.2
	Girl	15	9	15	100%	78.2-100	60%	32.3-83.4
Symptom score	0 - 4	22	8	22	100%	84.6-100	36%	17.2-59.3
	5 - 10	10	5	10	100%	69.2-100	50%	18.7-81.3
	≥ 11	7	7	7	100%	59.0-100	100%	59.0-100

Table 3 (continued)

Respiratory pathogen	subgroups	NPA	NS	Total	NPA		NS	
		(*n*)	(*n*)		Sensitivity	95% CI[a]	Sensitivity	95% CI[a]
Multiple pathogen	No	11	10	11	100%	71.5-100	91%	58.7-99.8
	Yes	28	10	28	100%	87.7-100	36%	18.6-56.0
CT NPA[b]	0-20	4	4	4	100%	39.8-100	100%	39.8-100
	20-25	11	10	11	100%	71.5-100	91%	58.7-99.8
	25-30	4	4	4	100%	39.8-100	100%	39.8-100
	30-35	7	1	7	100%	59.0-100	14%	0.4-57.9
	35-40	10	0	10	100%	69.2-100	0%	0-30.8
	40-45	3	1	3	100%	29.2-100	33%	0.8-90.6

NPA: nasopharyngeal aspirate; NS: nasal swab; RSV: respiratory syncytial virus; CT: Cycle threshold value.
[a]One-sided 97.5% confidence interval was used in case sensitivity was 100%.
[b]The CT value of the NPA was used as a reference to compare with NS, therefore the sensitivity of the NPA is 100% for all categories.

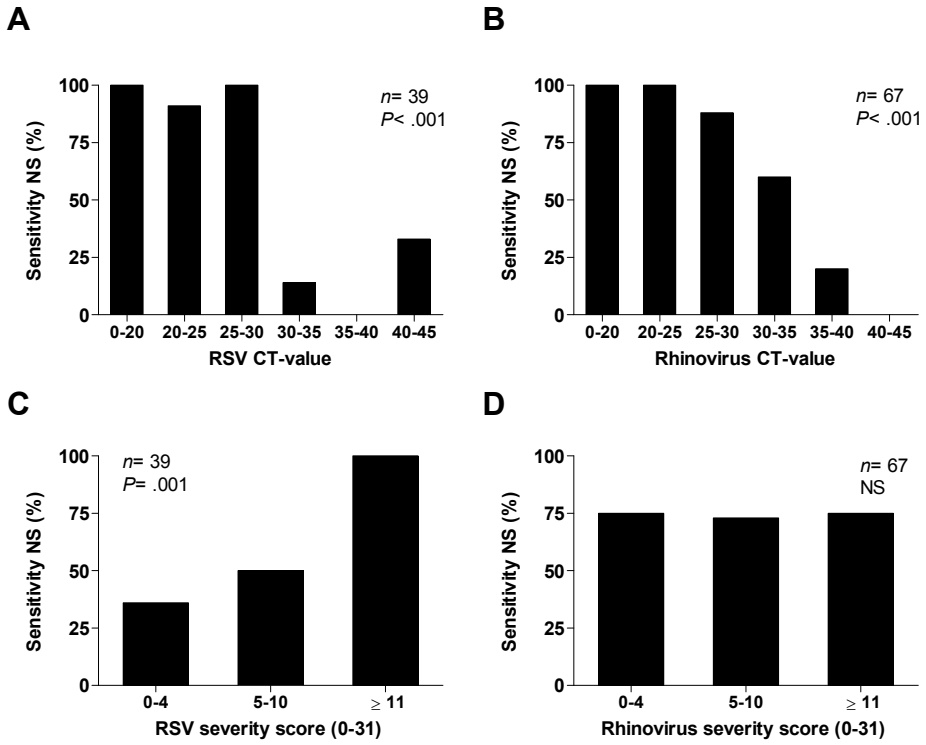
In the logistic regression analysis age and gender did not significantly predict the detection of RSV or rhinovirus. Symptom score predicted RSV detection in both the NPA (OR: 1.21; $CI_{95\%}$ 1.07-1.39) and the NS (OR: 1.28; $CI_{95\%}$ 1.12-1.48), while an inverse relationship was observed for symptom score and rhinovirus detection in the two samples (OR: 0.87; $CI_{95\%}$ 0.78-0.98). The presence of more than one pathogen predicted RSV (OR: 8.98; $CI_{95\%}$ 3.03-26.7) or rhinovirus detection (OR: 3.66 $CI_{95\%}$ 1.33-10.08) in the NPA. When the same analysis was performed as a backwards regression with $P < .20$, results did not change.

DISCUSSION

This study presents that the proportion of infants where a respiratory pathogen detected was high (96%), and co-infections were common. In twenty children more than two pathogens were detected during the first episode of respiratory symptoms. Co-infections were observed frequently for RSV (72%), coronovirus (71%) and bocavirus (91%) in particular.

High rates of co-infection in young children have been described recently for childhood pneumonia in particular in children aged less than 12 months,[22] and in children hospitalized with acute respiratory tract infection.[23,24] The most frequently detected virus was RSV followed by human bocavirus, and rhinovirus.[25,26] A common combination has been reported to be RSV and bocavirus.[27] Even though high occurrence of co-infections

Figure 1. Nasal swab sensitivity for RSV and rhinovirus infections according to CT value and disease severity



Sensitivity of nasal swab according to CT value (A+B) and disease severity (C+D), for RSV infections (A+C) and rhinovirus infections (B+D).

has been reported, ranging from 14-16%[28,29] to 27%,[30] our study presents an even higher rate of co-infection. Human bocavirus is a newly identified virus and has been detected in respiratory tract secretions in patients with acute respiratory symptoms in 2 to 19% of the samples.[31] Co-infection with another virus has been observed in 40% of the bocavirus-positive children.[32] The frequent associations of bocavirus with other respiratory viruses might be explained by the persistence of bocavirus in the respiratory tract.[33]

Furthermore, we investigated the sensitivity of NPA and NS tested by a real-time PCR method. The sensitivity of the NPA was 92%, while for the NS this was 67%. In particular for the detection of rhinovirus and RSV, the NS had a lower sensitivity (75% and 51%, respectively) compared to the NPA (97% and 100%). Sensitivity of the NS for RSV was 100% for children with high symptom scores. For both RSV and rhinovirus, viral load, indicated by the CT value, was the major determinant of sensitivity of NS in a dose-dependent fashion. Symptom score predicted RSV detection in both the NPA and the NS, while an inverse relationship was observed for symptom score and rhinovirus detection in the two samples.

The use of a swab has been considered as a suitable replacement in community-based research or epidemiological studies. The major advantage of a swab is that collection is less painful, and more convenient than an aspirate as no additional devices are needed.[34] These factors may outweigh some reduction in sensitivity. The advantage of molecular methods in the detection of respiratory viruses has been reported,[35,36] and Lambert et al. reported that using these methods seemed to overcome the previously observed sensitivity reduction when less invasive specimens were combined with the conventional laboratory methods.[37] With the recently developed flocked swabs, sensitivity is even further improved and the flocked swabs have the advantage of being rapid, and less traumatic for paediatric patients.[38] However, the sensitivity of the flocked swab in outpatient respiratory tract infection may be lower than in hospitalized patients. Further studies are required considering different types of swabs and patient populations, and should test for a broad spectrum of respiratory pathogens.

Our findings demonstrated a lower sensitivity of the NS, in particular for RSV. Similar results were reported in other studies where conventional, non-amplification based-methods were used.[39,40] Lambert et al. did not test for rhinovirus, and this was the most frequently detected virus in our study and elsewhere.[41,42] No influenza detections were found in our study. This is not explained by sampling bias, because most swabs were taken during the winter season, during which both RSV and influenza had their peak incidence. A possible reason may be related to the patient population and the small population size. Another study showed similar results with rhinovirus and RSV being most frequently detected.[43]

There were a number of limitations of this study. First, one limitation was the timing of sampling. For five cases sampling occurred 10 days after the onset of illness. Since viral shedding of RSV is highest between days 0 and 6 sampling should preferably occur in this period.[44] The high proportion of positive samples, however, indicates this effect was not a major drawback of this study. Second, in this study pain and discomfort of the collection of the samples was not assessed, but other studies provided reference for this.[45] Finally, it is unknown whether the order of obtaining the specimens may have resulted in a lower detection rate in NS. It is possible that by suctioning both nostrils for the NPA, the secretions with virus or viral nucleic acids were reduced. One of our findings was that sensitivity of the NS dropped with lower symptoms score and higher CT values. The lower sensitivity of the swab compared to the NPA may be partly biased by the specimen collection.

RSV and rhinovirus were commonly detected in infants during primary respiratory infection, and co-infections occurred in about half of the children. The sensitivity of NPA was higher than NS, in particular for detection of RSV and rhinovirus. Although sensitivity of a method is important, one must also take into account the advantages that different sampling methods offer. The great advantage of the NS is that this method can

be performed in outpatient settings without needing special devices, is less costly and causes less distress in the patient than the NPA. Although there is a reduction in sensitivity for RSV particularly in infants with mild symptoms, the NS is convenient for sampling patients in community studies and can be used for surveillance purposes.

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Chapter 6

The association between intrauterine inflammation and spontaneous vaginal delivery at term: a cross-sectional study

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ABSTRACT

Background

Different factors contribute to the onset of labor at term. In animal models onset of labor is characterized by an inflammatory response. The role of intra-uterine inflammation, although implicated in preterm birth, is not yet established in human term labor. We hypothesized that intra-uterine inflammation at term is associated with spontaneous onset of labor.

Methods

In two large urban hospitals in the Netherlands, a cross-sectional study of spontaneous onset term vaginal deliveries and elective caesarean sections (CS), without signs of labor, was carried out. Placentas and amniotic fluid samples were collected during labor and/or at delivery. Histological signs of placenta inflammation were determined. Amniotic fluid pro-inflammatory cytokine concentrations were measured using ELISA.

Results

A total of 375 women were included. In term vaginal deliveries, more signs of intra-uterine inflammation were found than in elective CS: the prevalence of chorioamnionitis was higher (18 versus 4%, $P = .02$) and amniotic fluid concentration of IL-6 was higher (3.1 versus 0.37 ng/mL, $P < .001$). Similar results were obtained for IL-8 (10.93 versus 0.96 ng/mL, $P < .001$) and percentage of detectable TNF-α (50 versus 4%, $P < .001$).

Conclusions

This large cross-sectional study shows that spontaneous term delivery is characterized by histopathological signs of placenta inflammation and increased amniotic fluid pro-inflammatory cytokines.

INTRODUCTION

Normal human delivery is initiated by the spontaneous onset of labor at term.[1] The role of inflammation in the onset of labor at term in humans is poorly understood, although it has been implicated in the physiological onset of parturition.[2–5] Inflammation at term comprises the gold standard of histologically proven infiltration of the chorioamniotic tissue, but also presence of inflammatory mediators in the amniotic fluid.[6] Microbial invasion of the amniotic space is found in up to 19% of term pregnancies.[7] However, infection contributes only to a small proportion of cases with signs of chorioamnionitis; amniotic fluid appears sterile in the majority of these cases. In general, histopathological inflammation, including chorioamnionitis develops sub-clinically.

The clinical relevance of sterile inflammation in normal delivery is intriguing. Inflammation may provide a danger signal by the innate immune system, resulting in the onset of labor.[8] Pro-inflammatory molecules stimulate the maternal myometrial cells and induce cerival ripening.[1] There is evidence in literature showing that sterile inflammation at term is indeed associated with the onset of labor in the animal model.[9–12] The relevance of these findings for humans is not clear, since mechanisms of parturition are species-dependent. Human data showing that inflammation is associated with term delivery are rare.[6,7,13,14] In the current study we aimed to establish the relationship between peripartum signs of inflammation and spontaneous onset of term delivery in humans. We hypothesized that intra-uterine inflammation at term is associated with spontaneous onset of labor. We compared the inflammatory response in normal vaginal deliveries with elective caesarean sections (CS) at term. To our knowledge, this is the first large cross-sectional study of normal term deliveries integrating both histological studies and amniotic fluid cytokine measurements, showing extensive inflammation in placenta tissue and in amniotic fluid in women with spontaneous onset of labor.

METHODS

Study population

A cross-sectional study was conducted in one secondary and one tertiary hospital in Utrecht, The Netherlands, between January 2006 and November 2007. Women delivering vaginally at term after an uncomplicated pregnancy and with spontaneous onset of labor were recruited. In addition, women who delivered by elective CS were recruited. Elective CS was defined as a planned CS, performed in the absence of signs of labor or rupture of membranes. Women were excluded if they did not comprehend the Dutch language, if the gestational age was below 37 weeks, or if the infant had major congenital organ abnormalities (such as spina bifida and congenital heart disease). Recruitment was performed

by one of the researchers. Shortly before or after delivery, parents were informed about the study. Collected samples of the placenta and amniotic fluid were only analysed when parents consented. The study was approved by the ethical review boards of both institutions and all parents provided written informed consent for study participation.

Clinical characteristics

Clinical characteristics of the mother (maternal age, gestational age, parity, meconium stained amniotic fluid, fever during delivery, assisted delivery, antenatal corticosteroid administration) and her child (birth weight, gender, Apgar score five minutes after birth, newborn infection) were collected. Gestational age was determined with the use of clinical history and the results of the earliest ultrasound examination. Secundary CS was defined as a CS performed after the onset of labor. Maternal fever during delivery was defined as a temperature of 38.0°C or higher, as measured during labor and delivery. Newborn infection was defined as a strongly suspected or proven early onset neonatal sepsis, within 48 hours after birth.

Collection of samples

Placentas were stored at +4°C and processed within 72 hours. In case of vaginal delivery, amniotic fluid was collected vaginally, in a non-sterile manner, at the moment of artificial rupture of membranes or after spontaneous rupture of membranes. When spontaneous rupture of membranes occurred outside the hospital or unwitnessed, the collection of amniotic fluid was delayed until the moment during delivery on which a large volume of amniotic fluid spontaneously passed the vagina. During elective CS, amniotic fluid was collected with a syringe directly after incision of the membranes. Amniotic fluid samples were stored at +4°C and processed within 72 hours.

Placenta histology

Placenta examination was performed by an experienced pathologist (PN).[15] The pathologist was masked for clinical information. Two sections of umbilical cord, at the fetal and placental side, a membrane roll, one sample from the umbilical cord insertion, and three slides of normal placental parenchyma, including both decidua and chorionic plate, were collected and stained with standard haematoxylin and eosin. Histological chorioamnionitis was diagnosed based on the presence of polymorphonuclear cells (neutrophilic granulocytes) in the chorionic plate or the extraplacental membranes. Funisitis was diagnosed in the presence of neutrophils in the wall of the umbilical vein and / or arteries or Wharton's jelly and villitis was diagnosed as an infiltration of lymphocytes and macrophages in the placental villi. In addition to the presence or absence of chorioamnionitis, funisitis or villitis, the severity of inflammation was graded mild, moderate or severe, with slight modifications comparable to the staging and grading system of Redline (**table 1**).[16]

Table 1. Histological classification of chorioamnionitis, funisitis and villitis

Grade	Chorioamnionitis	Funisitis	Villitis
0	No inflammation	No inflammation	No inflammation
0.5	Sporadic PMN in chorionic plate / membranes		
1	Frequent PMN in chorionic plate / membranes	Inflammation present in the wall of 1 vessel (vein)	1 section with 1 focus of chronic inflammation of > 5 villi
2	Invasion of chorionic plate, large infiltrate in chorionic plate/ membranes	Inflammation present in the wall of 2 vessels (vein and artery)	2-3 sections with each 1 focus of chronic inflammation of > 5 villi
3	Same as 2, including micro-abscesses in chorionic plate	Inflammation present in the wall of 3 vessels (vein and 2 arteries)	3 sections with each > 2 foci of chronic inflammation of > 5 villi

PMN polymorphonuclear cells.

Amniotic fluid analysis

After macroscopic assessment, the samples were purified by filtration (70 µl filter, Falcon BD) and centrifugation (1500 RPM, 10 minutes) to remove debris and blood clots. The supernatant was filtered through a 0.2 µL sterilization filter (Falcon, BD) to remove leukocytes and other contaminants. The sterile, acellular fraction of amniotic fluid was stored at –80°C until further analysis. Enzyme linked immunosorbent assays (ELISA) were used to determine interleukin-6 (IL-6), IL-8 and tumor necrosis factor-α (TNF-α) concentrations (CLB, Sanquin, Amsterdam, The Netherlands). The lower limits of detection for IL-6, IL-8 and TNF-α were 0.006 ng/mL, 0.01 ng/mL and 0.014 ng/mL, respectively. IL-6 and IL-8 concentrations were never undetectable. Amniotic fluid cellular composition was not systematically determined.

Amniotic fluid pilot experiments

Several pilot experiments were conducted to validate the methods of amniotic fluid analysis. (a) The influence of storage at +4°C during 72 hours was studied in paired fresh and stored samples. (b) To minimize the effect of aspecific binding proteins, spiking of diluted amniotic fluid with synthetic cytokines was performed to find the optimal dilution for ELISA. (c) Intra-assay and interassay reproducability were determined by comparing the results of duplo amniotic cytokine measurements within and between ELISA assays. (d) The effect of different intervals from labor until amniotic fluid sampling or from sampling until delivery between individuals was studied by collection and analysis of serial samples. (e) The influence of delay of amniotic fluid sampling after spontaneous rupture of membranes was analysed by comparing deliveries with different delay intervals. (f) To study whether transvaginal collection of amniotic fluid affects cytokine concentrations, paired samples were collected transvaginally and using an intra-uterine catheter simultaneously.

Statistical analysis

The association between intra-uterine inflammation and term vaginal delivery was studied. This cross-sectional study was part of a larger birth cohort study, for which a sample size of 500 participants was calculated; a sample size calculation for this study was not performed. Logarithmic transformation was used for amniotic fluid IL-6 and IL-8 concentrations, to provide a normal distribution. TNF-α was dichotomized into detectable and undetectable, because a large proportion of the samples contained undetectable levels of TNF-α. The frequency of chorioamnionitis, funisitis and villitis was compared between term vaginal deliveries and elective CS using the Pearson's χ^2 or Fisher's exact test. The geometric mean amniotic fluid IL-6 and IL-8 concentrations were compared using the Student's T test. The proportion of detectable TNF-α was compared using the Pearson's χ^2 test. Unadjusted odds ratios (OR) of chorioamnionitis, funisitis, villitis, amniotic fluid IL-6, IL-8 and detectable TNF-α for term vaginal delivery were calculated. With multivariable logistic regression analysis, ORs were adjusted for potential confounders, i.e. maternal age, gestational age, parity, meconium-staining. These potential confounders were selected for their biological plausibility and for their correlation to both intra-uterine inflammation and term vaginal delivery. Collinearity between amniotic fluid cytokine concentrations was studied by calculating correlation coefficients. All data were analysed in the Statistical Package for Social Sciences (SPSS) version 15.0.

RESULTS

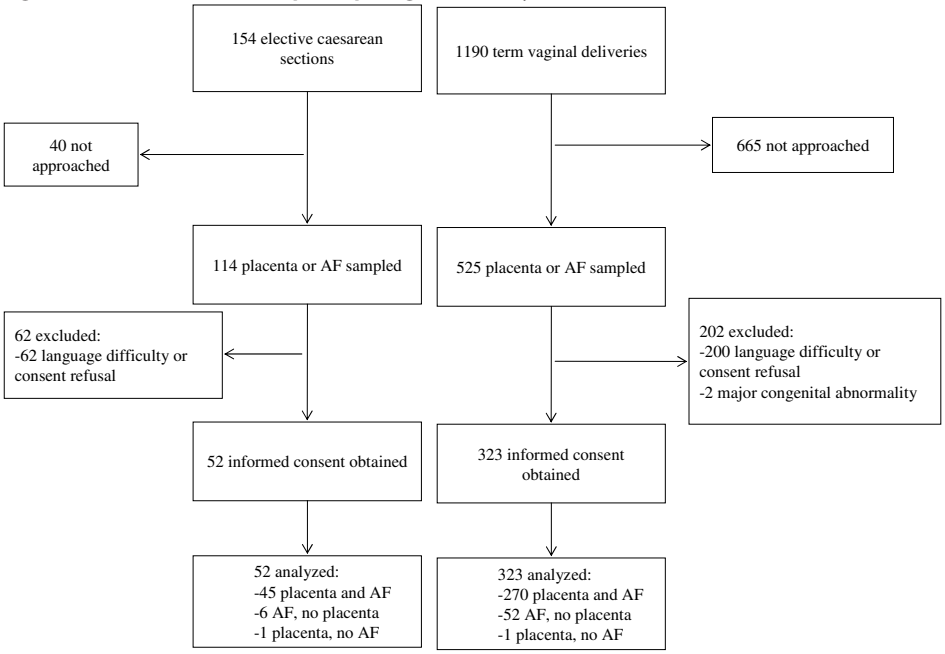
Study population

During the study period, 1190 term vaginal deliveries occurred and 154 elective CS were performed. Informed consent was obtained from 375 mothers (**figure 1**). Clinical characteristics of the excluded mothers and their children were similar to the characteristics of those included (data not shown).

Clinical characteristics

The women with spontaneous onset of labor, were younger, had a higher gestational age and a higher proportion of meconium stained amniotic fluid, as compared to the women in the elective CS group (**table 2**). Maternal fever during labor only occurred in 4 cases (1%). Newborn infection within 48 hours after birth did occur in one child (0.3%). Within the group of women with spontaneous onset of labor, 18 of the 323 women (6%) were delivered by vacuum extraction and another 12 (4%) by secondary CS. No mother reported antenatal corticosteroid administration. The majority of elective CS was performed because of a previous CS in the past obstetric history (18/52 (35%)), breech presentation (17/52 (33%)) or placenta or pelvic pathology, such as placenta previa (10/52 (19%)).

Figure 1. Flow chart of women participating in the study



Amniotic fluid (AF)

Table 2. Clinical characteristics of deliveries

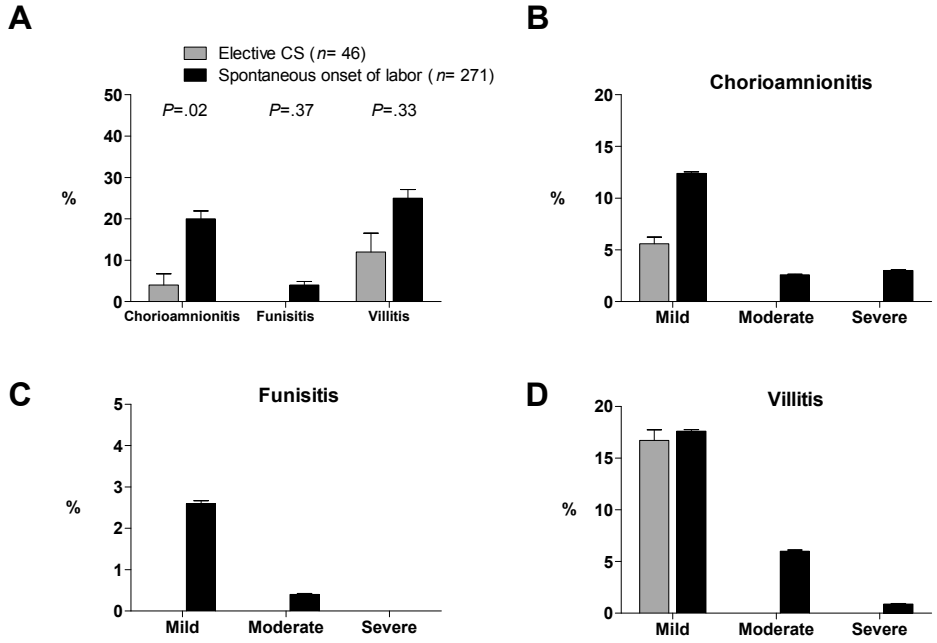
Characteristic	Elective CS (*n*=52)	Spontaneous onset of labor (*n*=323)	*P*-value
Mother			
Age mother (yrs)	34.3 (30.6 – 36.7)	31.9 (28.3 – 34.8)	.03
Gestational age at delivery (wks)	39.0 (38.5 – 39.3)	40.1 (39.3 – 40.9)	<.001
Nulliparity	15 (29%)	143 (44%)	.04
Meconium stained amniotic fluid	0 (0%)	57 (18%)	.001
Fever during delivery	0 (0%)	4 (1%)	.999
Child			
Birth weight (g)	3571 (3278 – 4006)	3580 (3265 - 3835)	.46
Male gender	23 (44%)	178 (55%)	.14
Apgar score <7 after 5 minutes	3 (6%)	1 (0.3%)	.008
Newborn infection	0 (0%)	1 (0.3%)	.999

CS caesarean section, Yrs years, Wks weeks. Values represent frequency (%) or median (interquartile range). Values were compared using the Mann-Whitney U test, the χ^2 test or the Fisher's exact test, as appropriate.

Placenta histology

Placenta histology was established in 317 samples. The remaining 58 placentas were not collected for non-specific reasons. Chorioamnionitis was present in 50 of 271 placentas (18%) in the group of women with spontaneous onset of labor and in 2 of 46 placentas

Figure 2. Prevalence and severity of signs of placenta inflammation



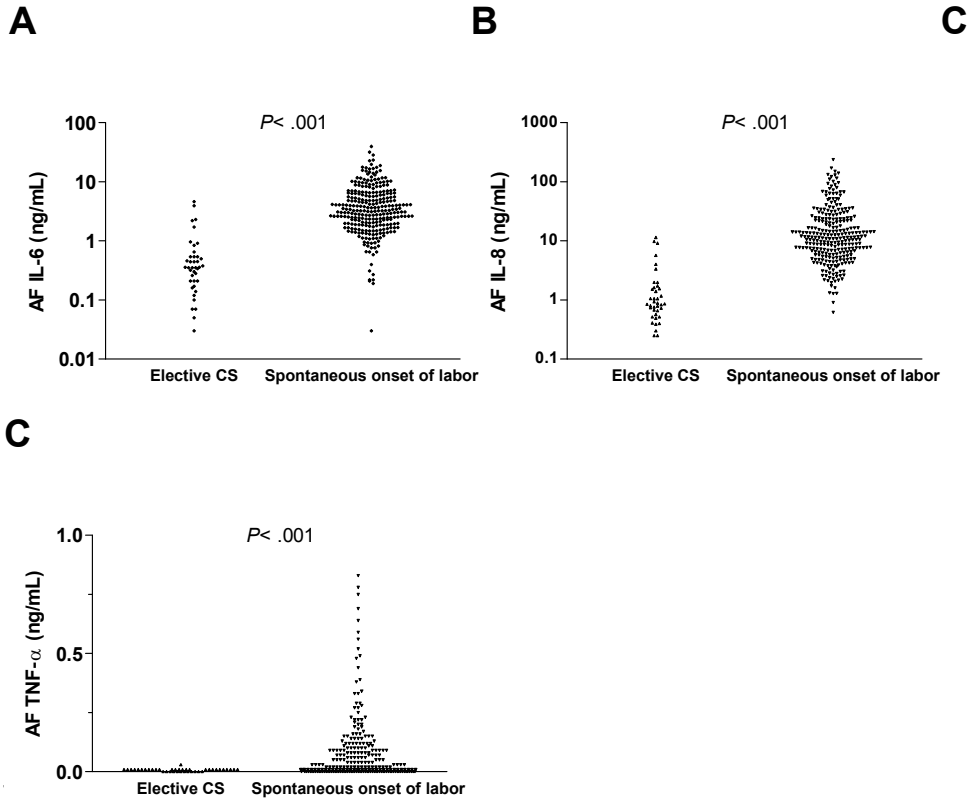
Presence (A) and severity of chorioamnionitis (B), funisitis (C) and villitis (D) of the placenta in elective caesarean sections (CS, $n = 46$) and spontaneous onset of labor ($n = 271$).

(4%) in the elective CS group (difference 14.1%, 95% confidence interval (CI) 6.6–21.6, $P = .02$, **figure 2**). Funisitis was rare in this study, but associated with chorioamnionitis in all cases (fraction funisitis in chorioamnionitis: 9/50 (18%)). Placentas with chorioamnionitis showed villitis in 27% (14/52). The prevalence of villitis was not related to gestational age at delivery. The prevalence of chorioamnionitis was higher in women who delivered for the first time as compared to multiparous women (27% versus 13%, $P = .002$).

Amniotic fluid inflammation

Amniotic fluid cytokines were determined in 373 samples. In the group of women with spontaneous onset of labor, amniotic fluid IL-6 concentrations were higher than in elective CS (3.1 versus 0.37 ng/mL; ratio 8.41, 95% CI 6.15-11.51, $P < .001$, **figure 3**). Similar differences were found for amniotic fluid IL-8 concentrations (10.93 versus 0.96 ng/mL; ratio 11.34, 95% CI 7.90-16.27, $P < .001$) and proportion of detectable TNF-α (50 versus 4%; difference 45.8%, 95% CI 38.1-53.4, $P < .001$). Similar results were found when women with maternal fever and/or instrumental vaginal delivery or secondary CS were excluded from the analysis. Subgroup analysis did not reveal differences in cytokine concentrations for gestational age, meconium-staining or gender (data not shown). IL-6 was higher in women who delivered for the first time as compared to multiparous women (3.97 vs 2.77

Figure 3. Amniotic fluid pro-inflammatory cytokine concentrations



Interleukin-6 (IL-6, A), IL-8 (B) and tumor necrosis factor-α (TNF-α, C) concentration in amniotic fluid samples of elective caesarean sections (CS, $n = 51$) and spontaneous onset of labor ($n = 322$).

ng/mL, $P = .001$). This marginal difference could not explain the contrast between the spontaneous onset of labor group and the elective CS group.

Multivariable analysis of the association between intra-uterine inflammation and term spontaneous onset of labor

Multivariable logistic regression analysis of placenta and amniotic fluid inflammation associated with term vaginal delivery was performed (**table 3**). Crude ORs were adjusted for potential confounders, i.e. maternal age, gestational age, parity and meconium-staining. Chorioamnionitis and villitis were not independently associated with term vaginal delivery. Funisitis was not analysed, because of the low prevalence. Amniotic fluid IL-6, IL-8 and TNF-α were independently positively associated with term vaginal delivery.

Table 3. Multivariable analysis of placenta and amniotic fluid inflammation associated with term vaginal delivery

	Odds ratio (95% CI)	
Factor	Unadjusted	Adjusted[a]
***Placenta* (*n*= 317)**		
Chorioamnionitis	4.98 (1.17 – 21.22)	3.30 (0.73 – 14.81)
Funisitis	NA	NA
Villitis	1.50 (0.67 – 3.38)	1.79 (0.72 – 4.46)
***Amniotic fluid* (*n*= 373)**		
Log IL-6	31.01 (13.37 – 71.94)	31.45 (11.83 – 83.67)
Log IL-8	33.74 (14.17 – 80.33)	26.48 (10.22 – 68.61)
TNF-α detectable	24.20 (5.79 – 101.18)	18.40 (4.32 – 78.33)

OR odds ratio, CI confidence interval, NA not applicable, IL interleukin, TNF-α tumor necrosis factor-α.
[a]Adjusted for potential confounders: maternal age, gestational age, parity, meconium-staining.

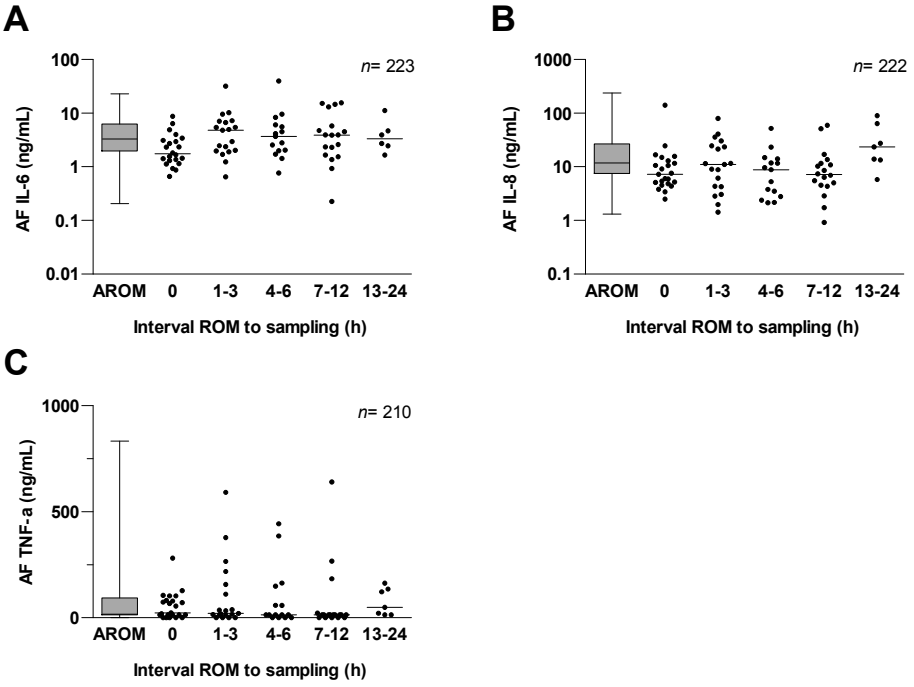
Correlation between placenta and amniotic fluid inflammation

Amniotic fluid IL-6 and IL-8 concentrations were correlated (Pearson's $\rho = 0.61$, $P < .001$), suggesting that these cytokines play a role in the same inflammatory event, but do not have an identical expression pattern. Chorioamnionitis was only weakly correlated with amniotic fluid IL-6, IL-8 and TNF-α (Spearman's $\rho = 0.23$, $P < .001$, $\rho = 0.22$, $P < .001$, and $\rho = 0.17$, $P = .003$, respectively).

Amniotic fluid pilot experiments

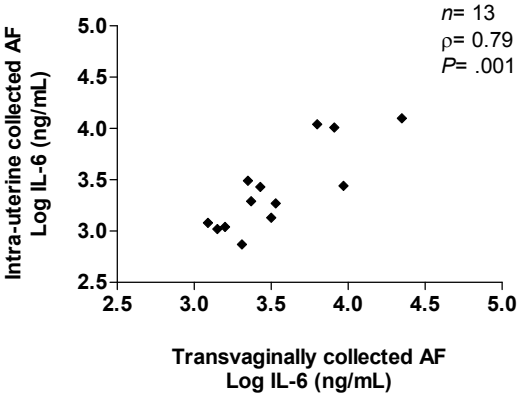
(a) Amniotic fluid cytokine concentrations measured in fresh samples were similar after storage ($n = 4$, Wilcoxon's $P = .72$). (b) Spiking of amniotic fluid with synthetic cytokines showed good reliability of dilution of amniotic fluid ($n = 7$, $\rho = 0.93$, $P = .003$). (c) Amniotic fluid cytokine measurements had a high intra-assay and inter-assay reproducibility (intra-assay duplicates $n = 145$, $\rho = 0.99$, $P < .001$, interassay duplicates $n = 6$, Wilcoxon's $P = .12$). (d) We did not find an association between duration of labor until sampling, or duration from sampling until delivery and cytokine concentrations in amniotic fluid (labor until sampling $n = 229$, $\rho = 0.06$, $P = .36$, sampling until delivery $n = 251$, $\rho = -0.01$, $P = .83$). (e) Delay of sampling after spontaneous rupture of membranes did not result in different amniotic fluid cytokine concentrations (**figure 4**). (f) Amniotic fluid cytokine concentrations in samples simultaneously collected transvaginally and using an intrauterine catheter were similar and highly correlated ($n = 13$, $\rho = 0.79$, $P < .001$, **figure 5**).

Figure 4. Interval between ROM and amniotic fluid sampling in relation to amniotic fluid inflammation



Relation of the interval between spontaneous rupture of membranes (ROM) and sampling and amniotic fluid interleukin-6 (IL-6, A), IL-8 (B), and tumor necrosis factor-α (TNF-α, C) concentrations in women with spontaneous onset of labor. The results of samples collected at artificial ROM (AROM) are shown in a boxplot ($n = 142$). ANOVA with Bonferroni correction did not show differences between the groups shown.

Figure 5. Comparison of amniotic fluid IL-6 between transvaginally collected and intra-uterine collected samples



Amniotic fluid samples were simultaneously collected transvaginally and via an intra-uterine catheter. Amniotic fluid concentrations of IL-6 were compared using Spearman's correlation.

DISCUSSION

In this cross-sectional study of 375 term vaginal deliveries and elective CS, we found that signs of intra-uterine inflammation are associated with spontaneous onset of labor. Histological signs of chorioamnionitis were found in 18% of placentas of term vaginal deliveries, whereas acute inflammation was virtually absent in elective CS. Amniotic fluid IL-6 and IL-8 concentrations and the proportion of deliveries with detectable amniotic fluid TNF-α were approximately tenfold higher in term vaginal deliveries than in elective CS.

Intra-uterine inflammation has been shown to induce preterm labor in a primate model. Intra-amniotic injection of TNF-α and IL-1β rapidly induced onset of labor.[9] The current study suggests that intra-uterine inflammation might play a role in the physiological process of onset of labor at term gestation in humans. Presumably, the contractility, excitability and connectivity of uterine myometrical cells is upregulated upon triggering by amniotic pro-inflammatory cytokines.[17] In addition, cervical ripening and dilatation can be promoted by exposure to increased levels of inflammatory cytokines.[18–20] For example, IL-8 increases activity of collagenase and metalloproteinases 8 and 9, which are required for softening of cervical tissue. Towards the end of the pregnancy in humans, maternal expression of circulating corticotropin-releasing hormone (CRH) increases exponentially, which is accompanied by increased excitablity the myometrium allowing the initiation of contractions.[21] It is conceivable that intra-uterine inflammation has a direct positive effect on these processes that characterize the pre-partum phase.

The origin of intra-uterine inflammation at term is uncertain but might be infectious. An estimate of 18% of women will have microbial invasion of the amniotic cavity at term, increasing to 30% when there is prolonged rupture of the membranes.[6,7,13,14] Indolent pathogens, like *Ureaplasma Urealyticum,* have been associated with the onset of labor.[22–24] Many of these pathogens are not isolated by conventional bacterial cultures.[25,26] Placenta histological examination showed chorioamnionitis and occasionally funisitis, predominantly in the spontaneous onset of labor group. These cases might represent the maternal and fetal response to a microbial invasion of the amniotic cavity. During preterm labor, chorioamnionitis has been associated with increased amniotic fluid inflammatory cytokine concentrations, but not during preterm prelabor rupture of membranes.[27] By contrast, a relation between villitis and the onset of labor is not likely, because of the non-acute character of villitis.[28–30] Moreover, the group of women with spontaneous onset of labor and the elective CS group showed similar villitis prevalences. Inflammation at term could also be of fetal origin. Surfactant protein A (SP-A) is produced by macrophages in the fetal airways. SP-A in the amniotic fluid promotes an inflammatory response in the chorioamniotic membranes. In the mouse model, intra-uterine SP-A administration results in prompt labor.[31] More research is required to establish the relative maternal and fetal contribution to inflammation at term.

The major strengths of this study are that it examined evidence of inflammation in different intra-uterine compartments and the large sample size. Amniotic fluid samples were collected vaginally during active labor. Other studies have collected amniotic fluid by amniocentesis or in pregnant women presenting with preterm contractions.[2,3,7,14,23,25,27,32,33] Furthermore, our sample of women with spontaneous onset of labor essentially consisted of uncomplicated pregnancies (e.g. no use of antenatal steroids) and deliveries (e.g. 90% unassisted physiologic deliveries).

Some possible limitations should also be discussed. The cross-sectional design of data collection does not allow definite conclusions on causality. The inflammation might also be the result of birth process, including maternal stress by labor activity or fetal stress by exposure to contractions during labor and passage through the birth canal.[34] However, the presence of histological signs of inflammation in the placenta suggests that inflammation precedes delivery. The modes of amniotic fluid sampling that were distinct for the two groups studied might have contributed to the differences found. But women with secondary CS after the onset of labor and women who delivered vaginally had similar high levels of amniotic fluid inflammation (e.g. IL-6 3.49 ng/mL versus 3.25 ng/mL, $P = .82$). We excluded the potential bias that vaginal collection of amniotic fluid, as compared to the gold standard of intra-amniotic collection, might have introduced. A delay in sampling of amniotic fluid after spontaneous rupture of membranes did not influence cytokine concentrations. A number of possible neonatal correlations (e.g. tracheal aspirates, cord blood acidosis) were not studied, for essentially this study sample consisted of healthy newborns.

The results of this study may have clinical implications. Better insight in the physiological role of intra-uterine inflammation may eventually result in strategies to intervene in normal labor and delivery, including induction of labor in postterm pregnancies.[20,35] We have considered the potential physiological role of intra-uterine inflammation for both the onset of spontaneous labor at term and the development and maturation of fetal and neonatal airways.[9,32,34,36–39] The candidate role of intra-uterine inflammation as a downstream step of the corticosteroid pathway, which is known to be involved in airway maturation, is intriguing.

In conclusion, human term spontaneous labor and delivery are associated with a high level of intra-uterine inflammation, and intra-uterine inflammation is a consistent finding in all intra-uterine compartments. This large cross-sectional study shows that normal labor at term is characterized by chorioamnionitis in a considerable number of cases and high amniotic fluid concentration of pro-inflammatory cytokines in all cases, providing insight in the pivotal role of intra-uterine inflammation in normal parturition.

Acknowledgements

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Chapter 7

High concentrations of amniotic fluid pro-inflammatory cytokines in healthy neonates are associated with low risk of RSV bronchiolitis

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Submitted for publication

ABSTRACT

Objective

To determine whether high amniotic fluid interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) protect against RSV bronchiolitis in healthy term infants.

Methods

Prospective birth cohort study of healthy term newborns, born after uncomplicated pregnancy. Amniotic fluid was collected during labour. In case of medical attention for respiratory symptoms during the first year of life, a nose-throat swab was taken to establish the presence of respiratory viruses by PCR.

Results

Physician-attended RSV infection was observed in 27 (9.3%) of 292 children at median age 6 months. Amniotic fluid concentrations of IL-8 were higher in children without physician-attended RSV infection than in children with physician-attended RSV infection (11.1 versus 5.5 ng/mL, $P = .002$). Similarly, in children without physician-attended RSV the proportion of detectable amniotic fluid TNF-α was higher (159/265 (60%) versus 8/27 (30%), $P = .002$). Among children with physician-attended RSV infection, amniotic fluid IL-8 was inversely correlated to the number of wheezing days during the first year of life ($\rho = -0.38$, $P = .048$).

Conclusions

High concentrations of amniotic fluid IL-8 and TNF-α are associated with low risk of RSV bronchiolitis in healthy infants. We hypothesize that direct exposure of fetal lungs to pro-inflammatory signals induces local protection against viral infection during infancy.

INTRODUCTION

The global burden of respiratory syncytial virus (RSV) infections in individual children and their families, the medical system, and society is considerable.[1,2] Annually, more than 2 million children under the age of 5 years in the USA require medical care for RSV infections.[3] Risk factors for severe course of disease are preterm birth, cardiopulmonary disease, Down's syndrome, immunodeficiency, and neuromuscular disease.[4-6] About half of children with RSV bronchiolitis will develop recurrent wheeze during early childhood.[7]

The pathogenesis of RSV bronchiolitis is not fully elucidated. Most children with RSV bronchiolitis were born at term and healthy until infected.[8,9] Because RSV targets the airways, pre-existent airway abnormalities have been considered in the pathogenesis of disease. The fetal airways are continuously exposed to amniotic fluid. In a cohort of 761 preterm infants, chorioamnionitis protected against the development of chronic lung disease.[10] Moreover, in preterm lambs, experimental intra-amniotic injection of bacterial endotoxin induced a twofold increase of fetal lung compliance and a threefold increase of lung volume following preterm birth.[11,12]

Our primary aim was to study whether fetal exposure to high concentrations of amniotic fluid pro-inflammatory cytokines protects against RSV bronchiolitis in the first year of life in a healthy birth cohort. Our secondary aim was to study whether the concentrations of amniotic fluid pro-inflammatory cytokines are related to recurrent wheeze.

METHODS

Study design and population

We carried out a prospective, observational, birth cohort study of healthy term newborns, born after an uncomplicated pregnancy of ≥37 weeks and spontaneous onset of labor.[13] From January 2006 to December 2008, amniotic fluid samples were collected from 882 of 2,323 eligible newborns (38%). The parents of 292 newborns (33%) gave written informed consent to participate in our study. The most frequent reason (94%) for non-participation was reluctance of parents to perform follow-up measurements according to the study protocol. Baseline characteristics were prospectively assessed by a standardized questionnaire.[14] Baseline characteristics of non-participating children and their parents were similar to the characteristics of those participating (data not shown). The study protocol was approved by the institutional review board of the University Medical Center Utrecht, Utrecht, The Netherlands.

Amniotic fluid collection and measurements

Amniotic fluid was sampled during labor. Collection and storage of amniotic fluid and measurement of interleukin-8 (IL-8, CXCL8) and tumor necrosis factor-α (TNF-α) by ELISA have been described previously.[13] The concentration of TNF-α was below the limit of detection (0.014 ng/mL) in 125 out of the 292 (43%) children using 1:10 diluted samples. We therefore dichotomized TNF-α into detectable or undetectable. Pilot experiments showed that <1:10 dilution of amniotic fluid resulted in duplicate variation. The concentrations of IL-8 and TNF-α were moderately correlated (Spearman's $\rho = 0.53$, $P < .001$).

Outcomes

The primary outcome was physician-attended RSV infection. Parents were instructed to obtain a nose-throat swab sample at the start of every respiratory episode during the first year of life.[15] The samples were sent to the researchers in a single vial containing 2 mL of viral transport medium and frozen at −80°C. The presence of RSV RNA was determined with real-time polymerase chain reaction (PCR) as described previously.[16] In case of a positive RSV PCR, the presence of co-infection with influenzavirus (A and B), parainfluenzavirus (1-4), coronavirus (OC43, NL63, 229E), rhinovirus, human metapneumovirus, adenovirus, enterovirus, or *Chlamydia pneumoniae* was assessed. Criteria for physician-attended RSV infection were: (1) respiratory tract illness that led to a visit to the general practitioner or pediatrician, and (2) the simultaneous presence of RSV RNA in the nose-throat swab irrespective of co-infections. Sensitivity analysis was performed using physician-attended single RSV infection, in which RSV was the only pathogen detected by PCR. The secondary outcome was recurrent wheeze, defined as the number of wheezing days in the first year of life. Parents kept a daily log and recorded days with wheeze, as described previously.[7,15]

Statistical analysis

Baseline characteristics, and the concentrations of amniotic fluid IL-8 and the proportion of detectable TNF-α were compared between the groups of children with and without the primary outcome using the Student's T test and the X^2 test. Generalized linear models were used to adjust for potentially confounding baseline characteristics. The numbers of wheezing days in the first year of life were compared using the Mann-Whitney U test. Among children with physician-attended RSV infection, the relation between the amniotic fluid IL-8 and TNF-α and the number of wheezing days was assessed using Spearman's correlation and the Mann-Whitney U test. There were no participants lost to follow-up or missing data by the nature of the study design. All analyses were performed in SPSS 15.0.

RESULTS

Physician-attended RSV respiratory tract infection occurred in 27 children (cumulative incidence 9.3% (95% confidence interval (CI) 5.2-12.6)), at a median age of six months (IQR 5-9). Children with and without physician-attended RSV infection in the first year of life had similar baseline characteristics (**table 1**). Fourteen children had fever (>38.5°C, 52%) and signs of expiratory airflow limitation (wheezing) were found in 15 (56%). A co-infection was detected in 9 (33%) children with physician-attended RSV (6 double infections, 3 triple infections; frequency rhinovirus 6, adenovirus 4, coronavirus 2). Children with physician-attended RSV infection had more wheezing days in the first year of life than children without (median 3 vs 0 (interquartile range 0-13 vs 0-3), $P = .02$).

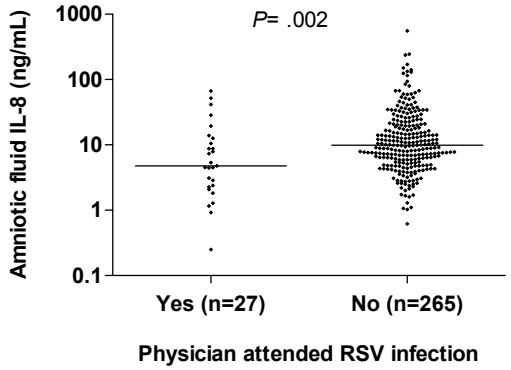
Table 1. Baseline characteristics of infants with and without physician-attended RSV infection in the first year of life (*n* = 292)

	Physician-attended RSV infection		
Variable	**Yes *n* = 27**	**No *n* = 265**	***P*-value**
Boys	11 (41)	138 (52)	.26
Birth weight, mean (SD), kg	3.76 (0.40)	3.60 (0.45)	.08
Gestational age, mean (SD), wk	40.3 (1.1)	40.1 (1.0)	.17
Siblings	17 (63)	156 (59)	.68
Antepartum smoke exposure	2 (7)	31 (12)	.75
Birth in April to September	17 (63)	135 (51)	.23
Parental atopy	18 (67)	135 (51)	.12
Parental asthma	8 (30)	51 (19)	.20

Values represent frequency (%), unless indicated otherwise.

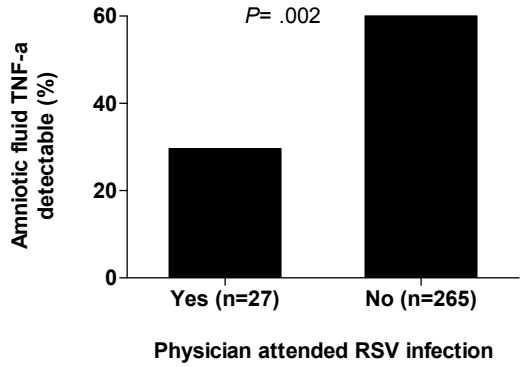
Amniotic fluid concentrations of IL-8 and TNF-α were not associated with parity, antepartum smoking, intrapartum administration of antibiotics, assisted delivery, season of birth, gender, Apgar score or birth weight. Children with detectable amniotic fluid concentrations of TNF-α had a higher mean gestational age than children with undetectable TNF-α (40.2 versus 39.9 weeks, $P = .02$). Amniotic fluid concentrations of IL-8 were twofold higher in children without physician-attended RSV infection as compared to children with physician-attended RSV infection in the first year of life (geometric mean 11.07 versus 5.49 ng/mL, ratio 2.02 (95% CI 1.31-3.11), $P = .002$, **figure 1**). Similar results were found for TNF-α, with a twofold higher fraction of detectable TNF-α in children without physician-attended RSV infection as compared to children with physician-attended RSV (159/265 (60%) versus 8/27 (30%), $P = .002$). Sensitivity analysis showed comparable results using the alternative outcome physician-attended single RSV infection. Results did not change after adjustment for potential confounders (ie gestational

Figure 1. Association between amniotic fluid concentration of IL-8 and physician-attended RSV infection



Amniotic fluid concentration of IL-8 for children with and without physician-attended RSV infection (Student's T test).

Figure 2. Association between the proportion of detectable amniotic fluid TNF-α and physician-attended RSV infection



Proportion of detectable amniotic fluid TNF-α for children with and without physician-attended RSV infection (X^2-test). TNF-α limit of detection: 0.014 ng/mL. Spearman's correlation between amniotic fluid IL-8 and TNF-α: $\rho = 0.53$, $P < .001$.

age). Two children were hospitalized for RSV bronchiolitis (amniotic fluid IL-8: 0.9 and 7.8 ng/mL, and TNF-α: undetectable in both).

Among children with physician-attended RSV infection, amniotic fluid concentrations of IL-8 were inversely correlated with the number of wheezing days in the first year of life ($n = 27$, $\rho = -0.38$, $P = .048$). For amniotic fluid TNF-α no significant association was found with recurrent wheeze. In children without physician-attended RSV infection, amniotic fluid cytokine concentrations were not associated with wheeze during the first year of life.

DISCUSSION

The current study reveals a previously unrecognized mechanism of infant airway morbidity. In a birth cohort of healthy infants, we found that high levels of amniotic fluid IL-8 and TNF-α are associated with low risk of RSV bronchiolitis in the first year of life. The concentration of amniotic fluid IL-8 and the fraction of detectable TNF-α were twofold higher in children without medically attended RSV infection as compared to children with medically attended RSV infection. We demonstrated an inverse relation between the concentration of amniotic fluid IL-8 and recurrent wheeze in the first year of life, suggesting that both RSV bronchiolitis and recurrent wheeze in healthy infants have common underlying pathophysiology. To our knowledge this is the first study that relates amniotic fluid chemokine and pro-inflammatory cytokine concentrations to respiratory disease in healthy children.

Our study increases the insight in the mechanisms underlying RSV bronchiolitis in otherwise healthy children.[3] The current study shows that the "acute inflammation gene expression signature" in the amniotic cavity, which is characteristic of normal parturition, provides protection against the development of RSV bronchiolitis during infancy.[17,18] The fetal airway mucosa is directly exposed to high chemokine and cytokine concentrations, which may provide a direct instructive signal to the airway mucosal immune system to prepare for post-partum life. We speculate about the mechanisms by which this occurs. This signal may be aimed at the generation of a mature lung dendritic cell population. First, chemoattraction of circulating monocytes into the lung is promoted by chemokines, including IL-8.[19,20] Second, monocytes differentiate into mature myeloid dendritic cells under influence of IL-4 and granulocyte-macrophage colony-stimulating factor, which is present in amniotic fluid, but may also be produced locally.[17] Third, maturation of dendritic cells occurs under the influence of pro-inflammatory cytokines, including TNF-α, which are abundantly present in amniotic fluid.[17,21] Alternatively, exposure to pro-inflammatory cytokines may also enhance lung development, which could explain a reduced incidence and / or severity of RSV infection. This hypothesis is supported by experiments in animals and by a recent birth cohort study with reduced prevalence of chronic lung disease in preterm infants exposed to chorioamnionitis.[10-12] Notably, the opposite, harmful effects of chorioamnionitis reported by others were absent after adjustment for gestational age and other potential confounders.[22,23] Of course, these mechanisms in preterm deliveries cannot directly be extrapolated to healthy term infants.

Potential study limitations deserve discussion. First, residual confounding cannot be excluded, although adjustment for confounders did not alter our results.[24] Second, not all eligible infants participated in this study. Although we were not able to detect baseline differences between participating and non-participating infants, selection bias may have occurred. Third, although the CXC chemokine IL-8 and the inflammatory cytokine TNF-α have an important role in the development of airway dendritic cells, many other

molecules in amniotic fluid have to be considered to fully understand the mechanisms by which normal amniotic fluid affects fetal lung development.[17] Fourth, due to the non-sterile collection of amniotic fluid samples, microbial cultures were not performed.[13] We have previously demonstrated high reproducibility of amniotic fluid cytokine measurements in these samples, and the distribution in our cohort is in coherence with previous measurements during term delivery by others.[13,17,18] Fifth, this study was not powered to determine the relationship between amniotic cytokine concentrations and recurrent wheeze, which may explain why no association between amniotic fluid TNF-α and post-bronchiolitis wheeze was found. Sixth, misclassification due to parental incompliance with sample collection cannot be ruled out. However, this may only have diluted the effects found, since an association between parental incompliance and amniotic fluid cytokine concentrations is highly improbable.

Our study increases the insight in the mechanisms underlying RSV bronchiolitis in otherwise healthy children.[3] Apparently, children with an "acute inflammation gene expression signature" gain protection against RSV infection later in life, possibly via maturation of the airway mucosal immune system.[17] Further study is required to determine whether the protective effect is also be involved in other respiratory conditions in early childhood, such as asthma development.[9,25,26] Finally, this study could have implications for the care of healthy term infants. The use of amniotic fluid IL-8 or TNF-α as biomarkers, might eventually enable targeted primary prevention of RSV bronchiolitis in healthy term infants.[15,27-30]

This birth cohort study shows that high concentrations of amniotic fluid IL-8 and TNF-α at term are associated with protection against RSV bronchiolitis in the first year of life. The similar inverse correlation between high amniotic fluid IL-8 concentrations and low risk of recurrent wheeze suggests both wheezing illnesses have similar underlying mechanisms. We speculate that local exposure of the fetal airway mucosa to pro-inflammatory amniotic fluid stimuli during parturition has a beneficial effect on the foetal mucosal immune system.

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Chapter 8

Newborn airway compliance is correlated with amniotic fluid soluble leukocyte-associated Ig-like receptor-1

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CK van der Ent, JLL Kimpen, L Meyaard, L Bont

ABSTRACT

Background

Poor airway function at birth is an important risk factor for childhood asthma and airflow limitation in adulthood. The mechanisms that determine newborn airway function are unclear. In preterms, intra-uterine inflammation has been associated with enhanced lung maturation. To date, pro-inflammatory cytokines have been studied as markers of intra-uterine inflammation. Leukocyte-associated Ig-like receptor-1 (LAIR-1) is a membrane-bound collagen receptor that increases the threshold for activating signals on several immune cells. The secreted form of LAIR-1 (sLAIR-1) is considered a distinct marker of immune activation.

Hypothesis

Based on a pilot study showing high concentrations of sLAIR-1 in amniotic fluid, we hypothesized that high sLAIR-1 in amniotic fluid is associated with normal newborn airway function and low infant airway morbidity.

Methods

In a healthy birth cohort, 152 newborns successfully underwent lung function measurement. Amniotic fluid was collected during labor and sLAIR-1 was measured by sandwich ELISA. To determine whether amniotic fluid sLAIR-1 could be spill-over from the neonatal blood system, in a subgroup of infants cord blood and amniotic fluid sLAIR-1 were measured in parallel. The expression of LAIR-1 on amniotic fluid neutrophils and its secretion upon stimulation were measured. Placenta tissue was stained for LAIR-1. At age 1 month compliance and resistance of the respiratory system were assessed with the single occlusion technique. Wheeze during follow-up was determined using a parental log for respiratory symptoms.

Results

sLAIR-1 was detected in all amniotic fluid samples. We found a clinically relevant correlation between airway compliance and the amniotic fluid concentration of sLAIR-1 ($\rho = 0.29$, $P = .001$). This correlation was independent from sex or maternal antepartum smoking. Amniotic fluid sLAIR-1 was lower in children who wheezed at ages 6 and 9 months ($P = .04$ and $P = .05$). We did not find the cellular source of amniotic fluid sLAIR-1. Cord blood and amniotic fluid sLAIR-1 concentrations were not correlated. LAIR-1 was expressed on amniotic fluid neutrophils, but sLAIR-1 secretion upon stimulation was not detected. LAIR-1 positive cells were absent in placenta tissue.

Conclusions

High amniotic fluid sLAIR-1 concentrations provide support of a strong intra-uterine immune activation during normal delivery. The association between sLAIR-1 in amniotic fluid and newborn airway compliance suggests a beneficial effect of intra-uterine immune activation on neonatal lung function.

INTRODUCTION

Asthma affects about 20% of all children and is among the most prevalent chronic diseases in childhood.[1,2] Poor newborn airway function is an important risk factor for the development of childhood asthma and airflow limitation in adulthood.[3-5] Accordingly, the occurrence of viral lower respiratory tract infections (LRTI) and wheezing symptoms in early childhood are associated with reduced newborn airway function.[6-8] The mechanisms influencing airway development in healthy term infants are largely unknown. Epidemiologic studies have identified birth weight and length, sex, maternal antepartum smoking, a family history of asthma, and parental airway function as determinants of newborn airway function.[9-11] Furthermore, it is suggested that intra-uterine inflammation, in absence of a fetal inflammatory response syndrome, promotes fetal lung maturation.[12] In a cohort of 761 preterm infants, chorioamnionitis protected against the development of chronic lung disease (CLD).[13] Moreover, in preterm lambs, experimental intra-amniotic injection of bacterial endotoxin induced an inflammatory response in membranes and in amniotic fluid, resulting in increased fetal lung compliance and lung volume.[14,15]

Leukocyte-associated Ig-like receptor-1 (LAIR-1) is a transmembrane glycoprotein that is expressed on several peripheral blood leukocytes, including T, B, and NK cells, eosinophils, monocytes and dendritic cells.[16,17] Activation of T and B cells results in down-regulation of membrane LAIR-1 expression.[18,19] On neutrophils, however, the cell-surface expression of LAIR-1 is low during steady-state conditions, while expression is induced upon stimulation with granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and N-formyl-methionyl-leucyl-phenylalanine (fMLP).[20] Collagens are ligands for LAIR-1 and cross-linking results in inhibited immune cell function.[21] Phosphorylation of the two immune receptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail of LAIR-1 leads to down-regulation of cell activation.[16] Thus, collagen binding of LAIR-1 increases the threshold for activating signals on several immune cells.[21] Interestingly, its family member LAIR-2 is expressed as a soluble receptor which also binds collagen molecules, and may function as a natural competitor of membrane-bound LAIR-1, serving as a regulator of LAIR-1.[22] Ouyang and colleagues have demonstrated soluble LAIR-1 (sLAIR-1) in serum of healthy individuals and at increased concentrations in patients with renal disorders.[23] Recently, we demonstrated sLAIR-1 in plasma and urine of healthy individuals and rheumatoid arthritis patients.*[Olde Nordkamp et al, Manuscript Submitted For Publication]* Conceivably, the high levels of intra-amniotic sLAIR-1 during term spontaneous onset of labor delivery, which we found in a pilot study, may reflect a general state of immune activation.[24,25] We hypothesized that the intra-amniotic amount of sLAIR-1 is associated with fetal lung maturation. In a birth cohort of healthy term newborns, we assessed the association

between amniotic fluid sLAIR-1 and newborn airway function. In addition, we studied the specific intra-amniotic origin of amniotic fluid sLAIR-1.

METHODS

Population and baseline characteristics

A birth cohort was founded in two large urban hospitals and followed up through the first year of life. From January 2006 to December 2008, a total of 372 healthy newborns were included after an uncomplicated pregnancy of ≥37 weeks and delivery by spontaneous onset of labor or elective caesarean section. Exclusion criteria were major congenital anomalies and limited parental Dutch language skills. The most frequent reason (>90%) for non-participation was reluctance of parents to perform follow-up measurements according to the study protocol. Baseline characteristics were prospectively assessed by a standardized questionnaire.[26] Baseline characteristics of non-participating children and their parents were similar to the characteristics of those participating (data not shown). The study protocol was approved by the institutional review boards of the University Medical Center Utrecht and the Diakonessen Hospital (both in Utrecht, The Netherlands) and written informed consent was provided by the parents of all participating children.

Collection of amniotic fluid, cord blood plasma and placentas

Amniotic fluid was sampled during labor, purified by filtration (70 μm filter, Falcon BD) and centrifugation (10 min, 1500 RPM), and stored at −80°C, as described previously.[27] Amniotic fluid cell immunophenotyping and stimulation were performed on fresh amniotic fluid samples. Cord blood was collected directly after birth and anticoagulated using sodium heparin. Plasma was prepared by centrifugation (5 min, 500 *g*), and stored at −80°C. Placentas were stored at +4°C and processed within 72 hours, as described previously.[27] Amniotic fluid lecithin-sphingomyelin (L/S) ratios were determined by thin layer chromatography according to Gluck and Kulovich.[28,29]

sLAIR-1 ELISA

The concentration of sLAIR-1 in amniotic fluid, in cord blood plasma, and in supernatant of stimulated amniotic fluid cells was measured by sandwich ELISA (in-house manufactured; limit of detection 1.95 ng/mL).*[Olde Nordkamp et al, Manuscript Submitted For Publication]* The intra-assay and the inter-assay correlations were high (Spearman's $\rho = 0.98$, $P = .005$, $n = 5$; Pearson's $\rho = 0.81$, $P < .001$, $n = 21$).

Immunophenotyping of amniotic fluid neutrophils

Amniotic fluid samples were stained with PE-labelled α-hLAIR-1, APC labelled α-CD11b, Pacific Blue labelled α-CD16 and APC-Cy7 labelled α-CD14 antibodies. IgG1 isotype controls were carried out using PE labelled mouse IgG1κ, to rule out non-specific binding (all antibodies were purchased from BD Pharmingen). Neutrophils were selected from the live cell gate of the forward-sideward scatter plot as $CD11b^+$ / $CD16^+$ / $CD14^-$ cells. Macrophages were selected accordingly as $CD14^+$ / $CD16^-$ cells. Flow cytometry was performed using a LSRII flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using FlowJo version 7.6 software (Tree Star, Ashland, USA).

Stimulation of amniotic fluid cells

Fresh amniotic fluid was filtered twice through a 70 μm filter. Cells were isolated by centrifugation, and cultered in RPMI 1640 (Gibco, Invitrogen, The Netherlands) supplemented with 10% fetal calf serum (Integro, Dieren, the Netherlands) and antibiotics at 37°C, 5% CO_2. Cells were stimulated with LPS (1 ng/mL and 10 ng/mL), and PMA (50 ng/mL) and ionomycin (1 μM) for 24 hours. The supernatant was harvested and stored at −20°C untill further use in a sandwich ELISA for the presence of sLAIR-1.

Placenta immunohistology

Placenta tissue was analyzed for the presence of membrane-bound LAIR-1. Two sections of the umbilical cord, at the fetal and placental side, a membrane roll, one sample from the umbilical cord insertion, and three slides of normal placental parenchyma, including both decidua and chorionic plate, were collected and stained with standard haematoxylin/eosin and with anti-LAIR-1 antibody. To differentiate between cells of trophoblastic (fetal) and decidual (maternal) origin, the samples were stained for the presence of keratin. CD68 staining was used to detect macrophages. Histological chorioamnionitis was diagnosed based on the presence of polymorphonuclear cells (neutrophilic granulocytes) in the chorionic plate or the extraplacental membranes.[27]

Newborn lung function

Newborn lung function was measured before the age of two months (median age 34 days, IQR 30-39), during natural sleep and without the use of any sedation, as described previously.[9,30,31] Lung function was assessed from measurement of passive respiratory mechanics (compliance and resistance of the total respiratory system) using the single occlusion technique (SOT). The results of airway compliance and resistance were standardized by correction for length, weight and age during lung function measurement.

Clinical definitions

Antepartum exposure to tobacco smoke was defined as maternal smoking of at least one cigarette per day during pregnancy. Parental atopy was defined as the presence of any atopic diagnosis (asthma, eczema or hay fever) made by a physician in one or both parents. Parental asthma was defined accordingly.[26] Data on wheezing and cough during the first year of life were collected in daily parental logs, as described previously.[32] Rhinovirus LRTI was defined as an episode with (1) rhinovirus RNA in a nose-throat swab, irrespective of detection of other pathogens, and (2) simultaneous symptoms of an LRTI as reported by parents in a daily log (wheeze and / or moderate to severe cough).[32,33] Upper respiratory tract infections (URTI) were defined by the absence of wheeze and cough or the presence of mild cough. The diagnosis of airway hyperreactivity was reported by the general practitioner at the age of one year.

Statistical analysis

Baseline and lung function characteristics were compared between groups using Student's T test, Mann-Whitney U test or X^2 test, as appropriate. After logarithmic transformation, the concentration of amniotic fluid sLAIR-1 was normally distributed. To assess the association between the amniotic fluid concentration of sLAIR-1 and the standardized airway compliance and resistance, Pearson's correlation coefficient was calculated. Linear regression analysis was carried out to adjust for baseline characteristics. Spearman's correlation was calculated for the association with L/S ratios.

For the associations with rhinovirus LRTI, we excluded the children without exposure to rhinovirus during the first year of life. We compared the group of children with one or more rhinovirus LRTIs with those with only rhinovirus URTIs. Finally, we compared the amniotic fluid concentrations of sLAIR-1 between children who ever had wheezing symptoms during their first year of life or a physician's diagnosis of airway hyperreactivity and those who had not. Reported sample sizes for the different measurements vary due to variable frequencies of missing data.

RESULTS

Newborn lung function is correlated with gestational age and anthropometry

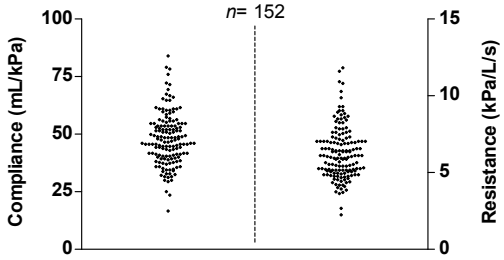
Successful lung function measurement was performed in 152 infants. The major reasons for failure of lung function measurement were: technical (insufficient quality and / or number of occlusions, 42%) and no sleep or too short period of sleep (52%). Children with successful lung function measurements and those who failed had similar baseline characteristics (**table 1**). Airway compliance and resistance were normally distributed

Table 1. Baseline and lung function characteristics

	Lung function measurement			
	Total *n*= 372	Passed *n*= 152	Failed *n*= 220	*P*-value
Delivery and birth				
Gestational age (wk)	40.0 (1.0)	40.1 (1.1)	39.9 (1.0)	.07
Birth weight (kg)	3.61 (0.47)	3.65 (0.47)	3.58 (0.46)	.17
Male gender	51%	52%	51%	.77
Antepartum maternal smoking	10%	10%	11%	.90
Parental asthma	23%	24%	23%	.83
Parental atopy	56%	54%	57%	.61
Lung function measurement				
Weight (kg)	4.64 (0.62)	4.64 (0.60)	4.64 (0.63)	.93
Length (cm)	55.5 (2.6)	55.3 (2.4)	55.5 (2.8)	.47
Age (day)	36.4 (8.1)	35.5 (7.0)	37.0 (8.8)	.08
Compliance (mL/kPa)	NA	47.8 (11.3)	NA	NA
Resistance (kPa/L/s)	NA	6.27 (1.7)	NA	NA

Values represent mean (SD) or percentage. *P*-values for Student's T test or X^2 test. Missing values: parental atopy and / or asthma *n* = 7 (2%), maternal antepartum smoking *n* = 13 (3%). NA denotes not applicable.

Figure 1. Compliance and resistance of the respiratory system of healthy term newborns



Newborn lung compliance (left Y-axis) and resistance (right Y-axis) were assessed using the single occlusion technique during physiologic sleep.

after logarithmic transformation (**figure 1**). Compliance was positively correlated with gestational age and length during lung function, and resistance was negatively correlated with gestational age, birth weight, and weight and length during lung function (**table 2**).

Amniotic fluid sLAIR-1 is correlated with newborn airway compliance

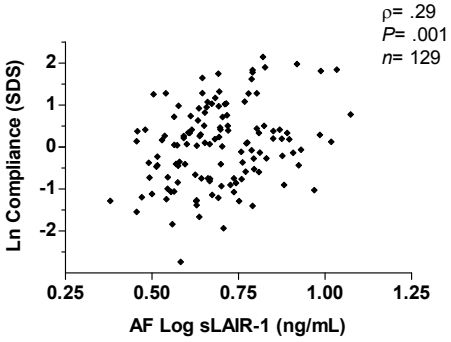
The mean concentration of amniotic fluid sLAIR-1 was 4.92 ng/mL (95% CI 2.7-9.1). sLAIR-1 was detectable in all amniotic fluid samples. There was a moderately strong, positive association between the amniotic fluid concentration of sLAIR-1 and newborn airway compliance ($\rho = 0.29$, $P = .001$, $n = 129$, **figure 2**). Adjustment for sex yielded identical results ($\rho = 0.28$, $P = .002$, $n = 129$; girls $\rho = 0.23$, $P = .07$, $n = 63$; boys $\rho = 0.33$, $P = .006$,

Table 2. Clinical determinants of newborn lung function

	Compliance (mL/kPa)		Resistance (kPa/L/s)	
n = 152	Rho	*P*-value	Rho	*P*-value
Gestational age (wk)	.22	.006	–.24	.003
Birth weight (kg)	.14	.09	–.20	.02
Weight (kg)	.11	.19	–.19	.02
Length (cm)	.23	.006	–.22	.008
Age (day)	.16	.06	–.56	.49
Maternal antepartum smoking	.05	.53	.13	.10

Natural logarithm of compliance and resistance. Missing values: weight and / or length $n = 7$ (5%), age $n = 1$ (0.7%). *P*-values for Pearson's correlation, except for maternal antepartum smoking (Spearman's correlation).

Figure 2. Correlation between amniotic fluid sLAIR-1 and newborn lung compliance



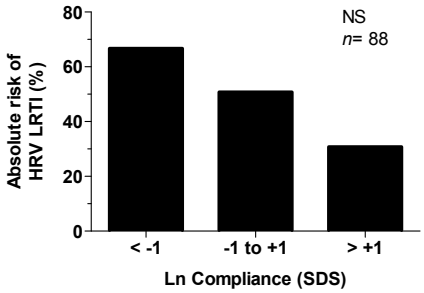
Amniotic fluid sLAIR-1 was measured by sandwich ELISA (limit of detection 1.95 ng/mL). Newborn lung compliance was assessed using the single occlusion technique during physiologic sleep. Compliance measurements were standardized, correcting for length, weight, and age during lung function measurement. Pearson's correlation was calculated.

$n = 66$). Adjustment for antepartum maternal smoking yielded similar results (correction: $\rho = 0.28$, $P = .001$, $n = 129$; no smoking $\rho = 0.28$, $P = .002$, $n = 113$). There was no association between the amniotic fluid concentration of sLAIR-1 and newborn airway resistance ($\rho = -0.08$, $P = .37$, $n = 131$).

Rhinovirus lower respiratory tract infection is associated with reduced airway compliance

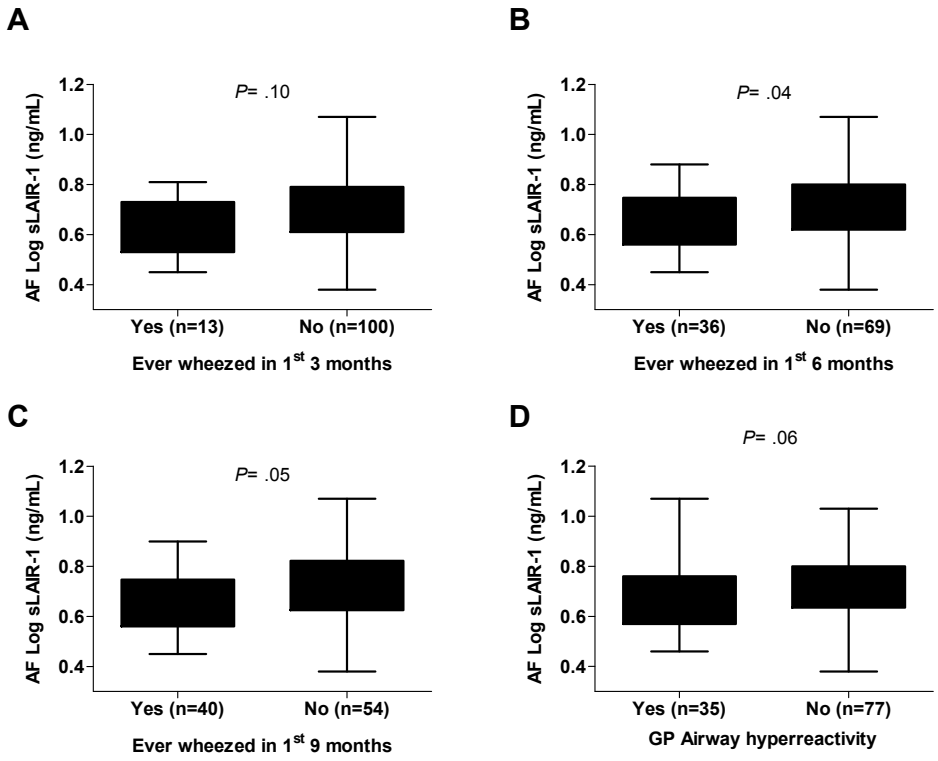
The amniotic fluid concentration of sLAIR-1 was similar in children with and without rhinovirus LRTI in the first year of life ($P = .91$, data not shown). However, the risk of rhinovirus LRTI tended to be inversely correlated with airway compliance (**figure 3**). Children in the lowest and the highest compliance tertile had 68% and 30% risk of rhinovirus LRTI in the first year of life, respectively. Adjustment for sex, gestational age, or antepartum maternal smoking yielded similar results (data not shown).

Figure 3. The risk of rhinovirus LRTI tends to be lower in children with high lung compliance



Newborn lung compliance was assessed using the single occlusion technique during physiologic sleep. All children with any human rhinovirus (HRV) respiratory tract infection during the first year of life were included. Lung compliance divided according to Z-score, logistic regression analysis with compliance as continuous variable ($P = .17$, $n = 88$). The total number of children is reduced due to missing data.

Figure 4. Infant wheeze and airway hyperreactivity are associated with low amniotic fluid sLAIR-1



Mean logarithmic transformed amniotic fluid concentration of sLAIR-1 for children that ever or never wheezed at the age of 3 (A), 6 (B), and 9 (C) months, or have or have not been diagnosed by their general practitioner (GP) with airway hyperreactivity in the first year of life (D). Varying total numbers of children due to variable frequencies of missing data. Boxes and whiskers represent interquartile and total ranges of measurements, respectively. *P*-values of Mann-Whitney U tests.

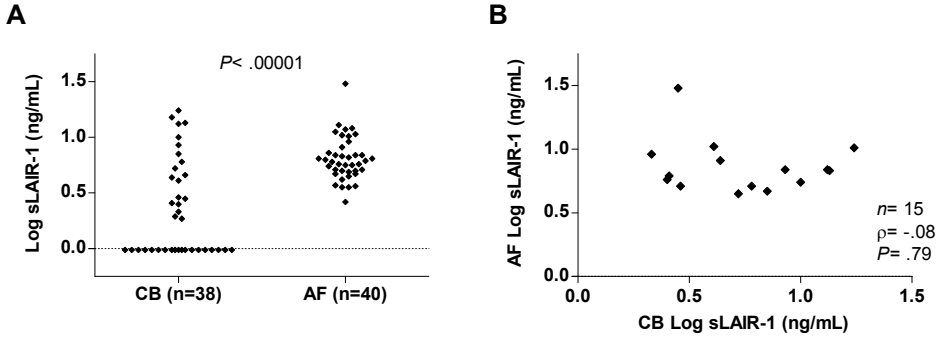
Infant wheeze and airway hyperreactivity are associated with low amniotic fluid sLAIR-1

The mean amniotic fluid concentration of sLAIR-1 was higher in children without wheezing symptoms during the first 3, 6 and 9 months of life (**figure 4A-C**). Similarly, amniotic fluid concentrations of sLAIR-1 tended to be higher in children without physician diagnosed airway hyperreactivity in the first year of life (**figure 4D**).

No correlation between cord blood and amniotic fluid sLAIR-1

We sought to identify the intra-uterine origin of amniotic fluid sLAIR-1. Therefore, we measured the concentration of sLAIR-1 in paired cord blood and amniotic fluid samples, to assess for spill-over of sLAIR-1 from cord blood to amniotic fluid. sLAIR-1 was detected in 50% of cord blood plasma samples versus 100% of amniotic fluid samples (**figure 5A**). There was no correlation between the concentrations of sLAIR-1 in paired samples of cord blood plasma (with detectable sLAIR-1) and amniotic fluid (**figure 5B**).

Figure 5. Concentrations of sLAIR-1 in amniotic fluid are higher than and not correlated with those in cord blood

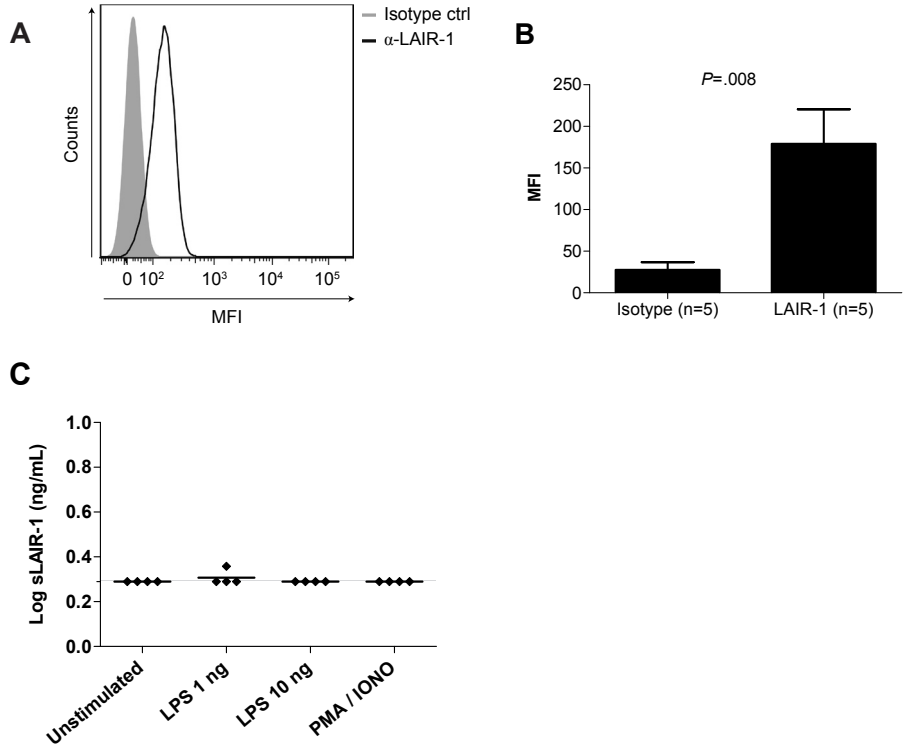


sLAIR-1 ELISA was performed in samples of cord blood (CB) and amniotic fluid (AF). Limit of detection 1.95 ng/mL. (A) Comparison of the concentration of sLAIR-1 in CB and AF samples. Student's T test for unpaired analysis. (B) Correlation between detectable sLAIR-1 in cord blood and amniotic fluid (Pearson's correlation coefficient).

Amniotic fluid neutrophils express LAIR-1 but do not secrete sLAIR-1 upon stimulation

LAIR-1 was expressed on the cell surface of neutrophils in amniotic fluid, as assessed by flow cytometry (**figure 6A-B**). However, upon ex vivo culture and stimulation of isolated amniotic fluid cells with LPS or PMA / ionomycin, no secretion of sLAIR-1 in supernatant was detected (**figure 6C**). Previous studies have revealed the presence of macrophages in amniotic fluid at term, next to neutrophil abundancy.[27] Flow cytometry did not consistently demonstrate the expression of LAIR-1 on the cell surface of amniotic fluid macrophages (data not shown).

Figure 6. LAIR-1 is present on amniotic fluid neutrophils, but sLAIR-1 is not secreted upon stimulation



LAIR-1 flow cytometry was performed on fresh amniotic fluid samples. Neutrophils were selected from the live cell gate of the forward-sideward scatter plot as $CD11b^+$ / $CD16^+$ / $CD14^-$ cells. (A) LAIR-1 expression on amniotic fluid neutrophils (representative of a series of $n = 5$). (B) Histogram of mean fluorescence intensity (MFI) of LAIR-1 staining versus IgG_1 isotype controls ($n = 5$, Mann-Whitney U test). (C) Amniotic fluid cells were stimulated with LPS or PMA / ionomycin ($n = 4$). After 24 hours supernatant was harvested and the concentration of sLAIR-1 was measured in a sandwich ELISA. The limit of detection of the ELISA was 0.29 (logarithmic transformed concentration). All values below the limit of detection were plotted as the limit of detection. Notably, flow cytometry did not consistently demonstrate the expression of LAIR-1 on the cell surface of amniotic fluid macrophages ($CD14^+$, $CD16^-$, data not shown).

No correlation between amniotic fluid sLAIR-1 and L/S ratio

Amniotic fluid L/S ratios were determined in 21 amniotic fluid samples. The mean L/S ratio was 9.2 (SD 4.1, range 3.1 to 18.2). Amniotic fluid L/S ratios were not correlated to the amniotic fluid concentrations of sLAIR-1 (Spearman's $\rho = 0.02$, $P = .94$, $n = 21$). In addition, L/S ratios were not associated with amniotic fluid pro-inflammatory cytokine concentrations, airway compliance or resistance, or gestational age or birth weight (data not shown).

No LAIR-1 positive cells present in placenta tissue during spontaneous onset of labor at term

The placentas of three children were analyzed. The two placentas without or with mild signs of histological chorioamnionitis were LAIR-1 negative (**figure 7A and B**). The placenta with signs of severe chorioamnionitis showed infrequent positive LAIR-1 staining of cells in the chorionic plate, stromal cells in Wharton's jelly of the umbilical cord, and the maternal side of the chorionic membranes (**figure 7C**). Within the chorionic membranes, LAIR-1 was only detectable in regions that stained negative for keratin, suggesting a maternal origin of the LAIR-1 positive cells (**figure 7D**). LAIR-1 was predominantly located on the membrane of cells, most consistently in the keratin negative regions (**figure 7D**). Cells that stained LAIR-1 positive were mononuclear on microscopy and stained CD68 negative (data not shown).

Figure 7. No LAIR-1 positive cells present in placenta tissue during spontaneous onset of labor at term (*next two pages*)

Immunohistology of placentas. Standard haematoxylin / eosin staining was applied to all samples.

(A) Placenta without histological signs of chorioamnionitis. LAIR-1 positive cells are absent in the chorionic plate (1+2), the umbilical cord, and the chorionic membranes (data not shown).

(B) Placenta with mild signs of chorioamnionitis. LAIR-1 positive cells are sparsely present in the chorionic membranes (1+2+4), the stromal cells of Wharton's jelly of the umbilical cord (3), and the chorionic plate (data not shown).

(C) Placenta with severe signs of chorioamnionitis. LAIR-1 positive cells are infrequently present in the chorionic plate (1), the stromal cells of Wharton's jelly of the umbilical cord (2), and the maternal side of the chorionic membranes (3+4).

(D) Placenta with severe signs of chorioamnionitis. LAIR-1 (left, 1+3) and keratin (right, 2+4) immunohistology. LAIR-1 positive cells are present in keratin negative regions (decidua) of the chorionic membranes. On these mononuclear cells, LAIR-1 staining is predominantly found on the cell membrane. CD68 staining (macrophage marker) of these cells is negative (data not shown). LAIR-1 positive cells are sparsely present in keratin positive regions (trophoblast) of chorionic membranes.

DISCUSSION

In a birth cohort of healthy term newborns, we demonstrated an association between intra-amniotic sLAIR-1 and newborn lung function. We found a positive correlation ($\rho = 0.29$) between the amniotic fluid concentration of sLAIR-1 and compliance of the total respiratory system at the age of one month. In addition, children with a high level of amniotic fluid sLAIR-1 and / or high airway compliance had lower risk of rhinovirus LRTI and recurrent wheezing during the first year of life. Our data suggest that during

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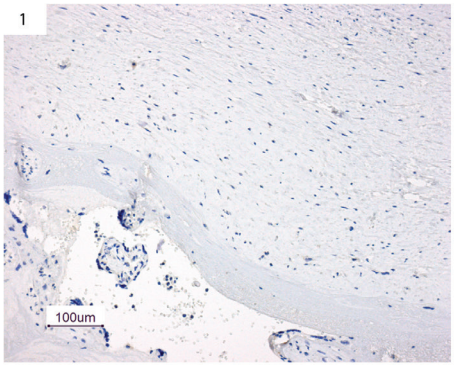

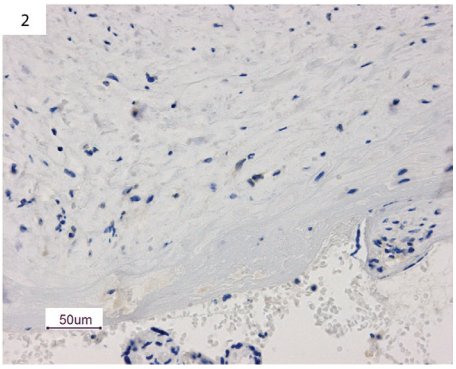

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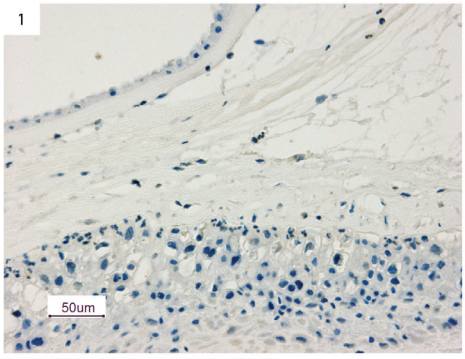

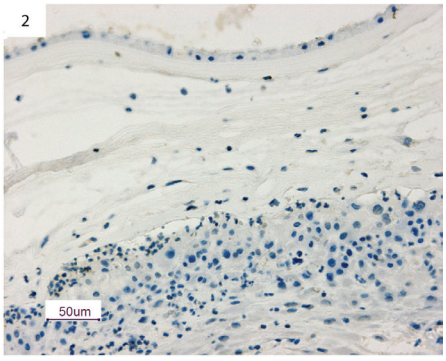

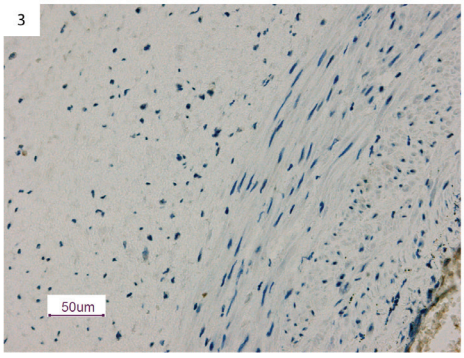

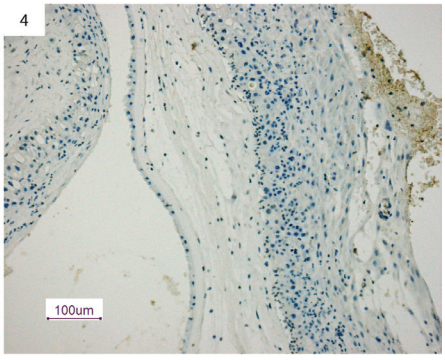

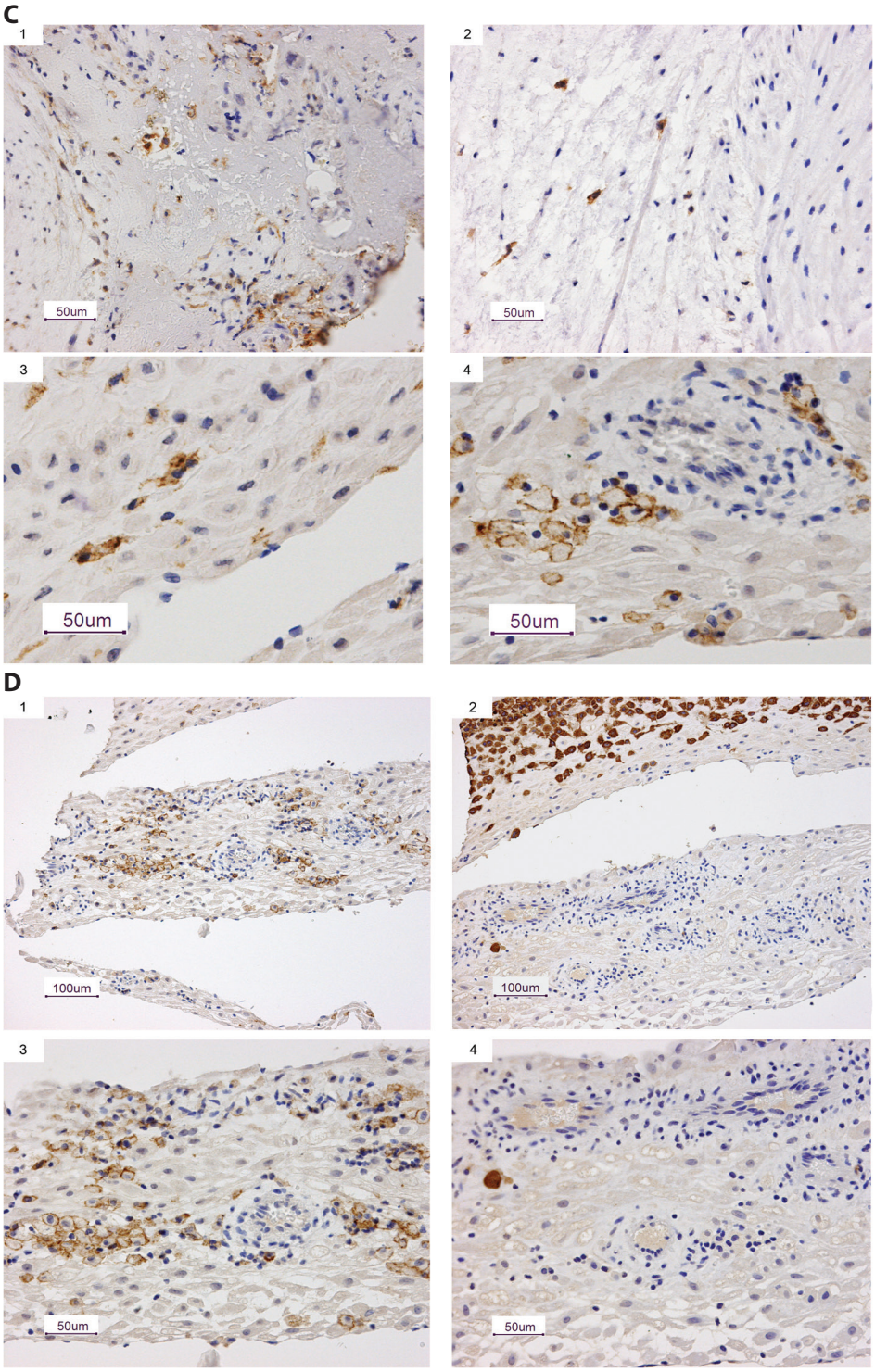
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term parturition LAIR-1 is highly expressed on amniotic fluid neutrophils. However, amniotic fluid sLAIR-1 is unlikely to originate from amniotic fluid cells or from spill-over from cord blood. Placenta tissue was generally negative for LAIR-1 staining.

Previously, only one single study described the soluble form of LAIR-1. Using an ELISA (limit of detection 1.5 ng/mL), Ouyang and colleagues have shown that sLAIR-1 was detectable in plasma of 68% of healthy individuals and in 96% of patients with hemorrhagic fever with renal syndrome (HFRS) and patients shortly after kidney transplant, both at increased levels.[23] Moreover, plasma concentrations of sLAIR-1 were higher in HFRS patients with oliguria and in kidney transplant patients with rejection. It was hypothesized that LAIR-1 is shed from the cell membrane of activated lymphocytes, possibly blocking the interaction with its ligand (ie collagen), thereby enabling persistent lymphocyte activation.[23] sLAIR-1 might serve as a predictor of immune response after kidney transplant. These hypotheses are consistent with our current findings, and with recent data of us, demonstrating increased amounts of sLAIR-1 in plasma and urine of rheumatoid arthritis patients.*[Olde Nordkamp et al, Manuscript Submitted For Publication]* We think that amniotic fluid sLAIR-1 reflects perinatal in utero immune activation.[24,25] Immune activation could contribute to increased lung maturation at term and amniotic fluid sLAIR-1 could be either a direct signal for lung maturation, or, more likely a reflection of the immune response, not directly involved in lung maturation.

Experimental studies in animals and observational studies in humans support the suggestion of a (complex) relation between fetal lung maturation, intra-amniotic inflammation and sLAIR-1. Intra-amniotic induction of a pro-inflammatory response doubled lung compliance and lung volume in prematurely delivered lambs.[14,15] Similarly, intra-amniotic injection of interleukin-1β (IL-1β), TNF-α, IL-6, and IL-8 in pregnant rhesus monkeys induced accumulation of neutrophils in the fetal lungs.[34] Studies on the relation between intra-amniotic inflammation and lung function in term newborns are lacking. However, in a recent study no differences were shown in the airway function of preterm infants exposed to histological chorioamnionitis and those not exposed.[35] In coherence, it was shown that the frequently reported adverse effect of chorioamnionitis on the risk of CLD of prematurity was consistently absent after adjustment for low gestational age.[36] Haddad and colleagues showed that an "acute inflammation gene expression signature" is generally present during physiologic term deliveries, and it was speculated that tissue homeostasis could be promoted by intra-uterine inflammation.[24] Previously, we have demonstrated that extensive intra-uterine inflammation is present during term delivery with spontaneous onset of labor.[27] Recently, the local expression of LAIR-2 in specific sites of the placenta was shown, adjacent to the extravillous trophoblast.[37] However, these findings were restricted to first trimester placentas and no data on LAIR-1 were presented.

Limitations of this study deserve careful discussion. First, due to the observational nature of this study, the complex relation between intra-amniotic inflammation, amniotic

fluid sLAIR-1 and newborn airway function remains unravelled. Second, in a pilot study among term spontaneous onset of labor deliveries, we demonstrated high correlations ($\rho > 0.6$) between amniotic fluid concentrations of IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, IL-23, TNF-α, MCP-1, MIF, sICAM, MIP-1α, eotaxin, IP-10, MIG, and sCD14 (data not shown), resembling the "acute inflammation gene expression signature" mentioned above.[24] Interestingly, the pattern of amniotic fluid sLAIR-1 concentration was distinct and poorly associated with before mentioned cytokines ($\rho < 0.3$). The effect of sLAIR-1 on neonatal lung function was not found for other pro-inflammatory cytokines nor for placenta histopathology (data not shown). Conceivably, sLAIR-1 is a unique representative of tissue inflammation or cell activation that does not overlap with established pro-inflammatory cytokines. Finally, the source of amniotic fluid sLAIR-1 remains unknown. Although we detected LAIR-1 expression on the cell surface of amniotic fluid neutrophils, secretion of sLAIR-1 could not be induced by vigorous stimulation. In addition, our data suggested that amniotic fluid sLAIR-1 is not the result of spill-over from cord blood. All amniotic fluid samples showed high L/S ratios, but no association was found with amniotic sLAIR-1 or airway compliance.[28] Furthermore, we did not demonstrate LAIR-1 positive cells in placenta tissue without or with mild signs of chorioamnionitis. In case of severe chorioamnionitis, mononuclear cells in the chorionic plate, the umbilical cord and the maternal side of the chorionic membranes were infrequently positive for LAIR-1. In future we will extend our studies of the source of intra-amniotic sLAIR-1 with measurements in lung tissue and fetal urine.

Our study has clinical implications. The association between intra-uterine sLAIR-1 and newborn airway function may extend our insight on the origin of childhood respiratory disorders.[2-5,38] Furthermore, it increases our insight in the mechanisms underlying fetal and newborn lung development.[14,15,35] In future, amniotic fluid proteins such as sLAIR-1 possibly may serve as biomarkers for early detection of the susceptibility to viral LRTIs or recurrent wheeze.[32,39,40] Eventually, such a biomarker can also be applied to target new preventive or treatment strategies to children at high risk of respiratory syncytial virus (RSV).[41,42]

In conclusion, newborn airway compliance is positively correlated with the amniotic fluid concentration of sLAIR-1 in healthy term infants. This effect is also associated with reduced risks of rhinovirus LRTI and recurrent wheeze. The source of intra-amniotic sLAIR-1 remains to be elucidated. The high level of amniotic fluid sLAIR-1 during term parturition probably reflects a general state of perinatal immune activation that is associated with lung maturation in term fetuses. These novel findings may improve our understanding of the origins of childhood and young adulthood respiratory disorders in general, and of the physiologic maturation of fetal and newborn lung function specifically.

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Conflict of interest statement

Prof.dr. L. Meyaard is named as inventor on a patent application on the LAIR–collagen interaction.

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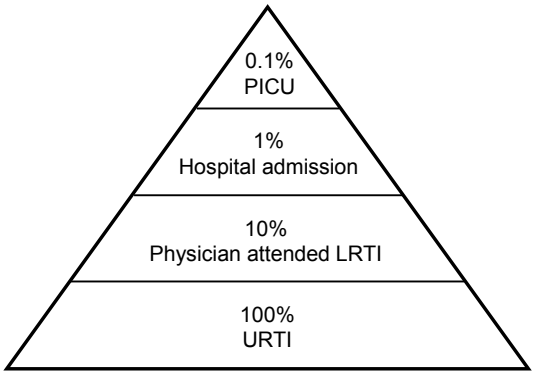
Chapter 9

General discussion

1. INTRODUCTION

The general aim of this thesis was to gain insight in the etiology of respiratory syncytial virus (RSV) bronchiolitis and to contribute to its prediction in healthy term infants. The majority of RSV infections occur in the community and involve otherwise healthy infants without any known risk factor, such as premature birth or congenital heart disease (**figure 1**).[1-5] The annual incidences of RSV associated hospitalizations, emergency department visits, and office visits in infants in the USA were estimated to be 2%, 11% and 31%.[2] Extrapolation of these numbers to the Dutch situation (16.7 million inhabitants; 185,000 births per year) demonstrates that in The Netherlands, yearly more than 40,000 children in the first two years of life (11%) visit a doctor's office because of a RSV infection **(figure 2)**.[2,6] Very similar estimates were found in two recent studies that used Dutch demographic, hospital discharge, primary care and viral surveillance data.[7,8] Given this very high incidence of RSV infection in the community, the impact and burden of this disease is highly relevant to children, parents, health-care workers, and society.[2,3,9]

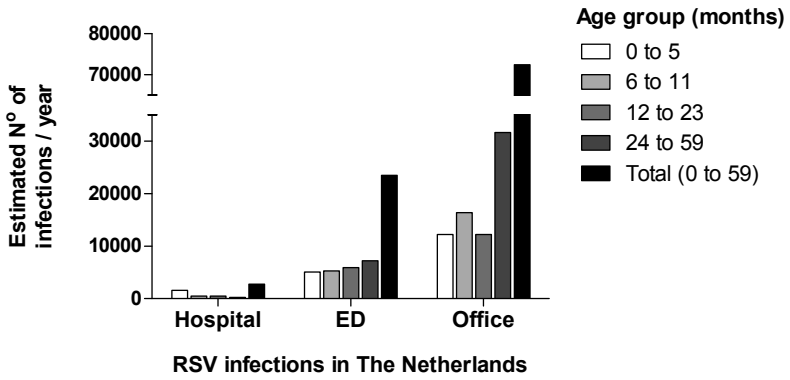
Figure 1. The RSV disease spectrum



Conceptual diagram of the incidence of different exponents of the RSV disease spectrum.

In this chapter, the results of the studies presented in this thesis are discussed in the context of what is known from previous studies on the prediction and the etiology of RSV bronchiolitis. The novel and distinct characteristics of our study domain (i.e. healthy term infants) and primary outcome (i.e. RSV bronchiolitis in the community, not requiring hospitalization) yield several new insights and conclusions, as compared to the well-studied domain and outcome of high-risk children admitted to hospital with severe RSV bronchiolitis. These contrasts are discussed and recommendations for future research are given.

Figure 2. Estimated annual number of RSV infections in The Netherlands



Dutch estimates of the incidence of RSV infection leading to hospital admission, emergency department (ED) visit and office visit. The numbers were extrapolated from USA estimates using Dutch demographic data (Hall et al, NEJM 2009; Netherlands Statistics 2010).[2,6]

2. PREDICTION OF RSV BRONCHIOLITIS

Early identification of newborns at high risk of RSV bronchiolitis is necessary for targeted prevention and timely therapeutic interventions.[10-14] In this section, the known risk factors for RSV hospitalization are summarized and compared to those we found for RSV bronchiolitis in the community in healthy term infants. In particular, the risk of RSV bronchiolitis that is conferred by birth shortly before the RSV season is discussed. In addition, the association with vitamin D deficiency at birth is shortly discussed. Finally, the novel prediction rule for RSV bronchiolitis in healthy term infants is highlighted and the need of validation is discussed.

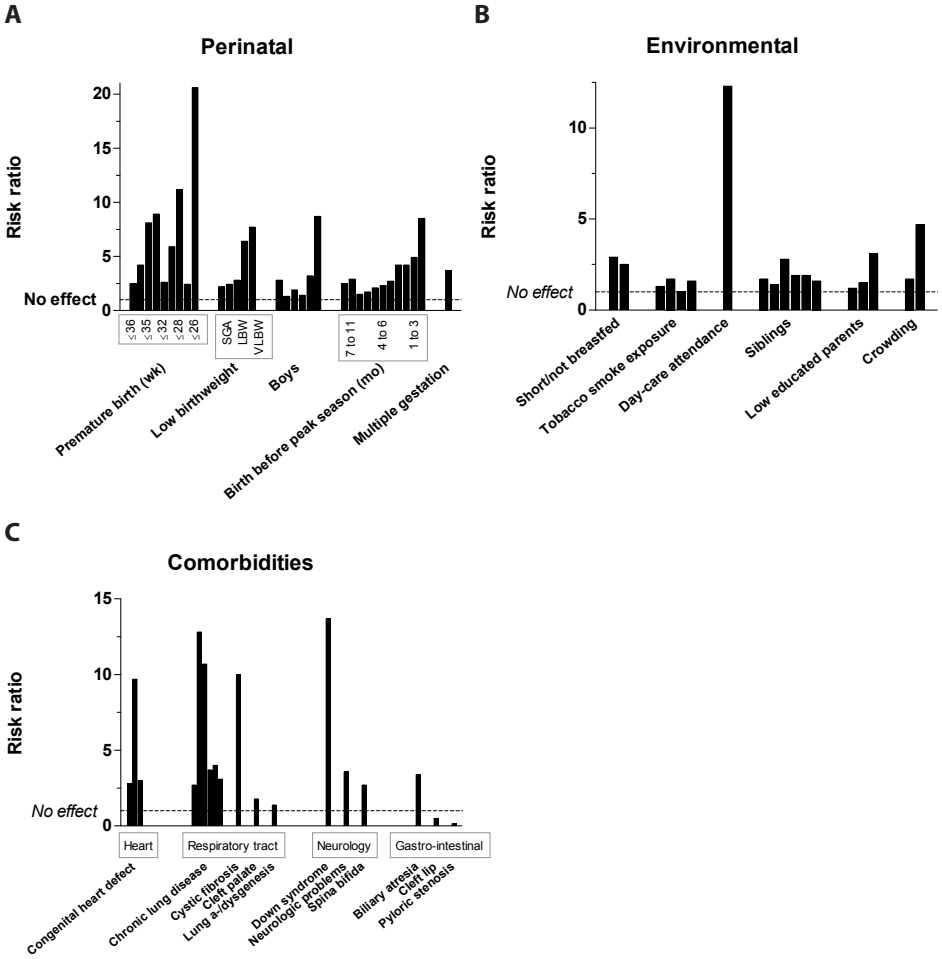
2.1. Known risk factors for RSV hospitalization

From large registry studies, we know that 50-90% of all children hospitalized for RSV bronchiolitis are healthy and born term.[1,4] However, in the past, several risk factors for hospitalization because of RSV bronchiolitis have been identified **(figure 3)**.

Perinatal factors

Premature birth and low birth weight are both dose-dependently associated with the risk of RSV hospitalization. Premature birth after <33 weeks gestation increases the risk with a factor two to eleven.[1,13,15-20] Very low birth weight (<1500 g) increases the risk of RSV hospitalization sevenfold.[21,22] In contrast, in a case-control study restricted to preterms of 33 to 35 weeks gestation, children with RSV hospitalization had higher birth weights than those without (2.20 versus 2.12 kg, $P = .04$).[23] Similarly, in our birth cohort, the children that developed RSV bronchiolitis had a higher birth weight as compared to children

Figure 3. Known risk factors for RSV hospitalization



Overview figure with (A) perinatal, (B) environmental, and (C) comorbidity risk factors for RSV hospitalization that have been published previously. The Y-axis represents the adjusted risk ratio for RSV hospitalization of children with a certain risk factor as compared to children without that risk factor.[1] In case only adjusted odds ratios were presented, the risk ratio was assumed to be approximated by the odds ratio, since the incidence of RSV hospitalization is generally low (<10%).[15,17-19,22,23,26,27,148] If no adjusted ratio was presented, crude risk ratios were used.[20,25,30,34] In case of absence of a control group, the average population risk of RSV hospitalization (1%) was used as denominator.[13,16,21,33] Potential risk factors that were only assessed in one single study yielding a small risk ratio were excluded, except for comorbidity risk factors. The author acknowledges that this overview is not exhaustive. Wk week, Mo month, SGA small for gestational age, LBW low birth weight, VLBW very low birth weight.

without (3.76 versus 3.59 kg, $P = .03$; **Chapter 2**).[24] In addition, birth shortly before or around the start of the RSV season increases the risk of RSV hospitalization two to eight-fold.[15,18-20,22,23,25-27] Multiple gestation has been associated with a fourfold increase of the

risk or RSV hospitalization.[20] Finally, male gender, white race and exposure to antenatal tobacco smoking generally impose a more modest risk of hospitalization because of RSV bronchiolitis.[1,15,23,25-27] A family history of eczema has been associated with a decreased risk of RSV hospitalization.[26]

Environmental factors

Day-care attendance is the environmental factor with the highest risk ratio for RSV hospitalization, namely twelve.[26] Children that have not been breastfed or for a short period and children with siblings at home have a two to threefold higher risk.[1,15,17,18,22,23,25,26] Low parental education, tobacco smoke exposure, rural residence, a higher number of persons in the child's household / sleeping in the same bedroom, and the presence of furred pets all impose a more modest risk of RSV hospitalization.[1,18,23,25,26] For the risk of RSV hospitalization (in the first five months of life), an interaction has been demonstrated between breastfeeding and cord blood anti-RSV antibodies.[25]

Comorbidities

Of all comorbidities, chronic lung disease of prematurity (CLD) is consistently found to confer the highest risk ratio for RSV hospitalization, ranging from three to twelve.[1,13,16,17,19,27] Second, congenital heart disease (CDH) markedly increases the risk of hospitalization because of RSV bronchiolitis.[28] These risks markedly decrease throughout the first year of life. Therefore, the relation between the month of birth and the start of the RSV season and the duration of study follow-up largely influence the observed incidences.[1,29] Children with Down syndrome, independent of the presence of CHD, have a fourteenfold increased risk of RSV hospitalization during their first year of life.[30] Furthermore, the high incidences of severe RSV disease among children with neuromuscular disease and children with congenital or acquired immunodeficiency suggest that these conditions are important risk factors.[20,31,32] Although studied in a small group of children, cystic fibrosis clearly is a risk factor of RSV hospitalization.[33] Finally, a number of congenital malformations has been associated with increased and decreased risks of RSV associated hospitalizations.[34]

Notably, in older studies immunofluorescence or viral culture was often used for the detection of RSV, while more recent studies use PCR. Therefore, the older studies may have had more information bias (i.e. classification bias), resulting in underestimated effects.[35,36]

2.2. Birth in relation to timing of the RSV season

The timing of birth plays a major role in the risk of RSV infection and the related disease severity. Although this thesis focuses on RSV bronchiolitis in the community, several mechanisms involved in RSV hospitalization apply, albeit modified. First, premature

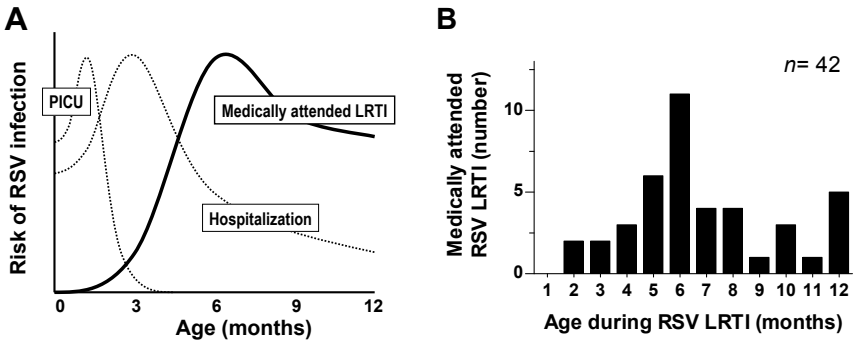
birth largely contributes to the risk of RSV bronchiolitis and its severity. An inverse correlation exists between gestational age and the risk of RSV bronchiolitis, independent of the presence of CLD.[1,13,15-20,37] A large proportion of preterms that develop RSV infection has severe disease that requires hospitalization, estimated to be 75% in a study by Hall and colleagues.[2]

Second, birth shortly before the RSV season is a dynamic risk factor, closely related to and dependant of gestational age. Preterm infants born at ≤30 weeks gestational age are at highest risk when born 1-2 months before the peak of the RSV epidemic. When exposed to RSV (after discharge from the neonatal ward), their chances of becoming symptomatic and severely affected are very high. A preterm infant born at 31-36 weeks gestational age is at highest risk when born around the start of the RSV season. Maternal antibodies probably give protection when born just before the peak of the RSV epidemic. If born around the start of the season, after some months of waning antibodies, during the peak of the epidemic, these children are at highest risk of becoming clinically ill. Similarly, infants born at term are protected by maternal antibodies.[38] When these children are born directly preceding the start of the RSV season, they will be protected during the peak of the epidemic. However, when these term infants are born three months before the start of the RSV season, they are aged six months during the peak of the epidemic. These term infants are at highest risk of symptomatic RSV disease, since at this age their anti-RSV antibodies have reached their nadir. Indeed, we found an increased risk of RSV bronchiolitis for children born in April to September (**Chapter 2**).[24] Consequently, the children that developed RSV bronchiolitis had a median age of six months (IQR 4-8). To conclude, the critical age at which exposure to the high viral pressure of the peak of the RSV epidemic confers the largest risk of severe RSV bronchiolitis depends on the gestational age. Of course, a low post-conceptional age represents both the risks of premature birth and low age during the peak of the RSV epidemic.[39]

Third, chronologic age during the RSV season not only determines the risk of RSV hospitalization. Age is also important for the disease severity associated with RSV infection.[2,40] Although children aged 6-12 months sometimes are hospitalized for RSV bronchiolitis, these children are far more likely to develop RSV bronchiolitis not requiring hospitalization or RSV upper respiratory tract infection (URTI).[2] **Figure 4** shows that outpatient-treated RSV bronchiolitis commonly develops at the age of six months and rarely occurs in children below age three months.

In conclusion, the timing of birth plays a crucial role in the chances of an infant to develop RSV infection and in the disease severity in case of infection. Premature birth confers the largest risk of RSV hospitalization during the first six months of life. Birth directly before the (start of the) RSV season is a dynamic risk factor, related to gestational age. Medically attended (but non-hospitalized) RSV bronchiolitis is most common in the second part of the first year of life of healthy term infants.

Figure 4. Age distribution of all medically attended RSV LRTI infants in comparison to other RSV patient populations



(A) Medically attended RSV LRTI in the first year of life in the population (this thesis) is compared to cohorts of hospitalized RSV LRTI patients.[2,24,39,149] (B) Age distribution of children with RSV LRTI in the first year of life (from data underlying **Chapter 2**).[24] PICU pediatric intensive care unit, LRTI lower respiratory tract infection.

Based on these data it is concluded that RSV bronchiolitis is very rare in young infants, but has a severe course when occurring.

2.3. Vitamin D deficiency at birth

Besides its well-known role in calcium homeostasis, vitamin D plays a role in several other biological pathways.[41] For example, vitamin D may facilitate antiviral responses by decreasing the inflammatory response of airway epithelial cells to viral infection (i.e. RSV) without jeopardizing viral clearance.[42] Cord blood concentrations of vitamin D vary according to the season of birth as a result of different levels of maternal sun exposure.[43] Since vitamin D has been implied in acute respiratory infections in general, and in RSV infections specifically, we hypothesized that infants born with a vitamin D deficiency are at increased risk of developing RSV bronchiolitis.[42,44-50] Indeed, we have demonstrated that children with cord blood vitamin D deficiency have a 43% increased risk of developing RSV bronchiolitis in their first year of life (**Chapter 3**). Children of non-Caucasian ancestry were more vitamin D deficient, but had a lower incidence of RSV bronchiolitis. Similarly, vitamin D deficiency was associated with birth in winter, but RSV bronchiolitis developed more often in children born in summer. Consequently, adjustment for ethnicity and month of birth strengthened the effects found (**Chapter 3**). Speculation on the mechanisms underlying this effect is outside the scope of this discussion. Recently, Camargo and colleagues demonstrated that cord blood concentrations of vitamin D were inversely associated with the risk of respiratory infections and childhood wheezing, which strongly supports our finding that in utero vitamin D exposure is inversely associated with risk of viral bronchiolitis.[51]

2.4. Prediction models for RSV infection

A number of prognostic models have been developed to predict RSV hospitalization.[15,22,23,26,37] In addition, we developed a prediction model for RSV bronchiolitis in the community (**Chapter 2**).[24] Although these studies vary considerably in study design and domain, there is a remarkable coherence in the demonstrated predictive factors (**table 1**). The majority of these studies indicate male gender, low birth weight, birth directly preceding the RSV season, and crowding (at home or attending day-care) as factors that predict RSV disease. Of note, the study of Simoes and colleagues and our prognostic study both indicate high birth weight as a risk factor (**Chapter 2**).[23,24] Apparently, children at the two extremes of the distribution of birth weight are at increased risk. Most probably, however, different mechanisms are involved. While low birth weight may be related to intra-uterine growth retardation and delayed lung maturation, high birth weight may be related to an altered immunologic phenotype associated with post-term labor and less effective response upon viral infections.[52,53] Although all prediction models include birth directly preceding the RSV season as a risk factor, our prognostic study differed with respect to the range of months that confer the highest risk (**Chapter 2**).[24] In the section above, this difference is explained by the nature of our domain (i.e. healthy term infants)

Table 1. Predictive variables of RSV bronchiolitis

1st Author	Doering	Law	Rietveld	Rossi	Simoes	Houben
Year	2006	2004	2006	2007	2008	2011
Sample size	1,158	1,832	140,661	437	553	298
Domain	Preterms 29-35 wks	Preterms 33-35 wks	Age <1 yr at start season (CLD <2 yr)	Children <4 yr	Preterms 33-35 wks	Term infants
Primary outcome (RSV)	Admission	Admission	Admission	Admission	Admission	LRTI
Birth early before start RSV season	Yes	Yes	Yes	Yes	Yes	Yes
Siblings / Birth order ≥2	Yes	Yes		Yes	Yes	Yes
Boys	Yes	Yes	Yes	No	Yes	No
Low birth weight	No	Yes	Yes	Yes		
High birth weight					Yes	Yes
Day-care attendance	No	Yes		No		Yes
ROC AUC	ND	ND	0.80	0.71	0.79	0.72

Predictive factors that were included in ≥2 RSV prediction models are presented in the table. In addition, the following factors were included in single models: Neurologic problems (Doering), ≥2 Smokers in household, ≥6 Persons in home, No eczema in 1st degree (Law), Premature birth, Chronic lung disease (Rietveld), Not breastfed / <2 months, Family atopy, Family wheeze (Simoes), and Highly educated parents (Houben).

"Yes" indicates a factor included in a prediction model. "No" indicated a factor not included in a prediction model. An "empty cell" indicates that a factor was not analyzed. CLD chronic lung disease, LRTI lower respiratory tract infection, ROC AUC receiver-operating characteristic area under the curve, ND not done

Table 2. Prediction of RSV LRTI in healthy term infants during the first year of life

Prediction rule		
Predictive factor	*Points*	
Siblings / daycare	2	
Birth in April to September	1	
Birth weight >4 kg	1	
Highly educated parents	1	
Total Sum	0 to 5	
Absolute risk of RSV LRTI		
Total sum	*Risk*	*Newborns with score*
0 to 2 points	3%	20%
3 points	7%	33%
4 points	22%	39%
5 point	32%	8%

(Upper part) Predictive factors that can be present or absent in healthy term newborns, added up in a prediction rule for RSV LRTI in the first year of life. (Lower part) Absolute risks of RSV LRTI in the first year of life, corresponding to the calculated prediction rule sums, with the proportion of newborns within each stratum. (From **Chapter 2**, *n* = 298)[24] LRTI lower respiratory tract infection

and primary outcome (i.e. RSV bronchiolitis in the community). Crowding (at home or attending day-care) is the most direct representation of exposure of the child to RSV (**Chapter 2**).[24] Conceivably, in our population, host factors contribute less to the risk of RSV bronchiolitis than in premature infants. Finally, in our cohort study and in contrast with studies of others, children with highly educated parents were at increased risk of developing RSV bronchiolitis (**Chapter 2**).[24] Possibly, these parents have a higher level of suspicion or a lower threshold to visit a physician, which reflects health care consumptive behaviour in daily practice.[9,54,55]

The prediction model that we developed for RSV bronchiolitis in healthy term infants was tested for over-fitness and further simplified to be used as a prediction rule by pediatricians and general practitioners (**table 2**, **Chapter 2**).[24,56] Although the overall discriminatory power of the prediction rule is moderate (ROC AUC 0.72), it can differentiate between a low risk of RSV bronchiolitis (3%) and a high risk (32%). However, the true value of a prediction rule needs to be estimated by validation in a remote population.[57] The Spanish model predicting RSV hospitalization in preterm infants has been validated in a German population.[23] Although the bootstrapping techniques that we used are a form of internal validation that adjusts for over-fitness of the derived model, true external validation is the optimal method. Therefore, we recommend validating our prediction rule prior to use in general practice. It would be important to choose another country, adhering to the unselected domain (i.e. healthy term infants). On the other hand, a country that is less well-educated or less economically developed would probably

introduce too much difference. In future, the validated prediction rule could be used to target preventive and / or therapeutic strategies to high risk populations, in daily practice or in randomized controlled trials.[10-12,14]

3. PATHOGENESIS OF RSV INFECTION

The pathogenesis of RSV bronchiolitis in healthy term infants is largely unknown. In absence of classic risk factors, such as CLD of CHD, it is impossible to define what determines the risk of a RSV bronchiolitis.[2] Certainly, epidemiologic research has identified several new risk factors, including day-care attendance and birth around the start of the RSV season.[58] However, RSV bronchiolitis also affects children with no risk factors, suggesting that other mechanisms are involved.

In this section, the causal roles of three potential mechanisms of RSV bronchiolitis are discussed: RSV viral load, intra-uterine inflammation, and newborn airway function. In particular, the potential effects of fetal lung exposure to intra-uterine inflammation in healthy term newborns are distinct and unprecedented. Although healthy term infants are generally not affected by immunodeficiencies, specific impaired immune responses or genetic polymorphisms affecting the immune system have been (suggested to be) related to the risk of RSV bronchiolitis and other respiratory infections.[59-74] However, a full discussion on immunodeficiencies and immunologic genetic polymorphisms underlying RSV bronchiolitis is outside the scope of this thesis.

3.1. Viral load

Disease severity of RSV infection is determined by a plethora of both host and viral factors.[75] As illustrated in **section 2.2**, the age of the child during infection and his / her gestational age play a major role. However, also viral load has repeatedly been listed as an important factor determining disease severity (**see table 3**).[76-79] In experimentally infected healthy adults, DeVincenzo and colleagues recently showed that RSV viral load is correlated with clinical signs and symptoms, as well as the production of pro-inflammatory cytokines and mucus.[80] It appears biologically plausible that the viral load in the respiratory tract measured during infection determines disease severity. First, a high viral load reflects the intensity of the infection. Second, viral load at a given moment during infection also represents the capacity of viral replication and thereby the temporal extent of the infection. In contrast, an inverse or absent effect of RSV viral load on disease severity has also been postulated. The extent and the nature of the antiviral immune response would be the major factor influencing disease severity.[81,82] However, although Legg and colleagues demonstrated similar initial viral loads in infants with RSV URTI and RSV bronchiolitis, they found impaired viral clearance in infants with bronchiolitis.[83] In a

well-described domain of primary RSV infections in the community, we showed that RSV viral load in nasopharyngeal aspirate was positively correlated to disease severity (**Chapter 4**).[84] This correlation was stronger in case of a single RSV infection and absent in rhinovirus infection.

Timing of sampling during the course of the infectious episode determines the detected RSV viral load.[11,85] In healthy subjects, after three days of nasal inoculation, RSV viral loads peaked after five to seven days, with a steep increase of the viral load over the first days of infection. Therefore, sampling by parents at home (e.g. day 2), at the general practitioner's office (e.g. day 3), upon hospital admission (e.g. day 4), at intubation (e.g. day 5), or during reconvalescence (e.g. day 10) markedly influences the detected viral loads.[85] Furthermore, viral load also depends on the absence or co-presence of other infections, both viral and bacterial (**Chapter 4**).[84,85] Other factors, such as exposure to tobacco smoking could also influence the viral load in the airways. Likewise, the nature (e.g. Th1 / Th2) and extent of the antiviral response should be assessed.[86] For example, high levels of cytokines in the respiratory tract can both be a consequence of a high viral load or a cause of a low viral load.

Besides, one could speculate on the level of disease classification at which RSV viral load may influence disease severity. We hypothesize that viral load does not contribute to the major differences in disease severity: viral load does not determine whether an RSV infection will be limited to mild symptoms that can be treated at home, or severe disease requiring mechanical ventilation.[83] Remarkably, we found similar RSV viral loads when comparing these extremes of severity (**see Chapter 4**).[84] However, several studies, including ours, have found correlations between RSV viral load and disease severity within the study domain. In other words, RSV viral load appears to modulate disease severity given a clinical setting (e.g. home, hospital, PICU).

3.2. Intra-uterine inflammatory signature

Healthy term infants that develop RSV bronchiolitis become ill in absence of host susceptibility factors or due to underlying asymptomatic susceptibility. Since some infants develop RSV bronchiolitis during the first month of life, it is likely that intra-uterine factors contribute to the susceptibility for RSV. The fetal respiratory tract is continuously exposed to amniotic fluid throughout pregnancy, due to amniotic fluid production in the lungs and due to fetal breathing movements.[87-89] Therefore, amniotic fluid content may play an important role in the development and maturation of the respiratory tract.

Intra-uterine inflammation and infection are associated with premature rupture of membranes, with preterm delivery, and with positive and negative perinatal outcomes after premature birth.[90-96] Of all potential causative organisms, *Ureaplasma urealyticum* is identified most frequently, either by tissue culture or by PCR.[97,98] Other common organisms include *Mycoplasma hominis, Streptococcus agalactiae, Escherichia coli, Fusobacte-*

Table 3. Association between RSV viral load and disease severity

1st Author, year	*Domain*	*n*	*Sampling*	*Detection*	*Severity*	*Difference / correlation*	*P*
Buckingham 2000	Admitted, <2 yr	39	Nasal aspirate	VC	MV vs No MV	+1.15 log pfu/mL	.02
Hall 1976	Admitted, <3 yr	19	Nasal wash	VC	Pulmonary consolidation vs Not	4.9 vs 4.1 log $TCID_{50}$/mL	<.025
					Hypoxia vs Not	4.6 vs 4.1 log $TCID_{50}$/mL	<.10
Fodha 2007	Admitted, <1 yr	81	NPA	rt-PCR	RR>60, LOS>7, PICU or MV	4.9 vs $1.7x10^6$ copies/uL	.02
Legg 2003	Birth cohort, ≥1 atopic parent	88	NP wash	rt-PCR	Acute bronchiolitis vs URTI	VL clearance over 1wk	.06
DeVincenzo 2005	Admitted, <2 yr	141	Nasal wash	VC	LOS	+0.8 day / log pfu/mL	.008
Wright 2002	Admitted, <2 yr	77	Nasal wash	VC	Illness score (Rodriquez 1997)	ρ = 0.16	>.05
Houben 2010	Birth cohort, <1 yr	82	NPA	rt-PCR	Severity score (Gern 2002)	ρ = 0.52	.003

NPA nasopharyngeal aspirate, NP nasopharyngeal, VC viral culture, rt-PCR realtime polymerase chain reaction, MV mechanical ventilation, LOS length of stay (days), RR respiratory rate (/min), PICU pediatric intensive care unit, URTI upper respiratory tract infection, $TCID_{50}$ 50% tissue culture infective dose, VL viral load. Hypoxia was defined as pO_2 ≤60 mmHg.

rium species, *and Gardanella vaginalis*.[90] In general, chorioamnionitis is considered to be the maternal response to an ascending urogenital infection, while funisitis is associated with a (more severe) fetal inflammatory response.[99,100] The occurrence of histological chorioamnionitis is inversely associated with gestation age.[95] However, in placentas of spontaneous onset of labor term deliveries, we also detected chorioamnionitis (18%) and funisitis (3%) (**Chapter 6**).[101] Similarly, we demonstrated high concentrations of interleuking-6 (IL-6), IL-8 and tumor necrosis factor-α (TNF-α) in transvaginally collected amniotic fluid samples of spontaneous onset of labor deliveries. In contract, in absence of spontaneous onset of labor, during elective caesarean section, funisitis was absent, the proportion of chorioamnionitis was fivefold lower, and the amniotic fluid concentrations of IL-6, IL-8 and TNF-α were tenfold lower.[101] We found no increase of amniotic fluid concentrations of pro-inflammatory cytokines for prolonged rupture of membranes, for progression of labor, or for acceleration to delivery. Neither did we find a difference between transvaginal collection of amniotic fluid and collection via an intra-uterine cathether (**Chapter 6**).[101] These findings suggest that high amniotic fluid concentrations of pro-inflammatory cytokines are not a product of labor, but rather play a role in the onset of spontaneous labor at term. This conclusion is in coherence with the findings of Haddad and colleagues, who demonstrated an "acute inflammation gene expression signature" in chorioamniotic membranes during labor at term.[102] Similarly, they showed

that the "differentially increased expression of the inflammatory response signature is independent of the duration of labor, as well as the interval after rupture".[102] The pro-inflammatory profile either reflects "hyperimmune responders" or represents a way of tissue homeostasis. Notably, Velez and colleagues have shown that polymorphisms of the IL-6 gene are associated with different amniotic fluid concentrations of IL-6. Moreover, a gene-by-infection interaction with microbial invasion of the amniotic cavity existed.[103]

The associations between intra-uterine inflammation and neonatal outcomes can be divided in effects on the respiratory tract and effects on other organ systems.[91-93] We will focus on the respiratory effects. Hitti and colleagues demonstrated that amniotic fluid infection and elevated TNF-α are associated with respiratory distress syndrome (RDS), intraventricular hemorrhage, necrotizing enterocolitis, and multiple organ dysfunction in infants born ≤34 weeks of gestation.[93] But in a large cohort study of preterms, histological chorioamnionitis without or with umbilical vasculitis had a protective effect for RDS.[94] In the same cohort, similar protective effects were found for CLD. In contrast, neonatal sepsis was strongly associated with an increased risk of CLD.[95] Been & Zimmerman reviewed the literature on this issue.[92] In a series of eighteen studies, there was ample evidence that chorioamnionitis protected against the development of RDS. An increased incidence of CLD was shown in six of the eighteen studies. However, this effect disappeared after adjustment for gestational age and other potential confounders.[92] Fetal inflammation was invariably associated with a decreased risk of RDS, after adjustment. There was no association with the development of CLD. Differences in study domain, definitions of determinants and outcomes, and trends in treatment practices (e.g. antenatal corticosteroids) complicate adequate comparisons.[92] In a recent prospective cohort study of the same group, chorioamnionitis was independently associated with a decreased risk of severe RDS.[91] In another recent study of preterm infants, there was no association between exposure to histological chorioamnionitis, with or without funisitis, and the development of CLD. Moreover, neonatal lung function at the post-menstrual age of 36 weeks did not differ between exposed and unexposed infants.[96]

We demonstrated very high concentrations of amniotic fluid pro-inflammatory cytokines during term spontaneous onset of labor (**Chapter 6**).[101] Apparently, the majority of term newborns are exposed to these high levels of inflammation and it is plausible that this exposure confers a beneficial effect for the newborn infant in general, and for the respiratory system specifically. In contrast with the situation in preterm delivery, there are few suggestions that exposure to intra-uterine inflammation may negatively affect the newborn. We therefore hypothesized that exposure to high levels of amniotic fluid pro-inflammatory cytokines protects against the development of RSV bronchiolitis in healthy term infants. Indeed, we found a reduced risk of physician attended RSV infection in the first year of life (**Chapter 7**). The amniotic fluid concentrations of IL-8 and the proportion of detectable TNF-α were twofold lower in children that developed

a medically attended RSV infection. This effect did not change after adjustment for potential confounders.

In addition, within the group of children with RSV bronchiolitis, the number of wheezing days in the first year of life was inversely correlated with the concentration of amniotic fluid IL-8 (**Chapter 7**). Thus, in healthy term infants, exposure to high intra-uterine pro-inflammatory signals decreases the susceptibility for and / or the severity of RSV infection and recurrent wheezing. The underlying mechanism most probably involves either the maturation of the innate mucosal immunity, or maturation of the newborn airways. Conceivably, a strong but orchestrated set of pro-inflammatory signals induces the recruitment of monocytes and the maturation of or differentiation into lung dendritic cells, which protect the developing infant respiratory tract.[102,104,105]

3.3. Intra-uterine determinants of newborn airway function

The risk of RSV bronchiolitis in healthy term infants can also be mediated by a suboptimal pre-existent airway function.[106,107] Reduced airway function preceding RSV acquisition can both increase the chance and the severity of RSV infection. First, infants with reduced airway function will exhibit diminished clearing of their lower airways in case of an RSV URTI.[108,109] Second, the clinical severity of RSV infection in infants with reduced airway function will be larger, as compared to infants with normal airway function. In a study of 39 preterm infants, a high airway resistance at 36 weeks post-menstrual age was associated with increased risk of RSV bronchiolitis and with increased prevalence of wheezing symptoms.[110] Apart from this study, direct evidence for the relation between newborn airway function and the risk or severity of RSV infection is lacking.

Several other studies have demonstrated that reduced airway function at birth is associated with more respiratory symptoms later in life. In a birth cohort of 802 healthy infants, Haland and colleagues showed that reduced airway function at birth is associated with a twofold increased risk of current and ever diagnosed asthma at the age of ten years.[111] Moreover, in the Tuscon birth cohort of 124 healthy newborns, Martinez and colleagues showed that diminished airway function at birth predisposes to a markedly increased risk of wheezing illnesses during the first year of life.[112] RSV was the most frequently detected pathogen.[113] In another birth cohort of healthy infants, reduced maximum flow at functional residual capacity (V'maxFRC) also preceded bronchiolitis and recurrent asthmatic symptoms.[114] A similar association was found for V'maxFRC and recurrent wheeze during the first year of life in term infants.[115] In a cohort of infants of atopic parents, lower V'maxFRC and more bronchial responsiveness were associated with increased risk of lower respiratory tract infection (LRTI) in boys and girls, respectively.[116] Katier and Van der Zalm and colleagues have related high airway resistance at birth with increased risk and duration of wheezing and other respiratory symptoms during the first year of life.(*Thesis chapters, Katier 2006, Van de Zalm 2009*) They also showed that high

airway resistance at birth confers a twofold higher risk of rhinovirus LRTI, as compared to low airway resistance.[117] Finally, tracking of airway function has recently been shown in participants of the Tuscon birth cohort, followed from birth until the age of 22 years.[118] In conclusion, reduced newborn airway function is associated with increased risk of LRTI and viral induced wheeze in infancy, recurrent wheeze at pre-school age, and asthma during childhood. In addition, there are suggestions that the risk and severity of RSV infection is determined by reduced airway function at birth.

What determines newborn airway function in healthy term infants? In a birth cohort study of 450 healthy term infants, the determinants of newborn airway function (measured at a mean age of 4.7 weeks) were studied.[119] In multivariable analysis, compliance of the total respiratory system was associated with birth weight and length, explaining 22% of the variance of compliance, and resistance was associated with birth length and gender, explaining 5% of the variance of resistance.[106] We found similar correlations between compliance and resistance and anthropometric measurements (**Chapter 8**). In addition, in animal experiments and in human observational studies, reduced airway function after term delivery has been associated with exposure to antenatal maternal smoking, parental lung function, parental asthma, and genetic polymorphisms.[107,107,120-122]*(Thesis chapter, Koopman 2011)* There is debate on the question whether parental lung function associations represent a genetic trait or environmental factors (nutritional, airway exposures).[123] Recently, it was shown that familial aggregation of body size only partially explains the association between lung function of newborns and their parents.[107] Furthermore, maternal respiratory tract infections during pregnancy were associated with reduced airway compliance in their newborns, independent of the presence of siblings and maternal smoking.[124] Finally, Kramer and colleagues have demonstrated increased lung volume and compliance of preterm lambs after intra-amniotic injection of LPS in pregnant sheep.[125,126] These experiments also showed markedly increased expression of pro-inflammatory cytokines, most evident in amniotic fluid and in pulmonary lavage fluid. Moreover, epidemiological studies have suggested that the prevalence of bronchiolitis and asthma is increased in children born via elective caesarean section, reflecting a less pro-inflammatory environment.[127-132] Apparently, fetal lung exposure to pro-inflammatory signals has a beneficial influence on fetal lung maturation and function.

In our birth cohort study, we were able to study the role of newborn airway function as a possible determinant or mediator of RSV bronchiolitis in healthy term infants. Moreover, we could analyse associations between intra-uterine inflammation and newborn airway function. Airway compliance was positively correlated with amniotic fluid concentration of soluble leukocyte associated IgG-like receptor-1 (sLAIR-1, **Chapter 8**). LAIR-1 is an inhibitory receptor that is present on the surface of immunologic cells.[133] Binding of its natural ligand, collagen, increases the threshold for immune activation.[133,134] To the contrary, secretion of LAIR-1, yielding sLAIR-1, is regarded to reflect general im-

mune cell activation.[135,136] Additionally, we found an increased prevalence of recurrent wheezing in infants with a low amniotic fluid level sLAIR-1 (**Chapter 8**). Finally, there was a trend of an inverse relation between the amniotic fluid level of sLAIR-1 and the risk of rhinovirus bronchiolitis in the first year of life (**Chapter 8**). We were unable to show that the protective effect of high amniotic fluid levels of pro-inflammatory cytokines against RSV bronchiolitis is mediated by enhanced lung development, possibly due to lack of statistical power. However, conceivably, pro-inflammatory signals can instruct the fetal lung to enhance maturation and / or growth of alveoli, bronchioli and interstitial tissue. Other amniotic fluid cytokines and chemokines might exhibit equal or distinct mechanisms resulting in enhanced lung development.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusions

The global estimated annual numbers of non-severe and severe (i.e. hospitalized) RSV infections in children younger than five years of age are 34 million and 3.4 million.[3] Most children with RSV bronchiolitis are treated outside the hospital and most children with RSV bronchiolitis are healthy term infants.[1-4] The Netherlands Amniotic Fluid Cohort Study was initiated to gain insight in the etiology of RSV bronchiolitis and to contribute to its prediction in healthy term infants. Based on the results of the studies reported in this thesis and previous studies, we were able to accomplish the objectives formulated in the introduction (**Chapter 1**).

Prediction of RSV bronchiolitis in healthy term infants

We found the following predictive factors for RSV bronchiolitis in healthy term infants: presence of siblings / day-care attendance, birth in April to September, birth weight >4 kg, and highly educated parents. Abundant viral exposure (i.e. siblings, day-care) appeared to be the strongest predictor of RSV bronchiolitis in healthy term infants. The derived prediction rule could differentiate between children with low risk (3%) of RSV bronchiolitis and high risk (32%). Children with RSV bronchiolitis had an increased prevalence of recurrent wheezing and reduced HRQoL during the first year of life.[24]

In addition, vitamin D deficiency at birth was also independently associated with an increased risk of RSV bronchiolitis.

Pathogenesis of RSV bronchiolitis in healthy term infants

RSV viral load was positively associated with disease severity in primary RSV infections. A high correlation with RSV viral load was found for single RSV infections and for RSV co-infections.[84] In line with these findings, the sensitivity of the nasal swab for the mo-

lecular detection of RSV infection in the community was generally moderate, but high in case of more severe RSV infection.[137]

Term spontaneous onset of labor vaginal delivery was associated with high amniotic fluid concentrations of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) and a high prevalence of histological chorioamnionitis.[101] Fetal exposure to higher amniotic fluid concentrations of pro-inflammatory cytokines (IL-8 and TNF-α) appeared to be associated with a decreased risk of medically attended RSV bronchiolitis in the first year of life. In addition, in the group of children that developed RSV bronchiolitis, the number of days with wheezing symptoms in the first year of life was inversely associated with the amniotic fluid concentration of IL-8. Finally, newborn airway compliance was positively correlated with the amniotic fluid concentration of sLAIR-1, which is considered a marker of general immune activation. Additionally, children exposed to a high level of amniotic fluid sLAIR-1 had a decreased incidence of recurrent wheezing.

Implications of intra-uterine inflammation during term vaginal delivery

Given the finding that a firm pro-inflammatory profile was present in amniotic fluid of the majority of physiologic term deliveries, one could speculate on the role of exposure of the fetal mucosa to these inflammatory signals.[102] Maybe these signals instruct the local mucosal immune system to prepare for the invasions of a plethora of pathogens, allergens and pollutants after birth.[68,138,139] Since the impressive inflammatory response is present during term deliveries on such a large scale, it is likely that the beneficial effect found is not specific for RSV bronchiolitis, but also holds true for other childhood respiratory condition, such as viral-induced wheezing and asthma. Maybe the origin of common human respiratory disorders should be sought antenatally.[140,141]

Conclusion

In conclusion, RSV bronchiolitis in healthy term infants has a large impact on children, their families, the health-care system, and society. It has distinct risk and predictive factors, resulting in a novel simple prediction rule. The pathogenesis of RSV bronchiolitis in healthy term infants is characterized by a high viral load and fetal lung exposure to low amniotic fluid pro-inflammatory cytokines. More study is required to determine whether the RSV-protective effect of exposure of the fetal airway mucosa to a firm inflammatory response during term vaginal delivery also applies to other respiratory conditions in childhood and young adulthood, such as asthma.

4.2. Recommendations for future research

Origins and effects of intra-uterine inflammation during term physiologic parturition

Future research into the specific origins of the extensive intra-uterine inflammation during term spontaneous onset deliveries will improve the insight in term and preterm human parturition.[102,142] Ultimately, novel preventive and therapeutic strategies to establish term physiologic delivery could be achieved. In line with the findings concerning the relationship between amniotic fluid cytokines and the risk of RSV bronchiolitis presented in this thesis, future research should attempt to expand this finding to childhood respiratory disease. Is exposure to the firm intra-uterine pro-inflammatory profile also associated with reduced occurrence of other common childhood respiratory conditions, such as viral-induced wheezing, rhinovirus LRTI or asthma?[118,140] In addition, is there also a beneficial effect on other mucosal sites, including the gastro-intestinal tract? Finally, can amniotic fluid proteins be used as biomarkers for early detection of respiratory disease in infancy?[143]

Validation, optimization and extension of the prediction model

The prediction rule that has been developed in this thesis requires external validation in a remote cohort of healthy term infants.[57] The prediction model should be further optimized, adjusting the relative weight of the predictive variables, and possibly deleting or inserting variables (e.g. breastfeeding, family atopy or tobacco smoke exposure).[23,26] Upon optimization and validation the prediction rule could be clinically applied.

Randomized clinical trials

Two randomized clinical trials (RCT) should be carried out to prevent RSV bronchiolitis. First, a RCT may be carried out to investigate whether use of supplemental vitamin D during pregnancy protects against RSV bronchiolitis.[51] A second RCT is related to prevention of RSV bronchiolitis by reduction of viral exposure. Since abundant exposure to RSV is the strongest risk factor for the development of RSV bronchiolitis in healthy term infants, minimizing this exposure could conceivably reduce the incidence and / or the severity of RSV LRTIs.[144-147] Surprisingly, a RCT testing this hypothesis has not yet been performed. Newborns at high risk of RSV bronchiolitis could be randomized to either instruction of strict hand hygiene and the provision of antiseptic agents, or no instruction or care as usual.

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Summary

Samenvatting

Summary

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis in infants. Annually, an estimated total of 34 million children under the age of 5 worldwide develop severe RSV infection, with 3.4 million being hospitalized. In the United States more than 2 million children under the age of 5 develop medically attended RSV infection each year, of which 97% are treated as outpatients. In half of the infants with RSV bronchiolitis recurrent wheeze during preschool years develops resulting in health-related quality of life (HRQoL).

Of all children that are hospitalized because of RSV bronchiolitis, approximately 70% have no underlying disorder. Moreover, most children that develop RSV bronchiolitis in general have no known risk factors, such as congenital heart disease, chronic lung disease, or premature birth. Establishing new predictive factors and a prediction rule for RSV bronchiolitis in healthy term infants could help to target preventive and early therapeutic interventions in future. Moreover, the etiology of RSV bronchiolitis in healthy term infants is largely unravelled and new insights may ultimately contribute to the development of new strategies to prevent or treat RSV bronchiolitis.

This thesis describes a series of studies on the prediction and the pathogenesis of RSV bronchiolitis in healthy term infants, all carried out in the Netherlands Amniotic Fluid (NAF) cohort, a healthy term birth cohort based in the University Medical Center Utrecht and the Diakonessen Hospital (both Utrecht, The Netherlands).

In **Chapter 1**, RSV bronchiolitis in healthy term infants is comprehensively introduced. The majority of medically attended RSV infections occur in children above the age of six months and do not require hospital admission. Nevertheless, the socioeconomic burden of RSV infections is substantial. The general aims of this thesis and the specific research questions of the studies on RSV prediction and pathogenesis in this thesis are discussed.

In **Chapter 2**, novel predictive factors for RSV bronchiolitis in the community and a simple clinical prediction rule are presented. In the NAF birth cohort study, 298 healthy term newborns were followed throughout the first year of life. Parents kept daily logs and collected nose-throat swabs during respiratory tract infections. The primary outcome was RSV lower respiratory tract infection (LRTI), which was defined as the combination of a positive RSV PCR and acute wheeze or moderate to severe cough. Of the participating children, 42 (14%) developed RSV LRTI. Independent predictors for RSV LRTI were: day-care / siblings, high parental education, birth weight >4 kg, and birth in April-September. The discriminative power of the prediction model was moderately high (ROC AUC 0.72). A clinical prediction rule was derived, with possible scores ranging from 0 to 5 points. The absolute risk of RSV LRTI was 3% in children with a score

≤ 2 and 32% for the children with score 5. Furthermore, 62% of the children with RSV LRTI suffered from wheezing during the first year of life versus 36% in those without. We concluded that a simple clinical prediction rule can identify healthy newborns at risk of RSV LRTI. Physicians can differentiate between children with high and low risk of RSV LRTI, and subsequently target preventive and monitoring strategies at high risk children.

In **Chapter 3**, the association between plasma concentrations of 25-OH vitamin D at birth and the subsequent risk of RSV LRTI was assessed. In the NAF birth cohort study, cord blood concentrations of 25-OH vitamin D were related to risk of RSV LRTI in the first year of life. Of 156 healthy term newborns, 18 (12%) developed RSV LRTI. The mean cord blood concentration of 25-OH vitamin D was 82 nmol/L. Overall, 27% of newborns had 25-OH vitamin D concentrations <50 nmol/L. Maternal vitamin D3 supplementation during pregnancy was positively associated with cord blood 25-OH vitamin D. Concentrations of 25-OH vitamin D were lower in newborns who developed RSV LRTI compared to those who did not (65 versus 84 nmol/L). Newborns with 25-OH vitamin D <50 nmol/L had sixfold increased risk of RSV LRTI in the first year of life compared to those with 25-OH vitamin D ≥75 nmol/L. We concluded that vitamin D deficiency at birth is associated with increased risk of RSV LRTI in the first year of life. Intensified routine vitamin D supplementation during pregnancy may be a useful strategy to prevent RSV LRTI during infancy.

In **Chapter 4**, we hypothesized that disease severity is correlated with viral load in primary RSV infection in the community. Eighty-two healthy term infants of the NAF birth cohort were included at signs of their first respiratory tract infection. Nasopharyngeal aspirate was obtained within 48-96 hours and disease severity was assessed with a previously published severity scoring model. PCR was applied to test the aspirates in a semi-quantitative way for the presence of ten respiratory pathogens. In case of multiple infection, the pathogen with the highest load was defined as the primary pathogen. Median age at first respiratory tract infection was 3 months. Pathogens were detected in 77 infants (94%); more than one pathogen was detected in 35 infants (43%). RSV was present in aspirates of 30 infants; in 16 aspirates RSV was the primary pathogen. A negative correlation between RSV CT-value and disease severity was found in all RSV cases ($\rho = -0.52$) and in cases with RSV as the primary pathogen ($\rho = -0.54$). This is the first report on viral loads in previously healthy infants with primary RSV infection in the community. We concluded that disease severity correlated positively with viral load during primary RSV infection.

In **Chapter 5**, we investigated the sensitivity of nasal swabs and nasopharyngeal aspirates and assessed whether viral load played a role in the sensitivity of either sampling method. Healthy term infants of the NAF birth cohort were included at signs of first respiratory tract infection. Paired nasopharyngeal aspirates and nasal swabs were collected and real-time PCR was carried out as before mentioned. Paired samples were collected

in 98 infants, with at least one respiratory pathogen identified in 94 cases. Rhinovirus ($n = 67$) and RSV ($n = 39$) were most frequently detected. The sensitivity of the nasal swab was lower than the nasopharyngeal aspirate, in particular for RSV (51% versus 100%) and rhinovirus (75% versus 97%). Sensitivity of the nasal swab was strongly determined by the CT-value. Sensitivity of the swab for RSV, but not rhinovirus, was 100% in children with severe symptoms (score ≥11). We concluded that the nasal swab may be used for community based studies on RSV infection, though its sensitivity is lower than of the aspirate, in particular for detection of mild cases.

In **Chapter 6**, we hypothesized that intra-uterine inflammation at term is associated with spontaneous onset of labor. Within the NAF birth cohort, a cross-sectional study of 375 spontaneous onset of labor term vaginal deliveries and elective caesarean sections (CS), without signs of labor, was carried out. Placentas and amniotic fluid samples were collected during labor and / or at delivery. Histological signs of placenta inflammation were determined. Amniotic fluid pro-inflammatory cytokine concentrations were measured using ELISA. In term vaginal deliveries, more signs of intra-uterine inflammation were found than in elective CS: the prevalence of chorioamnionitis was higher (18 versus 4%) and the amniotic fluid concentration of interleukin-6 (IL-6) was higher (3.1 versus 0.4 ng/mL). Similar results were obtained for IL-8 (10.9 versus 1.0 ng/mL) and the proportion of deliveries with detectable amniotic fluid tumor necrosis factor-α (TNF-α, 50 versus 4%). We concluded that spontaneous term delivery is characterized by histological signs of placenta inflammation and increased amniotic fluid pro-inflammatory cytokines.

In **Chapter 7**, we determined whether high amniotic fluid IL-8 and TNF-α protect against RSV bronchiolitis in healthy term infants, using the NAF birth cohort. In case of medical attention for respiratory symptoms during the first year of life, a nose-throat swab was taken for RSV PCR. Physician-attended RSV infection was observed in 27 (9.3%) of 292 children. Amniotic fluid concentrations of IL-8 were higher in children without physician-attended RSV infection than in children with physician-attended RSV infection (11.1 versus 5.5 ng/mL). Similarly, in children without physician-attended RSV the proportion of detectable amniotic fluid TNF-α was higher (60% versus 30%). Among children with physician-attended RSV infection, amniotic fluid IL-8 was inversely correlated to the number of wheezing days during the first year of life ($\rho = -0.38$). We concluded that high concentrations of amniotic fluid IL-8 and TNF-α are associated with low risk of RSV bronchiolitis in healthy infants. We hypothesized that direct exposure of fetal lungs to pro-inflammatory signals induces local protection against viral infection during infancy.

In **Chapter 8**, intra-uterine immune activation was studied as a possible determinant of newborn airway function. Leukocyte-associated Ig-like receptor-1 (LAIR-1) is a membrane-bound collagen receptor that increases the threshold for activating signals on several immune cells. The secreted form of LAIR-1 (sLAIR-1) is considered a distinct

marker of general immune activation. We hypothesized that high sLAIR-1 in amniotic fluid is associated with normal newborn airway function and low infant airway morbidity. In the NAF birth cohort, 152 healthy term newborns successfully underwent lung function measurement. At age 1 month, airway compliance and resistance were assessed with the single occlusion technique. sLAIR-1 was detected in all amniotic fluid samples. Airway compliance and amniotic fluid concentration of sLAIR-1 were positively correlated ($\rho = 0.29$). This correlation was independent from sex or maternal antepartum smoking. Amniotic fluid sLAIR-1 was lower in children who wheezed at ages 6 and 9 months. We concluded that high amniotic fluid sLAIR-1 concentration provide support of a strong intra-uterine immune activation during normal delivery. An association between sLAIR-1 in amniotic fluid and newborn airway compliance suggests a beneficial effect of intra-uterine immune activation on neonatal lung function.

Finally, in **Chapter 9**, a general discussion on RSV bronchiolitis in healthy term infants is given. First, the prediction of RSV bronchiolitis in healthy term infants is discussed. Abundant exposure to the virus is the most important predictive factor for RSV bronchiolitis in healthy term infants. Furthermore, birth approximately six months before the peak of the RSV epidemic also increased the risk, probably due to diminished maternal antibodies during the epidemic. Additionally, vitamin D deficiency at birth is associated with increased risk of RSV bronchiolitis. Upon external validation, the prediction rule for RSV bronchiolitis in healthy term infants could be used to target preventive and therapeutic strategies. Second, the pathogenesis of RSV bronchiolitis in healthy term infants is discussed. Viral load determines disease severity of RSV infections in the community, as in children hospitalized with RSV bronchiolitis. Remarkably, the absence of high amniotic fluid levels of pro-inflammatory cytokines confers an increased risk of RSV bronchiolitis. Possibly, this finding is mediated by an impaired maturation of the innate immune system of the airways. Future research should focus on the origins and effects of intra-uterine inflammation during term physiologic parturition, in order to understand whether exposure to high concentrations of pro-inflammatory cytokines is causally linked to infant lung function and respiratory disease.

Samenvatting

Respiratoir syncytieel virus (RSV) is de belangrijkste verwekker van virale lagere luchtweginfectie (bronchiolitis) bij zuigelingen. In een recent onderzoek werd geschat dat wereldwijd jaarlijks 34 miljoen kinderen onder de leeftijd van vijf jaar een RSV bronchiolitis ontwikkelen, waarvan er 3.4 miljoen in een ziekenhuis worden opgenomen. In Nederland bezoeken jaarlijks naar schatting 40.000 kinderen tot twee jaar een huisarts in verband met een RSV bronchiolitis en worden 2.000-3.000 in ziekenhuizen opgenomen. Na opname vanwege RSV bronchiolitis ontwikkelt de helft van de kinderen recidiverend piepen, wat samenhangt met een verminderde kwaliteit van leven.

De meeste kinderen die een RSV bronchiolitis ontwikkelen waren voorheen gezond en geboren na een voldragen zwangerschap. Ze hebben geen onderliggende ziekte, zoals een aangeboren hart- of longafwijking. Ontwikkeling van een model dat RSV bronchiolitis bij gezond kinderen voorspelt is belangrijk voor gerichte preventie en vroege behandeling in de toekomst. Bovendien is het mechanisme dat verklaart waarom sommige gezonde kinderen RSV bronchiolitis krijgen onbekend en zouden nieuwe inzichten op dat gebied uiteindelijk kunnen leiden tot nieuwe strategieën voor preventie en behandeling. In dit proefschrift wordt een aantal onderzoeken naar de predictie en de pathogenese van RSV bronchiolitis bij gezonde aterm (na een zwangerschapsduur van tenminste 37 weken) geboren kinderen beschreven, alle uitgevoerd in het Netherlands Amniotic Fluid (NAF) geboortecohort.

In **hoofdstuk 1** wordt het onderwerp RSV bronchiolitis bij gezonde aterm geboren kinderen ingeleid. De meeste RSV infecties die bezoek aan een arts vergen treden op bij kinderen van een half jaar of ouder en leiden niet tot ziekenhuisopname. De socio-economische impact van RSV infecties is echter aanzienlijk. De algemene doelen van dit proefschrift en de specifieke onderzoeksvragen van de verschillende onderzoeken naar de predictie (voorspellers) en de pathogenese (oorzaken) van RSV bronchiolitis worden besproken.

In **hoofdstuk 2** worden nieuwe voorspellende factoren en een nieuwe klinische predictieregel voor RSV bronchiolitis in de eerste lijn beschreven. Binnen het NAF geboortecohort werden 298 gezonde aterm geboren kinderen intensief gevolgd tot de leeftijd van één jaar. De ouders van deelnemende kinderen hielden luchtwegklachten bij en namen een neus-keelwat af ten tijde van luchtweginfecties. De primaire uitkomst RSV bronchiolitis werd gedefinieerd als een positieve RSV PCR met gepaard piepen en / of matig tot ernstig hoesten. Van de deelnemende kinderen ontwikkelden er 42 (14%) een RSV bronchiolitis. Onafhankelijke voorspellers waren: bezoek aan crèche / kinderdagverblijf en / of tenminste één broer / zus, hoog opgeleide ouders, geboortegewicht >4 kg

en geboortemaand van april tot en met september. Het onderscheidend vermogen van het ontwikkelde predictiemodel was matig hoog (ROC AUC 0.72). De afgeleide predictieregel heeft een score-range van nul tot en met vijf punten. Bij kinderen met een score van ≤2 was het absolute risico op RSV bronchiolitis 3%, bij kinderen met een score van 5 was het risico 32%. Bovendien had 62% van de kinderen met RSV bronchiolitis in het eerste jaar klachten van recidiverend piepen, tegenover 36% van de kinderen zonder RSV bronchiolitis. Indien deze eenvoudige klinische predictieregel wordt gevalideerd, is het mogelijk kinderen te identificeren met een hoog risico op RSV bronchiolitis. Artsen kunnen met behulp van deze predictieregel kinderen met een hoog en een laag risico op RSV bronchiolitis onderscheiden en vervolgens gericht preventieve en controle maatregelen nemen voor kinderen met een hoog risico.

In **hoofdstuk 3** wordt het verband tussen de plasmaconcentratie van 25-hydroxy (25-OH) vitamine D bij de geboorte en het risico op RSV bronchiolitis onderzocht. In het NAF geboortecohort werden navelstrengbloed concentraties van 25-OH vitamine D gerelateerd aan het risico op RSV bronchiolitis in het eerste levensjaar. Van de 156 deelnemende kinderen ontwikkelde er 18 (12%) RSV bronchiolitis. De navelstrengbloed concentratie van 25-OH vitamine D was gemiddeld 82 nmol/L en 27% van de pasgeborenen had een 25-OH vitamine D concentratie <50 nmol/L. Er was een positief verband tussen vitamine D3 suppletie van de moeder tijdens de zwangerschap en de concentratie 25-OH vitamine D in navelstrengbloed. De concentratie 25-OH vitamine D was lager in kinderen met RSV bronchiolitis in het eerste levensjaar dan in kinderen zonder (65 vs 84 nmol/L). Bij kinderen met 25-OH vitamine D <50 nmol/L bij geboorte was het risico op RSV bronchiolitis in het eerste levensjaar was zesmaal verhoogd in vergelijking met kinderen met een concentratie van ≥75 nmol/L. Wij concludeerden dat een vitamine D deficiëntie (tekort) bij geboorte geassocieerd is met een verhoogd risico op RSV bronchiolitis in het eerste levensjaar. Wij adviseren de huidige richtlijn van vitamine D inname tijdens de zwangerschap te volgen ter preventie van RSV bronchiolitis bij gezonde zuigelingen.

In **hoofdstuk 4** wordt de hypothese getoetst dat tijdens primaire RSV infecties in de eerste lijn de ziekte-ernst samenhangt met de virale load. Gezonde aterm geboren deelnemers van het NAF geboortecohort werden geïncludeerd bij het optreden van hun eerste luchtweginfectie. Er werd een nasofaryngeaal aspiraat (uitzuigmonster van achterin neus en keel) afgenomen en de ziekte-ernst werd gescoord met een gevalideerd scoremodel. De aspiraten werden met polymerase kettingreactie (PCR) semi-kwantitatief getest op de aanwezigheid van 10 verschillende respiratoire pathogenen (virussen en bacteriën). Als er meer dan één pathogeen werd aangetoond, dan werd als primaire pathogeen gedefinieerd het pathogeen met de hoogste virale load. Gedurende twee jaar werden in totaal 82 kinderen geïncludeerd. De mediane leeftijd ten tijde van de eerste luchtweginfectie was drie maanden. Bij 77 kinderen (94%) werd een pathogeen gedetecteerd en bij 35 (43%) twee of meer. RSV werd gedetecteerd in 30 monsters en in 16

gevallen was RSV het primaire pathogeen. Er bestond een negatief verband tussen RSV cycle threshold (CT) waarde (inverse van virale load) en ziekte-ernst bij alle gevallen van RSV infectie ($\rho = -0.52$) en in geval van een primaire RSV infectie ($\rho = -0.54$). Dit is de eerste beschrijving van virale loads tijdens een primaire RSV infectie in de eerste lijn bij gezonde kinderen. Dit onderzoek bevestigt dat ziekte-ernst tijdens een primaire RSV infectie ook in gezonde aterm geboren kinderen wordt bepaald door de virale load.

In **hoofdstuk 5** wordt de sensitiviteit van de neuswat en het nasofaryngeale aspiraat onderzocht. Er wordt bestudeerd in hoeverre virale load de sensitiviteit bepaalt van beide methoden. Gezonde aterm geboren deelnemers van het NAF geboortecohort werden geïncludeerd bij het optreden van hun eerste luchtweginfectie. Er werden gepaarde neuswatten en nasofaryngeale monsters afgenomen en detectie vond plaats met real-time PCR zoals bovenbeschreven. Bij 98 kinderen werden gepaarde monster afgenomen en bij 94 kinderen werd tenminste één pathogeen gevonden. Rhinovirus ($n = 67$) en RSV ($n = 39$) werden het meest frequent gedetecteerd. De sensitiviteit van de neuswat was lager dan die van het nasofaryngeaal aspiraat, met name voor RSV (51% versus 100%) en rhinovirus (75% versus 97%). De sensitiviteit van de neuswat hing sterk samen met de CT waarde. De sensitiviteit van de neuswat voor RSV was 100% bij kinderen met een grote ziekte-ernst (score ≥11); dit gold niet voor rhinovirus. Wij concludeerden dat de neuswat kan worden gebruikt voor RSV onderzoek in de eerste lijn, hoewel de sensitiviteit lager is dan bij gebruik van een nasofaryngeaal aspiraat, met name voor de detectie van milde infectie.

In **hoofdstuk 6** wordt onderzocht of spontane bevalling wordt voorafgegaan door intra-uteriene (in de baarmoeder) ontsteking. In het NAF geboortecohort werd een cross-sectioneel onderzoek uitgevoerd onder 375 aterme spontane vaginale bevallingen en electieve sectio's caesarea (geplande keizersnedes) zonder tekenen van partus (zoals weeënactiviteit, vliesscheur of ontsluiting). Tijdens en / of onmiddellijk na de bevalling werden placenta's en vruchtwatermonster verzameld. Histologische tekenen van ontsteking in de placenta werden microscopisch vastgesteld. De concentraties van pro-inflammatoire cytokines (ontsteking-inducerende signaalmoleculen) in vruchtwater werden met ELISA gemeten. Bij spontane vaginale bevallingen werd meer intra-uteriene ontsteking vastgesteld dan bij electieve sectio's caesarea: de prevalentie van chorioamnionitis (ontsteking van vliezen) was hoger (18% versus 4%) en de vruchtwater concentratie van interleukine-6 (IL-6) was hoger (3.1 versus 0.4 ng/mL). We vonden vergelijkbare resultaten voor IL-8 (10.9 versus 1.0 ng/mL) en de proportie van bevallingen met een detecteerbare concentratie tumor necrosis factor-α (TNF-α, 50% vs 4%). Wij kwamen tot de verrassende conclusie dat spontane aterme vaginale bevallingen worden gekenmerkt door histologische tekenen van placenta ontsteking en verhoogde concentratie van pro-inflammatoire cytokines in vruchtwater.

In **hoofdstuk 7** wordt onderzocht of blootstelling aan hoge concentraties IL-8 en TNF-α in vruchtwater gezonde aterm geboren kinderen beschermen tegen RSV bronchio-

litis, gebruikmakend van het NAF geboortecohort. Bij elke episode van luchtwegklachten in het eerste levensjaar waarvoor de huisarts (of een andere arts) werd geraadpleegd werd een neus-keelwat voor RSV PCR afgenomen. Bij 27 (9.3%) van de 292 kinderen werd RSV bronchiolitis vastgesteld. De IL-8 concentraties in het vruchtwater waren hoger bij kinderen zonder RSV bronchiolitis dan bij kinderen met RSV bronchiolitis. Bovendien was in kinderen zonder RSV bronchiolitis de proportie in vruchtwater detecteerbaar TNF-α groter (60% versus 30%). In de groep kinderen met een RSV bronchiolitis was er een omgekeerde relatie tussen de vruchtwater concentratie van IL-8 en het aantal dagen piepen in het eerste levensjaar ($\rho = -0.38$). Wij concludeerden dat hoge concentraties IL-8 en TNF-α in vruchtwater samenhangen met een laag risico op RSV bronchiolitis bij gezonde zuigelingen. Mogelijk beschermt directe blootstelling van de foetale longen aan pro-inflammatoire signalen tegen virale infecties bij zuigelingen.

In **hoofdstuk 8** wordt intra-uteriene immuunactivatie (activatie van het afweersysteem) bestudeerd als mogelijke determinant van de longfunctie van pasgeborenen. Leukocyte-associated Ig-like receptor-1 (LAIR-1) is een membraangebonden receptor voor collageen (bindweefselmolecuul) op verscheidene immuuncellen (afweercellen), die de drempel voor activerende signalen verhoogt. De gesecreteerde (afgescheiden) vorm van LAIR-1 (sLAIR-1) wordt beschouwd als een aparte marker van algemene immuunactivatie. Wij hypothetiseerden dat een hoge concentratie sLAIR-1 in vruchtwater geassocieerd is met een normale longfunctie van de pasgeborene en weinig luchtwegmorbiditeit (ziektes van de luchtwegen). Binnen het NAF geboortecohort werd bij 152 pasgeborenen succesvol een longfunctiemeting verricht. Op de leeftijd van één maand werd met de 'single occlusion techniek' de compliantie (~elasticiteit) en de weerstand van de luchtwegen gemeten. In alle vruchwater monsters werd sLAIR-1 gedetecteerd. De luchtwegweerstand en de vruchtwater concentratie van sLAIR-1 bleken positief samen te hangen ($\rho = 0.29$). Deze associatie was onafhankelijk van geslacht of antepartum (voor de bevalling) roken door de moeder. De vruchtwater concentratie van sLAIR-1 was lager bij kinderen die op de leeftijd van 6 en / of 9 maanden een piepende ademhaling hadden. Wij concludeerden dat de hoge concentraties van sLAIR-1 in vruchtwater de grote mate van intra-uteriene immuunactivatie bij spontane bevallingen lijken te bevestigen. Het verband tussen vruchtwater sLAIR-1 en luchtwegcompliantie bij pasgeborenen suggereert een voordelig effect van intra-uteriene immuunactivatie voor de longfunctie van zuigelingen.

Hoofdstuk 9 is een algemene discussie over RSV bronchiolitis bij gezonde aterm geboren kinderen. Enerzijds wordt het voorspellen van RSV bronchiolitis bij gezonde kinderen besproken. Overmatige blootstelling aan het virus is de belangrijkste voorspellende factor voor RSV bronchiolitis bij gezonde aterm geboren kinderen. Daarnaast verhoogt ook een geboorte circa zes maanden voor de piek van de jaarlijkse RSV epidemie het risico, waarschijnlijk door sterk gedaalde maternale antistoffen tijdens de epidemie.

Bovendien hangt vitamine D deficiëntie bij de geboorte samen met een verhoogd risico op RSV bronchiolitis. Na externe validatie kan de klinische predictieregel voor RSV bronchiolitis bij gezonde aterm geboren zuigelingen gebruikt worden voor gerichte preventie. Anderzijds wordt de pathogenese van RSV bronchiolitis bij gezonde aterm geboren kinderen bediscussieerd. Virale load bepaalt gedeeltelijk ziekte-ernst van RSV infecties in de eerste lijn, zoals eerder beschreven bij in het ziekenhuis opgenomen kinderen met RSV bronchiolitis. Bovendien is het risico op RSV bronchiolitis verhoogd bij afwezigheid van hoge concentraties pro-inflammatoire cytokines in vruchtwater. Mogelijk wordt is het mechanisme van dit effect een gestoorde rijping van het immuunsysteem van de luchtwegen. Toekomstig onderzoek zou zich moeten richten op de oorsprong en de effecten van intra-uteriene ontsteking tijdens aterme fysiologische bevallingen, om te verklaren of blootstelling aan hoge concentraties pro-inflammatoire cytokines causaal (oorzakelijk) samenhangt met de neonatale longfunctie en luchtwegziekten bij de normale zuigelingen.

Dankwoord

Het is onvoorstelbaar hoeveel mensen hebben bijgedragen aan het realiseren van dit promotieonderzoek. Een geboortecohort dat start op de verloskamers en dat zoveel dimensies heeft, ontstaat louter en alleen door de onuitputtelijke inzet van velen. Ik wil hiervoor iedereen – en een aantal mensen in het bijzonder – heel hartelijk bedanken.

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Michiel
Utrecht, april 2011

Curriculum vitae

Michiel Houben werd geboren op 25 augustus 1975 te Amersfoort. Hij woonde in Utrecht en in Nijmegen en verhuisde in 1984 naar Heerlen. In 1993 haalde hij daar aan het gymnasium van het Bernardinuscollege (cum laude) zijn eindexamen.

Vanaf 1993 studeerde hij Farmacie aan de Universiteit Utrecht en in 1995 begon hij met de studie Geneeskunde. Hij verrichtte een gecombineerd afstudeeronderzoek met als onderwerp de bewaking van interacties van geneesmiddelen in de ziekenhuisapotheek van het UMC Utrecht (Prof.dr. A.F.A.M. Schobben) en haalde in 2000 het doctoraalexamen van Farmacie en Geneeskunde. Van 2000 tot en met 2002 deed hij co-assistentschappen, waaronder twee keuzestages kinderinfectiologie: in het Great Ormond Street Hospital in Londen en in het Wilhelmina Kinderziekenhuis (WKZ, UMC Utrecht).

Na zijn artsexamen in 2002 startte hij met de opleiding tot kinderarts: eerst in het St Elisabeth Ziekenhuis te Tilburg (opleiders: Dr. J.M.Th. Draaisma, Dr. P.J. van Dijken), vervolgens in het WKZ (opleiders: Prof.dr. J.L.L. Kimpen, Dr. J. Frenkel). In oktober 2005, na het verwerven van een Fellowship Award van de European Society for Paediatric Infectious Diseases, startte hij als arts-onderzoeker met het Vruchtwateronderzoek, wat leidde tot dit proefschrift. Hij werd begeleid door Prof.dr. J.L.L. Kimpen (Divisie Kinderen), Prof.dr. G.H.A. Visser (Divisie Vrouw & Baby), Dr. L.J. Bont (Divisie Kinderen) en Dr. M.M. Rovers (Divisie Julius Centrum). Van 2006 tot 2009 volgde hij tevens de opleiding tot klinisch epidemioloog (MSc, Universiteit Utrecht). Per 1 januari 2010 is hij werkzaam als kinderarts / chef de clinique in het WKZ. In de toekomst hoopt hij zijn werk als kinderarts te blijven combineren met klinisch epidemiologisch onderzoek op het gebied van de kinderinfectiologie / -immunologie.

Michiel Houben is sinds 2008 getrouwd met Tamar Fischer. Zij hebben twee dochters: Hester (2007) en Titia (2009).

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Τα δέ μοι παθήματα εόντα αχάριτα μαθήματα γέγονε.

Herodotus (circa 485 – 425/420 voor Chr.), *Historiën I*