

Lung function measurements in children

I. Accuracy of Wholebody Plethysmography in preschool children

II. -III. Lung function measurements before and after Respiratory Syncytial Virus Bronchiolitis



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virus bronchiolitis

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Abbreviations

ANOVA	ANalysis Of VAriance
ASC	[Automatischer Schleifen-Computer]
BHR	Bronchial Hyper Responsiveness
BTPS	Body Temperature, barometric Pressure and Saturated with water vapour
CI	Confidence Interval
COPSAC	COPenhagen Studies on Asthma in Childhood
ELISA	Enzyme-Linked ImmunoSorbent Assay
F _E NO	Fractional Exhaled Nitric Oxide
FEV _{0.5}	Forced Expiratory Volume at 0.5 seconds
FEV ₁	Forced Expiratory Volume at first second
kPa	kiloPascal
MZ	MonoZygotic
OR	Odds Ratio
P	Pressure
P _{amb}	Ambient Pressure
P _{H₂O}	Pressure of Water vapour at body temperature
PtcO ₂	Transcutaneous Oxygen Pressure
PCR	Polymerase Chain Reaction
PD	Provocative Dose
PD ₁₅	Provocative Dose of methacholine producing a 15 percent fall in lung function
PD ₂₀	Provocative Dose of methacholine producing a 20 percent fall in lung function
PP	Observer Porntiva Poorisrisak
ΔP	Change in Pressure
RSV	Respiratory Syncytial Virus
RVRTC	Raised Volume Rapid Thoracic Compression
SD	Standard Deviation
SD _w	Within subject Standard Deviation
sRaw	Specific Airway Resistance
sRawTOT	Parameter line connecting the flow points at maximum change in plethysmographic volume (pressure)
V	Volume
\dot{V}	Airflow
Δ \dot{V}	Change in Airflow

Introduction

Symptoms of chronic respiratory disease as asthma or cystic fibrosis appear in early childhood. Clinical evaluation of chronic respiratory disease by auscultation and history of respiratory symptoms is of limited sensitivity and specificity, which makes diagnosing and monitoring the respiratory disease difficult. Therefore, lung function measurement is “the gold-standard” for diagnosing and monitoring chronic lung diseases in young children.

Lung function techniques in children

Lung function measurements in children can be measured with different methods according to their age and feasibility. In this thesis we used the raised volume rapid thoracic compression technique in infants, wholebody plethysmography in preschool children and spirometry in schoolchildren to measure lung function. In the following section the different lung function techniques are described briefly.

Lung function measurement in infancy

Raised volume rapid thoracic compression technique (RVRTC)

Infant lung function testing is increasingly applied in research. It is often necessary to sedate the infant during measurement since the infant while awake cannot cooperate to special breathing maneuvers. The RVRTC method requires relatively complex equipment for lung inflation. An external pressure is applied to the chest and abdomen to force expiration (Figure 1). The method allows measurements of volume-time parameters as for example forced expiratory volumes at 0.5 seconds.

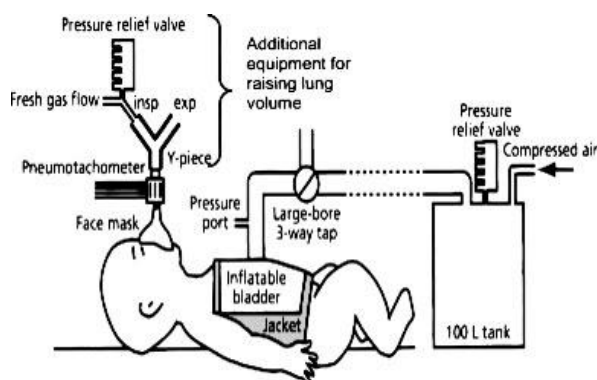


Figure 1: Equipment required for RVRTC (1)

Guidelines for raised volume forced expirations in infants has been published by the European Respiratory Society/American Thoracic Society (ERS/ATS) Task Force (2). The method can detect abnormal respiratory function in infants with chronic lung disease (3, 4). Studies also found impaired lung function in infancy in young children with recurrent wheeze compared to healthy controls (5, 6).

Other lung function tests in infants have been studied such as the gas washout technique measuring functional residual capacity (7), plethysmographic measurements of lung volume and airway resistance (8) and the occlusion technique measuring passive respiratory mechanics in infants (9).

Lung function measurements in young children (<6 years of age)

Wholebody Plethysmography

Specific airway resistance (sRaw) measurements require no active cooperation and are therefore feasible in children from 2 years of age. sRaw measured by wholebody plethysmography in young children is increasingly used in research and clinical practice. The method has been documented in the recent decade (10-16). Previous studies found sRaw values being significantly higher in 2-7 year old stable asthmatic children compared to healthy controls (17-19) and the sRaw method more sensitive compared to other techniques (16). By using the sRaw method a British study group found impaired lung function in children with personal and parental atopy even in the absence of respiratory symptoms. The same study group found that a poor lung function at age 3 was predictive for subsequent persistence of symptoms in children who had wheezed within the first 3 years (20, 21).

Other techniques can be useful when measuring lung function in preschool children (22) such as the forced/impulse oscillation (23) or the occlusion/interrupter technique (24) which measures respiratory resistance; or multiple-breath washout gas-mixing test measuring ventilation distribution in the lungs and the functional residual capacity (25-27). These techniques are mostly applied in research practice.

Lung function measurements in schoolchildren

Spirometry is the traditional method measuring forced expiratory volume in the first second (FEV₁); this is routinely applied in school children (28). It is the most frequent

pulmonary function test obtained by clinicians caring for children with respiratory diseases and invaluable as a screening test of general respiratory health. Younger children can rarely perform a maximal inspiration followed by a forced expiration sufficiently (29). Most schoolchildren are able to perform acceptable and repeatable spirometry that conforms to standard criteria (30, 31).

Bronchial hyperresponsiveness

Bronchial hyperreactivity (BHR) is a characteristic phenomenon in asthma, defined as an abnormal fall (FEV_1) in lung function (or rise in airway resistance) after direct or indirect airway challenge test with stimuli which induce increased airflow limitation. Test of BHR contributes to the asthma diagnose (32).

Direct airway challenge tests (e.g. methacholine, histamine) cause airway narrowing by acting directly on their respective receptors on the bronchial smooth muscles. Methacholine challenge test are well established (33, 34) and guidelines have been published by the American Thoracic Society (35). The subject inhale increasing dose of methacholine until the FEV_1 falls by 20% or more and the provocative dose causing a 20% fall (PD_{20}) is calculated. A low PD_{20} suggests severe bronchial responsiveness.

Indirect airway challenge tests (e.g. exercise, hypertonic saline, cold air, mannitol, and adenosine) use non-allergic irritants that have no direct effect on the smooth airway muscles. Hyperventilation leads to dehydration of the cells in the airways, which induces release of bronchoconstricting mediators. Dry/cold air hyperventilation testing has a similar mechanism of osmotic dehydration. School children and adults traditionally use exercise test. This is applicable from the age of 7-8 years where adequate cooperation may be expected. For younger children Danish paediatricians developed a simple test, cold air provocation. The method is documented and has shown good predictive value and repeatability for asthma or ongoing asthma in preschool children. The test is positive if sRaw increases $\geq 3SD$ corresponding 20% or more (18, 36-38).

Nitric oxide as an inflammatory marker

Direct monitoring airway inflammation in respiratory diseases with bronchoscope taking biopsies or making broncho alveolar lavage (BAL) is limited in children because

of the invasive nature. In the last decade fractional exhaled nitric oxide ($F_{E}NO$) has been studied as a non-invasive marker of eosinophilic airway inflammation (39, 40). $F_{E}NO$ has been correlated with BAL fluid and bronchial biopsies (41, 42). Studies have found higher concentration in subjects with asthma compared to healthy (43-46). Especially atopy seems to be a significant factor associated with a raised exhaled NO. In general $F_{E}NO$ has not correlated with lung function but positively associated with bronchial hyperresponsiveness (47, 48) and sensitive to changes in anti-inflammatory treatment (49). $F_{E}NO$ is now applied in the clinical setting as a supplement to the traditional lung function testing.

I. Accuracy of Wholebody Plethysmography in preschool children

Background

Quality control of wholebody plethysmography

The method is precise within center, and reference data are available (10, 12). The precision (within-observer and between-observer variability) and repeatability of sRaw measurements have previously been documented at one center (12, 13, 50) but the accuracy of the method has not been reported and needs quality assurance (11). Particularly using reference values generated by other centers is vulnerable to the accuracy of the methods used. Currently, there is no method available for calibration to compare sRaw between centers, which raise concern.

There have been attempts to develop a mechanical infant lung model analogue for quality control of a whole-body infant-plethysmograph (51, 52), but this turned out to be difficult because of the small pressure and flow changes and is not readily available. Flow and box leak are checked routinely, but the composite resistance measure is generated by algorithms hidden in the software with settings often inaccessible to the end-user. Thereby errors in software or mechanics could go unnoticed with potential impact on clinical evaluation. Therefore, it is of concern that the accuracy of sRaw measurements cannot be verified.

The sRaw method was established as part of this thesis at 6 Danish paediatric departments with implementation of a standard operation procedure for lung function measurement with a whole body plethysmograph in preschool children. Determination of inter-center reproducibility and variability of the technique has important relevance for its application to multicenter clinical trials. Harmonizing the standards for lung function test will develop an indicator for the quality in treatment of preschool children with asthma in Denmark.

The focus of study I was to standardize a protocol for sRaw lung function measurements between 6 Danish paediatric departments in order to conduct a multicenter trial.

Aims (study I)

Study I

- A. To determine the center agreement by comparing sRaw measurements in healthy preschool children between 6 centers in Denmark currently using identical hardware equipment, but different software versions.
- B. Provide sRaw values for non-asthmatic preschool children at 5 of the centers to expand normative data.

Material

Study I

We used 7 healthy preschool children as biological controls in the center agreement study. The children were between 4.9 to 6.6 years old. None of the children had a history of asthma or allergy. Three children previously had atopic dermatitis; three had parental atopy and none had smoking parents.

In the normative data we recruited healthy preschool children by random selection through the Central Person Registry from the local catchment area of 5 centers. Approximately 20% of the families responded positively to the posted invitations.

105 non-asthmatic preschool children (52 male) were measured locally at one of the 5 centers; mean age was 5.1 years (interquartile range 4.3-6.0). One child was of Latin-America descent and two of Arabic descent.

Children included were born at term, with no history of asthma related symptoms, other chronic lung symptoms or use of asthma treatment. If the child had a lower respiratory tract infection within the week before the appointment, the measurement was rescheduled.

Methods

Designs

Study I:

Six centers in Denmark currently use identical hardware equipment, but software versions differed between centers (JLAB 4.51, 4.53 with different sub versions, 4.65 and 4.67).

Center agreement

The seven children were brought to each center for sRaw measurements. The children were not trained in performing lung function measurement before entering the study. The order of center visits was randomized. At each center the children were measured by a center specific observer as well as one external observer visiting each center (PP). The two observers were blinded for each other's measurements. Measurements in the individual children were finished within a period of 3 months.

Normative data

The children attended their local center where duplicate measurements were done by a local observer. The center numbers in the center agreement study represent the same center numbers in the normative study. Center no. 1-5 provided data for the healthy cohort. Center no. 6 did not participate in the second part of the study because a local investigator was unavailable.

Wholebody Plethysmography

Protocol of sRaw measurement

Principle of measurement

Whole body plethysmography was introduced by DuBois in 1956 for specific airway resistance measurement (53).

Our measurements were conducted in a constant volume whole body plethysmograph (Master Screen Body; E. Jaeger GmbH, Würzburg, Germany). A transducer measured pressure changes in the sealed box generated by the thoracic and abdominal movements during tidal breathing. By calibration of the plethysmograph the changes in pressure

were expressed as changes of plethysmographic volume. A pneumotachograph simultaneously measured the flow swing at the mouth.

Specific airway resistance (sRaw) was calculated as the ratio between the change in volume (V) and the resulting change in air flow (\dot{V}): $sRaw = (\Delta V / \Delta \dot{V})$.

Flow and volume measurements were corrected to body temperature and pressure, saturated with water vapor (BTPS) conditions. $sRaw = (\Delta V / \Delta \dot{V}) \times (P_{amb} - P_{H_2O})$, where P_{amb} is ambient pressure and P_{H_2O} is the pressure of water vapour at body temperature (54). The equipment was calibrated daily for ambient conditions (room temperature, atmospheric pressure and humidity), box calibration (leak test result should be between 4 and 7 seconds and test for internal pressure which should result in a correction factor of <3%) and volume calibration (piston was pulled regularly 10 times with a 3 L piston and automatically accepted or rejected by the software).

Measurement procedure

The same standard operating procedure was followed by all observers. The child was seated in a comfortable position alone in the box with the door closed. Sitting position was relaxed without slouching and a footstool supported the feet. The box door was closed before starting the software program. The observer waited approximately one minute for stabilizing the temperature in the box before measurement (the heated air generated by the body temperature expands and create a little increase in pressure in the box). The child was not allowed to touch the sides of the plethysmograph as it could affect the pressure (volume) signal.

Preschool children often do not accept wearing a nose-clip and have difficulty in keeping the lips around a mouth-piece. Therefore the children in this study used a facemask with a large cushion, which ensured a good seal and stabilized the cheeks and chin. A built-in flexible tube with a 1 cm long internal metal ring ensured that the mouth remained open to avoid nasal breathing. The child's head was slightly tipped back and normal breathing aimed for a frequency of 30-45 breaths per minute (15, 55) encouraged by the observer. A microphone and loudspeaker ensured communication and instruction of the child during measurement.

"Loops" on the screen showed the relation between pressure (or volume) (x-axis) and flow (y-axis) i.e. the pressure driving the air flow in and out of the lungs. sRaw was estimated from the inclination of these loops using the line between points of maximum pressure (sRaw_{TOT}).

Technically acceptable loops were chosen as those that were "closed" in the middle. "Open" loops normally indicated insufficient BTPS correction. The loops assumed a straight line with a tendency to an S-shape, and symmetric around the inclination (Figure 2) (56).

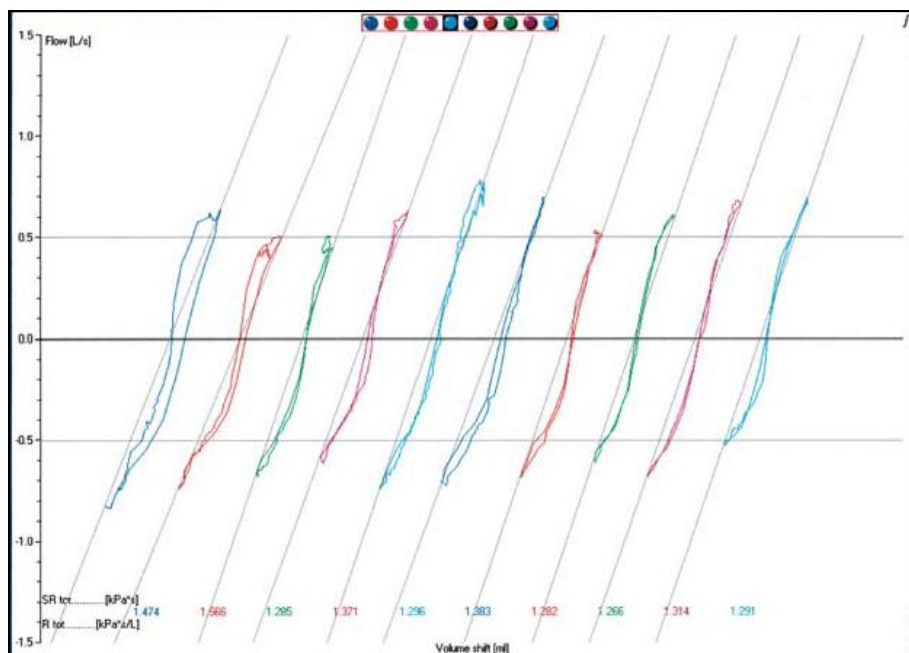


Figure 2: sRaw loops in a 3 year old healthy child

Low sRaw reflected as steep loops. The inclinations of the loops were "lying" down with rising resistance (Figure 3).

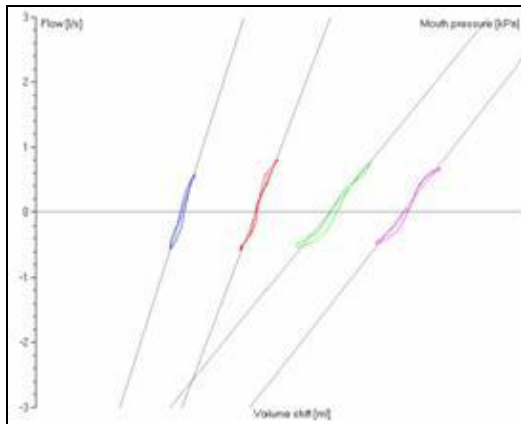


Figure 3: sRaw loops before and after provocation in a young child with asthma

BTPS correction was done automatically by the software when the result was analyzed. sRaw measurement was assessed during regular breathing free of artefacts caused by e.g. swallowing, vocalization, coughing or leakage around the facemask, which could be detected as abnormal (open or asymmetric) loops on the on-line display. The decision to whether accept or reject a measurement was immediately done after the measurement by the observer. sRaw from one run was calculated as the median value of at least five technically satisfactory loops with similar configuration and inclination (10-12).

In the center agreement study each of the seven children performed in total six runs. The local operator made the two first runs, and then switched the operator to PP which made two runs and finally did two runs with the local operator again.

In the normative study duplicate measurements (two runs) were done by a local observer.

Statistical analyses

SAS version 9.1 was used for statistical analyses.

Study I: We used ANOVA with unbalanced block design to analyze differences due to center, child, center specific observer, accompanying observer (PP) and age of the child.

We included age in the analysis of center agreement because of the small number of children, and preschool children could theoretically have a higher variation of sRaw values throughout the many visits. Our data were powered to detect a difference of 0.078 in expected log(sRaw) between two pre-specified centers. If centers were not pre-specified our data were powered to detect a difference of 0.117 using Bonferroni correction. For the normative data (incl. SD) we used mixed model with repeated measurements using log-transformed sRaw values adjusted for center number. A comparison between data from center no. 3 with previous reported normative values was done using a two sample t-test for means with logtransformed sRaw values. The figures were made by using Analyse-it for Excel.

Ethics

Study I was approved by the Local Ethics Committee as a quality assurance project and approved by the Danish Data Protection Agency (J.nr. 2005-2-11).

Results

Center agreement (study I)

All 7 children (3 boys) completed measurements at each of the 6 centers. Lung function measurements differed significantly between centers (Figure 4).

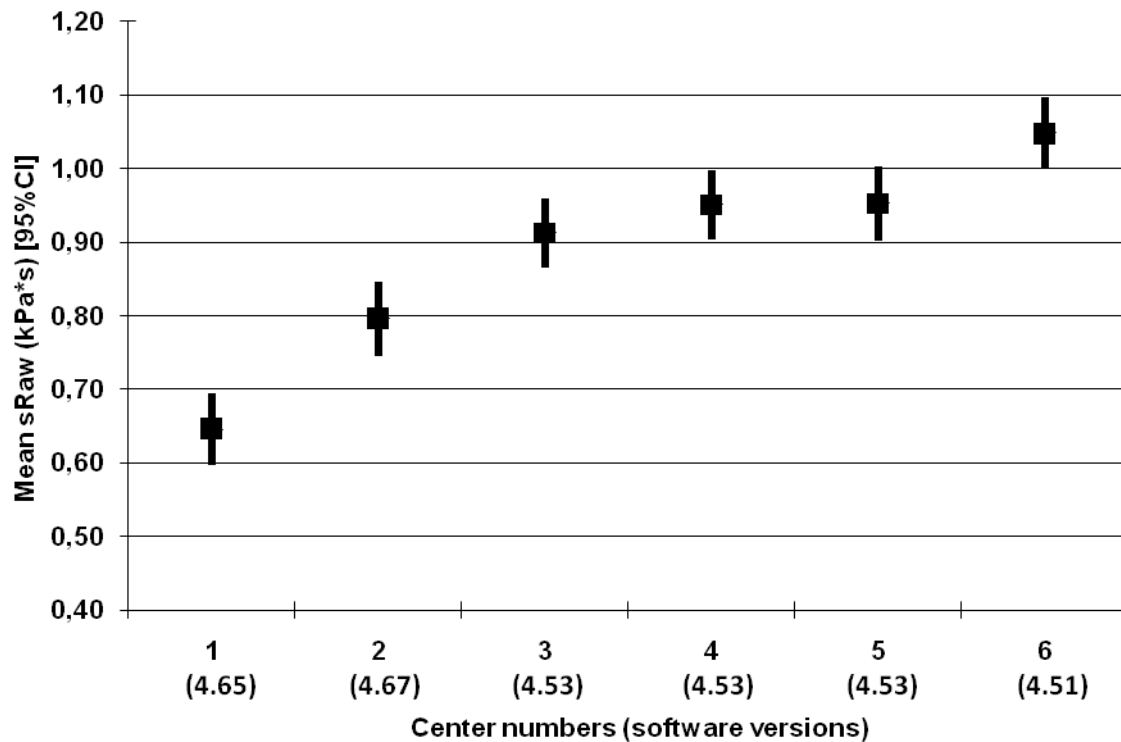


Figure 4: Center agreement study. Least squares mean for sRaw on 6 centers with 95% CI (software versions are indicated in parenthesis).

sRaw at center no. 1 and 2 were significantly lower compared to the other 4 centers and center no. 6 had significantly higher sRaw values than all the other centers. Mean sRaw (SD) for all 6 centers was 0.88 kPa*s (0.23). The within-subject SD was 0.01 and between-center SD for each child was 0.02. Observer and age of the child did not significantly affect the measurements ($p>0.5$).

There were no significant difference between the sRaw results obtained by PP and the local operator (table 1).

Table 1: Normative study: sRaw results obtained by PP and the local operator

Child no	1		2		3		4		5		6		7	
Center no	PP	Local operator	PP	Local operator	PP	Local operator	PP	Local operator	PP	Local operator	PP	Local operator	PP	Local operator
1	0.75	0.75	1.05	0.97	0.98	1.07	1.31	1.05	0.92	0.93	0.73	0.79	0.75	0.77
2	0.69	0.68	0.67	0.67	0.86	0.95	0.87	0.83	0.86	0.79	1.09	1.01	0.65	0.59
3	0.51	0.44	0.61	0.61	0.51	0.65	0.46	0.61	0.74	0.67	1.05	1.02	0.43	0.65
4	0.99	0.74	0.79	0.76	1.13	1.11	0.93	0.90	0.88	0.96	1.29	1.33	0.81	0.77
5	1.54	1.41	1.09	0.97	1.02	1.02	0.99	1.13	0.95	0.87	1.20	1.08	0.82	0.71
6	0.82	0.87	0.89	0.88	0.86	1.01	1.11	1.09	0.74	0.91	1.14	1.29	0.76	0.77

Normative data (study I)

Mean sRaw (SD) was 1.21 kPa * s (0.33) independent of height, weight, age and gender (Table 2); within-subject SD (variability) was 0.07 calculated by using mixed model with repeated measurements. There was no significant effect of center. Furthermore, there was no effect of the child's history of atopy, parental atopy or smoking (p-values > 0.05 for all estimates).

Table 2: Normative study: Clinical characteristics of the children from the 5 centers

Center no.	1	2	3	4	5	Total	Estimate [CI]	p- value
Number of children	21	28	29	16	11	105		
Mean sRaw (SD)	1.30 (0.32)	1.09 (0.24)	1.26 (0.31)	1.28 (0.46)	1.10 (0.23)	1.21 (0.33)		
Mean age (SD)	5.39 (1.16)	5.24 (1.04)	5.07 (1.18)	4.53 (1.19)	5.39 (1.03)	5.13 (1.15)	0.98 [0.93-1.03]	0.47
Gender/boys (%)	11 (52)	15 (54)	16 (55)	6 (38)	4 (36)	52 (49.5)	1.05 [0.98-1.13]	0.16
Weight/kg (SD)	22.0 (4.29)	20.0 (4.84)	20.4 (4.32)	18.9 (4.24)	19.4 (2.88)	20.3 (4.39)	1.01 [0.99-1.03]	0.26
Height/cm (SD)	115.2 (8.95)	110.2 (9.90)	112.3 (9.79)	106.2 (8.27)	111.2 (8.28)	111.3 (9.61)	0.99 [0.99-1.01]	0.52
Rhinitis (%)	4 (19)	0 (0)	4 (14)	1 (6.3)	1 (9.0)	10 (9.5)	0.91 [0.79-1.04]	0.16
Dermatitis (%)	2 (9.5)	10 (36)	5 (17)	4 (25)	1 (9.0)	22 (20.9)	1.05 [0.96-1.15]	0.29
Parental atopy (%)	12 (57)	13 (46)	21 (75)	11 (69)	5 (45)	62 (59.6)	0.99 [0.92-1.07]	0.85
Smoking (%)	4 (19)	8 (29)	4 (14)	3 (19)	4 (36)	23 (22)	1.02 [0.94-1.11]	0.64

We pooled the previous report on normative data by Klug and Bisgaard (121 children) (12) and the current normative data (105 children); mean sRaw (SD) 1.27 (0.25); 95% CI [0.78-1.76] (Figure 5).

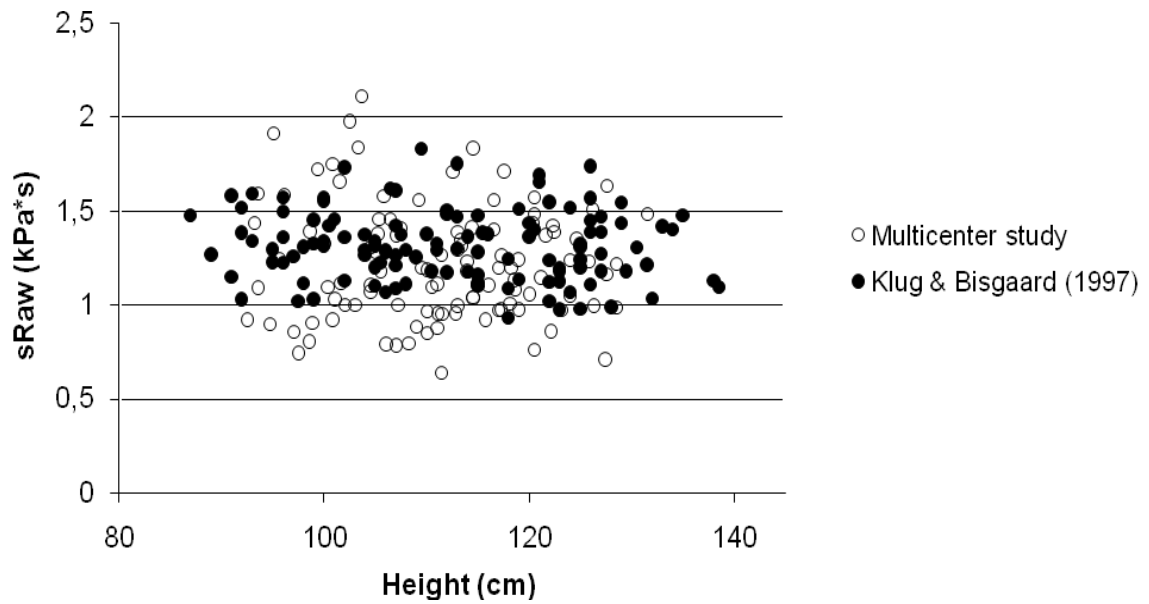


Figure 5: Normative data from the multicenter study (5 centers) and the previous study (Klug & Bisgaard, 1997)

The sRaw values fitted a normal distribution (Figure 6).

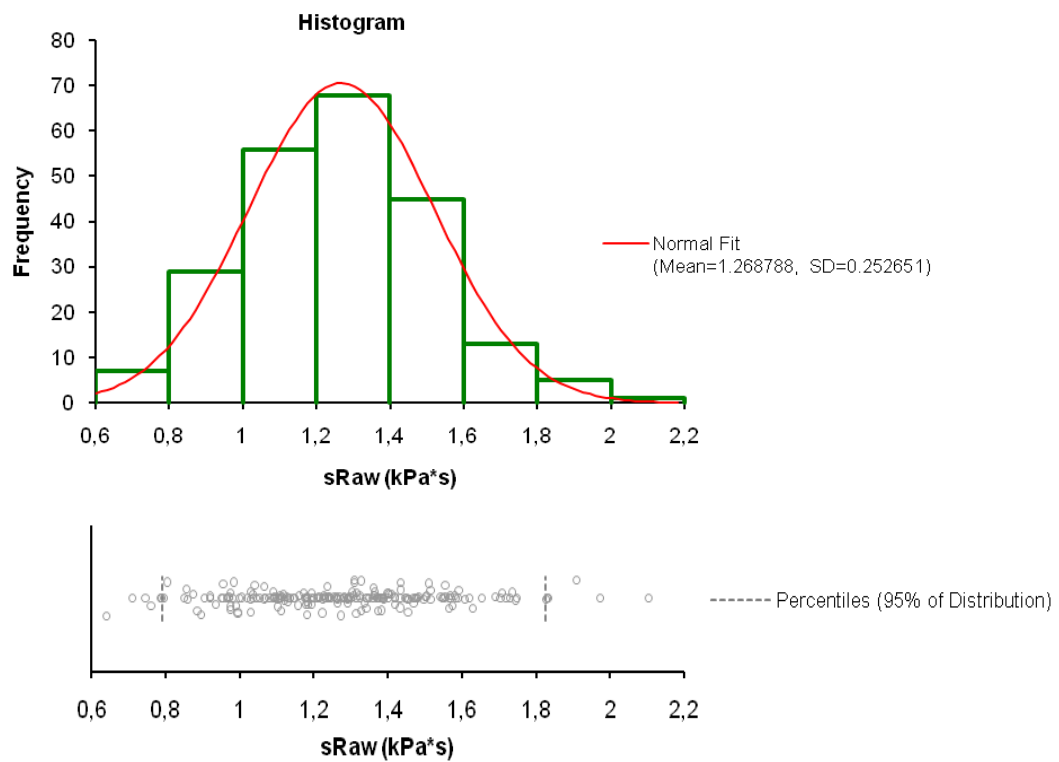


Figure 6: Normal distribution of sRaw values with 2.5 and 97.5 percentiles.

Additional results

sRaw measurement was established at center no. 2 during the year 2005. We registered the feasibility of the sRaw technique on untrained children. Figure 7 was based on 97 children trying sRaw measurement for the first time, consecutively examined; all sitting alone in the whole-body plethysmograph. The patient cases were mixed asthmatic children familiar with the facemask for asthma medication use and children with suspected asthma visiting the out-patients' clinic for the first time. The main problem in the youngest age group was being seated alone in the box and secondly accepting the facemask. We did not subdivide in gender.

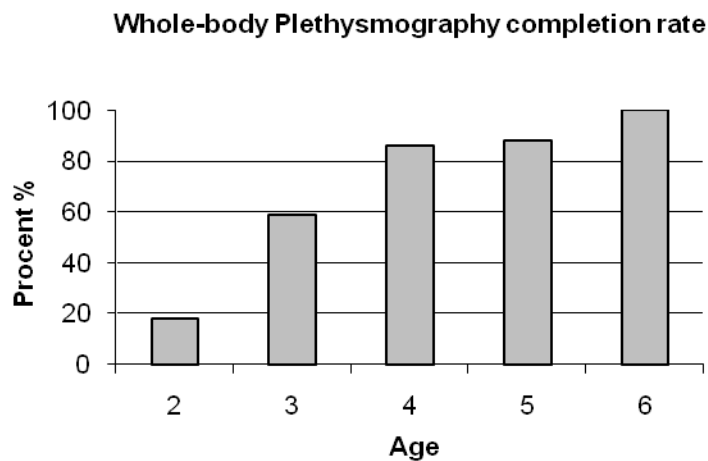


Figure 7: Age dependence on completion of sRaw measurement

Discussion

Between-center variation

sRaw offers a method for clinical monitoring and research during the critical period of growth and development early in life. The method is feasible from the age of two years and the precision is high (10-12). Our aim was to examine center agreement between six centers in Denmark currently using identical hardware equipment with different software versions (JLAB 4.51, 4.53 with different sub versions, 4.65 and 4.67). The study was conducted in a “real” setting of a multi-center study; therefore we did not update the centers software into the same version. We assumed the manufacturer had checked the equipments and made adjustments for possible variations in sRaw measurements (also electronic BTPS correction) after releasing new software. The starting point of the study was that there would be no difference in sRaw measurements between the centers despite different software versions.

However, the present study showed that the accuracy of sRaw measurements in young children was flawed from errors. A technician from the company (Cardinal Health) was sent to identify the problems in the deviating centers (center no. 1, 2 and 6). This revealed incorrect setting of the “ASC Compensation” at center no. 1. “Time delay for compensation” was set to 20 milliseconds and should have been 50, which resulted in 19-32% lower values. This was a factory setting not accessible for the operator. The technician found no reason for the deviating measurements at the other centers (center no. 2 and 6). Center no. 6 subsequently updated the whole-body plethysmograph software after this study. It was not possible to re-analyse the data because the software saved the sRaw values after the primary calculation of sRaw and the original loops was lost, which was a great limitation of the software. To optimize the study design we could have made prints of the original breath curves and have a third person evaluate the quality of the loops to avoid bias of the operator. In future software updates or development, it would be optimal if the software could determine the best breath curves from algorithms as flow and respiratory rates or encourage the child (with animated bio-feedback) to breath with optimal respiratory rates and flow. Currently, it relies on the operator who estimates by visual judgement when the breath curves are representatively and decides when the computer should analyze the loops.

After correcting the factory settings at the deviating center there were no longer differences between the centers and normative values were generated in this multi-center setting. The problem was not discovered by the standard calibration of flow, box leak and internal box pressure. Current calibration only assesses flow measured by the pneumotachograph, leak from the box and pressure transducer. Previous studies have shown that the electronic BTPS compensation may influence the accuracy of sRaw measurements (15, 55). Klug et al. found when using electronic BTPS compensation the sRaw measurements were systematically overestimated by 43% with increasing respiratory rate compared with true BTPS conditions (15). The electronic compensation (BTPS) was done identically by the different software versions. The available calibration does not assess the final resistance measure, which is generated by algorithms buried in the software with settings often inaccessible to the end-user. Thereby errors in software or mechanics may go unnoticed with potential impact on clinical evaluation and flawed accuracy as illustrated in our study. It is the key-message of our study that center-effects were seen and could only be explained by difference in the software hidden from the end-user.

We chose not to repeat the study with the same procedure after the correction; first of all because the travelling was time consuming and second the observers and children would be biased by the prior knowledge.

We could have used adults as biological controls, but they could be biased even if we blinded the results from each center and we would have to change the observers.

The local observers had different experience level in sRaw measurements. Two local observers had only measured approximately 5-10 children before performing the study and some had 5-10 years of experience.

The children had good repeatability and were cooperative. The mean sRaw for the biological controls was within but a little lower than the normative data (Figure 4+5) if we do not consider center no. 1. We consider the children physiologically representative for preschool children and the lower mean sRaw could be due to the low number of children.

A mechanical infant lung model analogue has previously been developed for quality control of infant whole-body plethysmographs (52) but a model testing for preschool children is not available to the end-user. This study suggests the need for development

of methods for control of the actual resistance measure for young children and not only the flow and box leakage. Without such proof of accuracy normative values generated at other centers may not be applicable. Until a mechanical standard becomes available biological standard (healthy subjects) is the only possible substitute.

We used a standardized protocol including standard calibration of flow, box leakage and internal box pressure in 6 Danish centers at secondary and tertiary referral hospital departments. The 6 centers included in the study of center-agreement were spread over the country, which prevented measurements the same day. Therefore the day-to-day variability reduced the sensitivity by which we could identify outliers among the centers. The visit order was randomized to ensure a possible difference between the 1st and 2nd visit did not bias the center variation.

In the current study the within-subject SD on the same day and center was 0.01 and the within-subject SD between centers was 0.02. The small difference between centers within-subject could be due to different flow and respiratory rates. A higher expiratory or inspiratory flow (turbulence) may cause higher resistance. Every center followed the same standard operating procedure where the aim of breathing frequency was 30-45 breaths per minute and the observers were trained to choose technically acceptable loops with minimal turbulence. We did not report the flow and respiratory rates because it was not possible with the current software to save the data.

In our previous study, the precision (repeatability) of sRaw measurements 9 days (mean) apart in young children with asthma (asymptomatic during the study period) was found to have an intra class coefficient of 0.87 (within-subject SD 0.03) for baseline measurements between occasions (50). The higher within-subject SD in the previous study could be explained by the asthma status of children.

The current study was designed to find a possible center effect. We were able to account for any possible observer bias by having a center specific observer as well as a common observer visiting every center. The order of measuring the biological control for the local and travelling observer was not randomized, but we found no effect of the investigator who travelled between the centers on the measurements (p-value >0.5) (table 1). A previous study found significant systematic difference in between-observer variability (7%) in sRaw measurements (13).

Normative data

In the second part of the study a center effect could not be found, probably because center no. 1 was corrected and center no. 6 did not participate. Atopy and smoking did not significantly differ between centers (Table 2).

A British cohort study found that children of atopic parents and those with personal atopy had impaired lung function at the age of 3 years, even in the absence of respiratory symptoms (20). The high incidence of parental atopy in our study could be a selection bias but we did not find a statically difference between atopic disposed and non-disposed children.

Most papers have reported association between parental smoking and impaired lung function in wheezing or asthmatic young children (57-59); some have investigated the effect of parental smoking in healthy children (60-62) and found reduced lung function in healthy infants. The ratio of smoking parents in the normative data corresponds the smoking habit in the Danish population (63). The complex interaction of genetic and environmental factors influence the development of airway disease and diminished lung function (20, 64). We did not find an effect of the child's history of atopy, parental atopy or smoking on lung function in our study, this could be biased by either the relatively small population, or the children with atopy, parental atopy or smoking could be a selected healthy population.

We previously reported normative values from a population of 121 children 2-5 years of age (12); mean sRaw (SD) was 1.31 (0.20). Z-score was -0.5 between the two data sets. No other reference value for sRaw_{TOT} has been reported, other studies have reported values of sRaw \dot{V}_{max} (54, 65, 66).

The variance was higher in the new collected data (SD 0.33) compared to previous reported (SD 0.2). This could be explained by a small non-significant center variance.

The previous population differs from the current in several following aspects: 1) Measurements were done at one center; 2) exclusion of children exposed to tobacco smoke and anyone with a history of eczema or doctor-diagnosed atopy in first-degree relatives; 3) The study included more 2- and 3-year-old children; many of these measurements had an accompanying adult in the whole-body plethysmograph. The current data only included children who performed a lung function measurement alone. The exact same equipment (unchanged hardware and software) at center no. 3 was used

in the previous report on normative data by Klug and Bisgaard (12). A comparison was made to ensure that time (10 years between the two studies) did not have an effect on sRaw measurements before we pooled the data. Result from center 3 was mean sRaw (SD) 1.26 (0.31). We compared the two data sets with a two sample t-test for means using logtransformed sRaw values. There was no significant difference between the previous data and the current normative data; p-value was 0.20 using the very same equipment. With no time and center effect we therefore pooled the previous data (121 children) and the current normative data (105 children) showing the normal sRaw in young children to be 1.27 kPa*s (0.25) independent of age, height and gender (Figure 5).

Parameters of specific airway resistance

Different estimates of sRaw can be calculated from the resistance loop: sRaw_{TOT} (inclination of the line between points of maximum pressure (volume) during inspiration and expiration), sRaw_{V_{max}} (flow point at the maximum flow during in- and expiration), sRaw_{50%} (flow point at 50% of the maximum during inspiration and expiration), sRaw_{0.5} or sRaw_{0.2} (flow points at which flow is 0.5 L/s or 0.2 L/s) and sR_{eff} (the slope resulting from calculation of area ratio by regression technique). sRaw_{TOT} is sensitive to partial obstruction of peripheral airways, sRaw_{0.5} reflects primarily the behavior of larger, more proximal airways and less sensitivity to peripheral airway abnormalities and sR_{eff} reflects larger central airways (67). Bronchial asthma may not only involve the large and the medium-sized airways, but the entire airway tree (68).

sRaw_{TOT} seems to be more sensitive or as sensitive as other methods (interrupter and impulse oscillation technique) of estimating the total resistance (10, 38) in asthmatic children, although this estimate may have a higher variability compared with sRaw_{50%} and sRaw_{0.2} (13). This must be a consequence of using only two points at the extremes of inspiratory and expiratory shift volume.

The previous study on normative data by Klug and Bisgaard (12) could only estimate sRaw_{0.5} in a third of the children due to flows lower than 0.5 L/s in the youngest children. We chose to report the normative data as sRaw_{TOT} in this study for comparison and pooling with the previous data.

II. -III.

Lung function measurements before and after RSV Bronchiolitis

Background

Respiratory syncytial virus infection in infancy

Respiratory syncytial virus (RSV) is a single-stranded RNA virus, a member of the family Paramyxoviridae, subfamily Pneumovirinae. RSV can be divided into two major antigenic groups, known as A and B, and each has several subtypes (69, 70). The virus was isolated first time in 1957, Baltimore, USA, from children with lower respiratory illness (71).

Epidemiology

Respiratory syncytial virus is a common cause of lower respiratory tract disease and hospitalization in otherwise healthy infants (72-75), and therefore a major cause of healthcare utilization (76). Recent data from the United States suggest that hospitalization rates caused by RSV infections are increasing (77). Two-three percent of infants react to RSV infection with bronchiolitis (inflammation of the small airways in the lung) before age 1 (77-80). A study in East Denmark estimated that 34/1000 infants below 6 months were hospitalized with RSV infection during one winter season (81). During the RSV-season from November to May approximately 1500 Danish children are hospitalized with RSV infection. High-risk groups for developing severe RSV infection include premature infants, children less than 2 years of age with congenital heart or chronic lung diseases or reduced immune-function. Most hospitalized infants are born at term and otherwise healthy; among this group the risk factors associated to RSV hospitalization are age under 6 months, male gender, month of birth, low socio-economic status, crowded living conditions, siblings, parental smoking, day care attendance and family history of asthma and atopy (69).

Passive prophylaxis (Palivizumab) against RSV has been available since 1998 and has reduced RSV hospitalization 55% among high-risk infants (82-84). Palivizumab is expensive and only given prophylactic to high-risk infants.

Clinical manifestations

RSV infection can be spread from an infected person up to three weeks after symptom debut by sending virus-containing droplets into the air when coughing or sneezing. The droplets remain contagious on environmental surfaces more than 6 hours and can infect a person when it comes in contact with mouth, nose or eye. Common symptoms of RSV infection are upper respiratory infection and coryza. Most recover from RSV infection within 1 to 2 weeks. However, in 25-40 % of the primarily infected infants a RSV infection manifests as a lower respiratory tract infection, bronchiolitis or pneumonia. Infants with bronchiolitis have tachypnea, wheezes, chest retractions and in the youngest infants risk of apnea (69, 85). Almost all children are infected with the virus by their second birthday, but only a small percentage develops severe disease.

Asthma in childhood

Asthma in young children is associated with high morbidity and the prevalence is increasing (86). Approximately 7% of Danish schoolchildren have asthma and 15-20 % of preschool children have episodes of asthmatic symptoms. This is the most common cause of hospitalization of young children in Denmark (87).

Definition

Asthma is a chronic inflammatory disorder of the airways. The mechanism involves several inflammatory cells (mast cells, eosinophils, Th2 etc.) which release mediators that contribute to airway narrowing: the airway smooth muscle contracts; increased microvascular leakage lead to airway oedema; mucus hypersecretion; and airway thickening (“remodelling”).

Asthma has significant genetic and environmental components, but since its pathogenesis is not clear, much of its definition is descriptive (86).

Clinical manifestations and diagnosis

The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. Following symptoms are highly suggestive

of a diagnosis of asthma: Frequent episodes of wheeze (more than once a month), activity-induced cough or wheeze, nocturnal cough in periods without viral infections, absence of seasonal variation in wheeze, and symptoms that persist after age 3.

Diagnosis of asthma in children 5 years and younger presents a particularly difficult problem, since the episodic wheezing and cough also are common in children with a viral respiratory illness. Global Initiative for Asthma (GINA) describes three categories:

- 1) Transient early wheezing, which is often outgrown in the first 3 years and often associated with prematurity and parental smoking.
- 2) Persistent early-onset wheezing (before age 3), typically children who have recurrent episodes of wheezing associated with acute viral respiratory infection, with no personal or parental atopy. Symptoms persist through school age. The cause of wheezing episodes is usually respiratory syncytial virus in children < 2 years of age.
- 3) Late-onset wheezing/asthma. These children have asthma that often persists into adult life. They typically have an atopic background.

In children younger than 5 years old, the diagnosis of asthma is mostly based on clinical judgment and assessment of symptoms. A useful method for confirming the diagnosis in this age group is a trial of treatment with short-acting bronchodilators and inhaled glucocorticosteroids. For older children (school age) lung function and airway responsiveness are recommended as diagnostic measures (86).

Risk factors

Genetic factors (sex or atopic predisposition) may influence the development of asthma, whereas environmental factors (allergens, tobacco smoke or infections) can trigger asthma symptoms or both. However, the mechanisms whereby they influence the development and expression of asthma are complex and interactive. The maturation of the immune response and the timing of the infectious exposures during the first years of life may be an important factor modifying the risk of asthma in the genetically susceptible person.

The “hygiene hypothesis” has been debated in the recent years and used as an explanation for the increasing incidence of asthma and allergy in the developed countries. The cornerstone in the hypothesis is that improvements in public health and

hygiene change the level of stimulation from the microbial environment. Newborn babies have immature immune responses characterized by a preponderance of cytokines of T helper 2 (Th-2) cells. It is hypothesized that the maturation of T-helper 1 (Th-1) cells after birth are triggered by microbes. The exclusion of such stimuli via microbe-free environment results in a skewed (high) Th-2 type response and lead to an exaggerated response to common allergens (88). Atopics have increased IgE production, eosinophilia, and exuberant Th-2 cell activity. By contrast, non-atopic people show mainly Th-1 immunity characterized by production of interferon gamma, which inhibits the growth of Th-2 cells. Studies suggest that a RSV infection stimulates a Th2 response (89-93); this may explain the reason why atopic predisposition is associated with severe RSV infection.

Causal direction between Respiratory syncytial virus bronchiolitis and asthma

Within the first year after RSV hospitalization, up to 20% of the children are re-hospitalized due to wheezing (75). Severe RSV bronchiolitis has been associated with later development of abnormal pulmonary function, wheezing, asthma and allergic sensitization (94-99).

However, the cause of severe RSV bronchiolitis has also been suggested to associate with pre-existing abnormal pulmonary function. Infants with impaired pulmonary function seem more prone to recurrent wheezing episodes (58, 100-108). Therefore it has been speculated that pre-existing abnormal airway resistance and/or bronchial hyperresponsiveness could account for the development of bronchiolitis in response to a RSV infection (109-111).

A genetic predisposition could play a role in the association between RSV hospitalization and hypersensitive airways; this is supported by the finding that atopic disposition is associated with increased risk of RSV hospitalization (112-116).

The risk of RSV bronchiolitis has also been associated with seasonality of birth (79, 117). A recent paper, based on 95,000 American children studied over five winters, found that being born 4 months before the winter virus peak had a 29% increase in odds of developing asthma. The authors suggest that bronchiolitis or some factor closely associated with bronchiolitis causes asthma (118) (Figure 8).

Causal Model

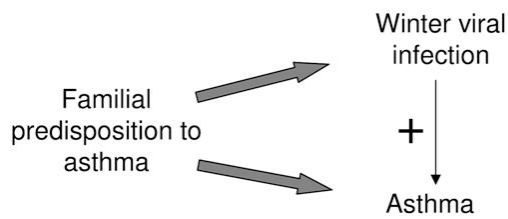


Figure 8: Familial predisposition to asthma relates to winter viral infection and asthma, winter viral infection is in the causal pathway of development of asthma (118).

Thus, many studies indicate an association between RSV hospitalization in infancy and wheezing and atopic disease, but it is unclear whether severe RSV bronchiolitis causes wheezing, or genetic predisposition or environmental risk factors increase the propensity to such exaggerated response to RSV.

Aims (study II-III)

Study II

To compare the long-term outcome of asthma, allergy and pulmonary function in monozygotic (MZ) twin pairs discordant for hospitalization with verified RSV bronchiolitis in infancy as a surrogate marker of the RSV disease severity. Any differential long-term effect from RSV disease severity in these genetically identical twins would suggest a causal role of RSV.

Study III

To compare the preexisting baseline lung function and bronchial responsiveness in infants who later develop RSV bronchiolitis with those who do not develop RSV bronchiolitis.

Material

Study II

Our study group of MZ twins discordant for severe RSV infection was subgroup of a larger twin cohort study previously presented by Thomsen et al. (2008) (115).

Four registries were used for recruitment of the study population: The Danish Civil Registration System (119); The Danish Twin Registry (120, 121); The Danish National Patient Registry (DNPR) and a research database recording RSV test from all hospitalizations in Denmark (122). The target population was identified by linking the personal identification number to 1) the twin status, 2) living address and 3) hospitalizations due to RSV infection during the period 1/1-1994 to 31/12-2003. The registry assured that the control twin had never been hospitalized with RSV infection.

During the period 12,349 twin pairs were born in Denmark. Fifty-seven MZ twin pairs (26 males) were discordant for RSV hospitalization (Figure 9). Nine pairs were unavailable due to death or address protection. Five pairs were excluded based on their hospital records because the RSV infection was found incidentally during hospitalization for other reasons (i.e. elective surgery) and not associated with lower respiratory symptoms. Forty-three pairs were invited of which 37 pairs accepted to participate (14 males; mean age 7.6 years; interquartile range: 5.9-9.2).

The twin status (monozygosity) was confirmed by DNA analysis with 10 highly polymorphic markers in our study population.

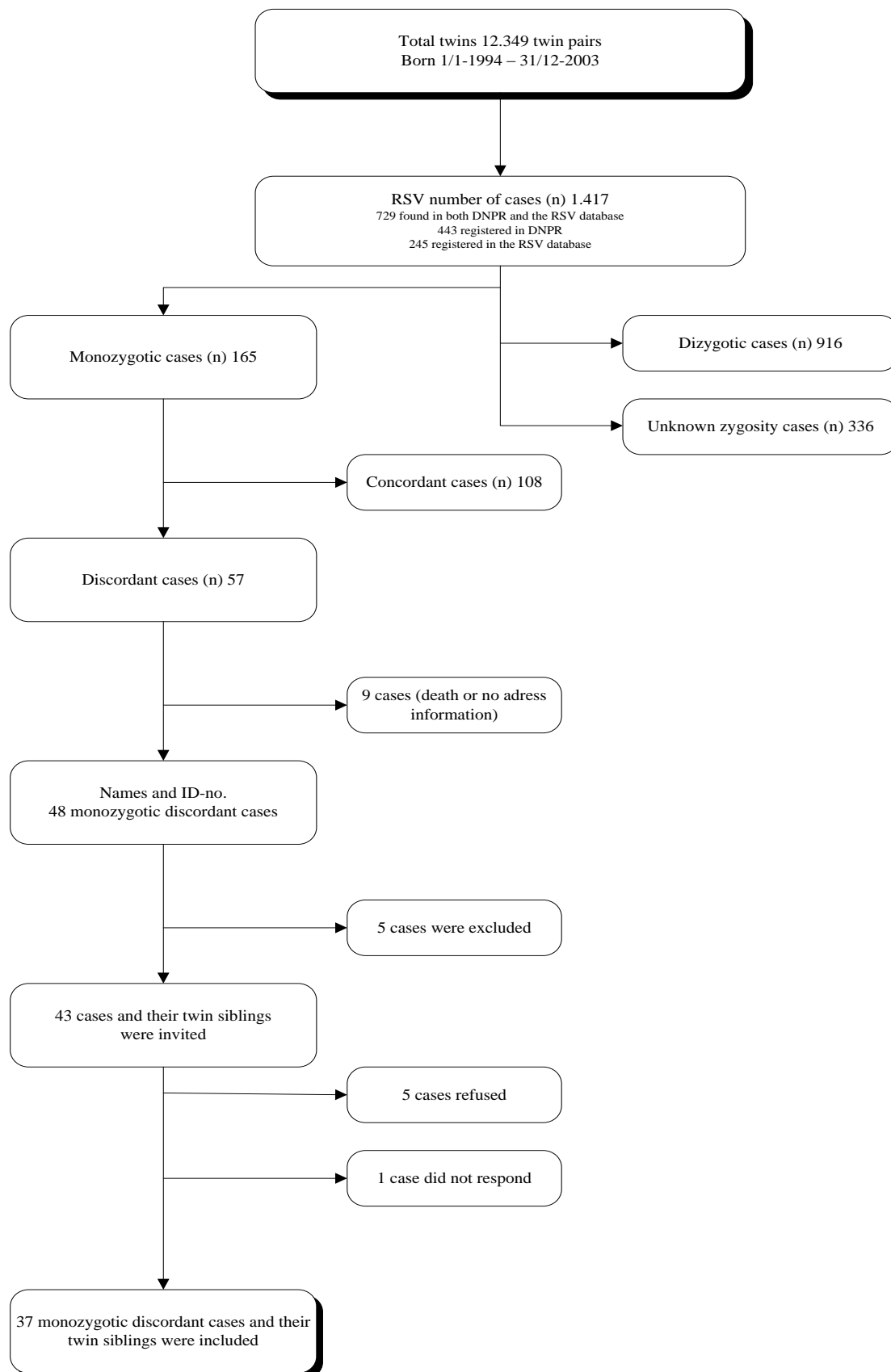


Figure 9: Study population (n = number of cases)

Study III

“Copenhagen Prospective Study on Asthma in Childhood” (COPSAC) is a prospective clinical study of a birth-cohort, which included measurements of baseline lung function and bronchial responsiveness to methacholine at 1 month by infant spirometry (123). The close clinical follow-up allowed prospective identification of infants who developed RSV bronchiolitis.

411 infants born at term (203 boys) of mothers with physician-diagnosed asthma were enrolled at the age of 1 month in the birth cohort study COPSAC (124-126).

Twenty-two infants developed RSV bronchiolitis before age 2 years; mean age 8 months (interquartile range 3-12 months). The control group consisted of 366 children, excluding the 22 with RSV bronchiolitis and 23 with acute severe wheezy exacerbations from the main cohort of 411 (Figure 10).

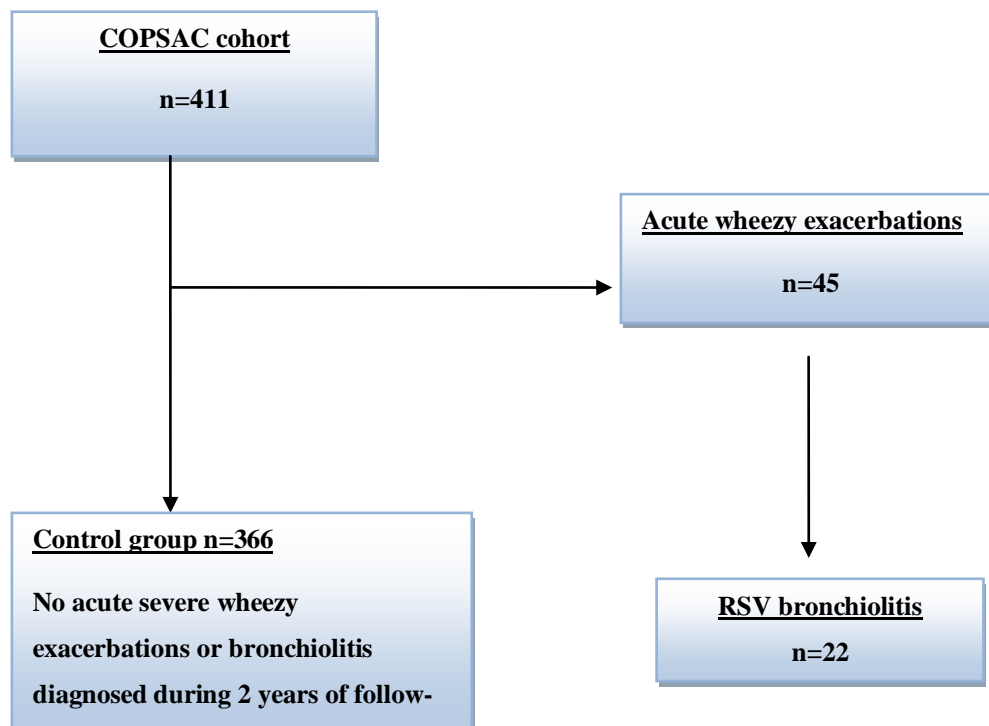


Figure 10: Flow chart

Methods

Designs

Study II:

Hospital records were retrieved to verify respiratory symptoms compatible with RSV bronchiolitis (severe cough, positive X-ray of thorax, use of β 2-agonist, crackles or wheeze by auscultation of lungs) (85, 127) and verified with an enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay test for RSV.

The twin pairs and their parents were summoned to the Danish Pediatric Asthma Center two centers in East- and West- Denmark for clinical examination including interviews on the child's asthma according to the GINA guidelines (128); information about medical history and objective assessments. The interviews and clinical examinations were done by one physician (PP). Twenty-two pairs were examined in Copenhagen and 15 twin pairs in Aarhus.

Skin prick test was done with standard inhalation allergens: birch, mugwort, grass, horse, dog, cat, dust mite and mould (Soluprick, SQ ALK-Abelló A/S) (129).

Study III:

The COPSAC cohort was assessed at one center (the Danish Pediatric Asthma Center, Copenhagen).

Bronchiolitis

Bronchiolitis was defined as an acute respiratory illness before the age of 2 years based on symptoms of coryza progressing over a few days to cough, tachypnea, chest retraction and wide spread crackles, wheezes or both (127) diagnosed by the COPSAC clinical research unit doctor or admitted acutely to the local hospital for such symptoms.

RSV bronchiolitis

RSV bronchiolitis was defined as bronchiolitis with a positive ELISA test for RSV infection within 10 days of the acute episode.

Acute severe wheezy exacerbations

Acute severe wheezy exacerbations was defined as an acute obstructive respiratory illness diagnosed by the COPSAC clinical research unit doctor and judged to be a severity requiring treatment with oral or high-dose inhaled corticosteroids (>800 micg budesonide/day) or hospitalizations at their local hospital caused by airway symptoms where an anti-asthmatic drug was prescribed (pseudocroup episodes were excluded).

Control group

Children who developed neither bronchiolitis nor acute wheezy exacerbations before age 2 were considered as controls.

Lung function measurements

Study II:

Whole body plethysmography

Children less than 7 years were tested using the whole body plethysmography to measure specific airway resistance (sRaw) as previously detailed (10, 11).

Spirometry

Children of 7 years and above used a spirometer to measure Forced Expiratory Volume in the first second (FEV₁) (Vitalograph Spirotrac) according to the criteria of the American Thoracic Society (130).

Bronchial responsiveness

Children of 7 years and above were tested for responsiveness to methacholine chloride with FEV₁ measurements before and 3 minutes after each dose. Methacholine was delivered in successively increasing doses according to a protocol previously validated for school children (33) by an automatic, inhalation synchronized, dosimetric jet-nebulizer (Spira Elektro 2; Respiratory Care Center, Hämeenlinna, Finland). PD20 value (μmol) was calculated by linear interpolation between the dose points bracketing the 20% fall in FEV₁ (131).

Children less than 7 years were tested with dry air hyperventilation as previously described and validated in this age-group (18, 36, 132).

Fractional exhaled nitric oxide

Fractional exhaled nitric oxide ($F_{E}NO$) was measured by the online single breath method according to European Respiratory Society/American Thoracic Society task force (40) using the NIOX equipment (Nitric Oxide Monitoring System; Aerocrine, Sweden).

Study III:

Lung function measurement in neonates

Lung function was assessed during sedation by infant spirometry at 1 month applying the raised volume rapid thoracic compression technique (RVRTC). The method, equipment, data collection and analysis was performed in agreement with ATS/ERS standards (2). Equipment was calibrated and tested prior to every lung function test. An inflatable “squeeze”-jacket was wrapped around the infant's chest and abdomen. A subsequent inflation of the jacket provided the passive expiration and flow was measured using a pneumotachograph with an aircushion facemask (123, 133, 134). The infant was administered three inflations reaching a transrespiratory pressure of 2 kPa with passive deflations between each. A compression force transmitting an additional pressure of 2 kPa was then applied via the squeeze-jacket to the thorax and abdomen, leading to an airway opening of pressure of 4 kPa for forced expirations. Baseline flow was measured as the Forced Expiratory Volume at the first 0.5 second ($FEV_{0.5}$) (123, 133). Other parameters not reported in this thesis comprised FVC, forced expiratory flow at 25% of FVC (FEF_{25}), forced expiratory flow at 50% of FVC (FEF_{50}) and forced expiratory flow at 75% of FVC (FEF_{75}). All parameters were measured five times at baseline and three times at each challenge step, using the median value for $FEV_{0.5}$ to decide on the continued provocation. Tidal flow was also measured but not reported in this thesis; indexes comprised tidal volume (V_T), respiratory frequency, peak tidal expiratory flow (PTEF), expiratory time as ratio of respiratory cycle time, time to reach PTEF (T_{PTEF}), and T_{PTEF} /expiratory time ratio (T_{PTEF}/T_E) (123, 133).

The software defined FVC as the first plateau on the volume-time curve, and only measurements with FVC appearing after 0.5 seconds, and with $FEV_{0.5}$ being smaller

than or equal to FVC were accepted. In addition, FVC was only accepted if correctly defined as the plateau appearing after a full expiration. The aim was collecting 3-5 curves at each dose step. The curve containing the median value for the FEV_{0.5} was used for the analyses of both volume and flow parameters.

Bronchial responsiveness to methacholine was assessed by FEV_{0.5} as well as continuous measurements of trans-cutaneous oxygen (PTcO₂) (TCM3; Radiometer; Copenhagen, Denmark). The methacholine was administered in quadrupling dose-steps by a dosimeter attached to a nebulizer (SPIRA 08 TSM 133; Respiratory Care Center; Hämeenlinna, Finland) as previously detailed (133). The result was assessed by PTcO₂ in accordance with previous sensitivity analyses (10, 133) and the provocative dose causing a 15% drop in PTcO₂ was estimated from the dose response curves fitted with a logistic function.

Statistical analyses

SAS version 9.1 was used for statistical analyses.

Study II: The continuous data were log-transformed and presented as geometric means in the table. Odds ratios were calculated for the dichotomous data with 95% confidence intervals. Data were analyzed with a paired t-test for continuous outcome measures, Fisher's exact test for dichotomous outcomes and Wilcoxon's (paired) test for dry-air hyperventilation outcome. All hypotheses tests were 2-sided and used a significance level of 0.05.

Study III: Lung function measurement data (FEV_{0.5} and PD15) were calibrated with lifespan and birth length in accordance with previous lung function analyses of the newborn (135) calculated by a generalized linear model. Lifespan at examination date was calculated as the sum of estimated gestational age in weeks and weeks since birth. The lung function measurements were log transformed and we used a multivariable logistic regression model to calculate adjusted odds ratios and 95% confidence intervals. Outcome variable was RSV bronchiolitis compared to control group. Confounder adjustment included mothers smoking during 3rd trimester (dichotomized) and gender.

Ethics

The two studies were approved by the local ethics committee (KA-20060022 and (KF)01-227/97) respectively) and by the Danish Data Protection Agency (J.nr. 2005-41-5163, J.nr. 2005-2311-0121 and 2008-41-1754, respectively).

The children were enrolled after written consent was obtained from the parents or guardians.

Results

Twin study: Lung function after RSV infection (study II)

Mean gestational age of the twin pairs was 35.5 weeks (median 36 weeks; interquartile range 34-38 weeks). There was no significant difference between RSV hospitalized and non-hospitalized twins with respect to birth weight (mean 2369g; interquartile range 2034-2750g), neonatal treatment for lung complications (Continuous Positive Airway Pressure, surfactant or pneumothorax) and birth order (22/37 probands were first born).

Forty-nine % of the twin pairs had parental atopic predispositions. The average age when hospitalized for severe RSV bronchiolitis was 10.6 months (interquartile range: 5.1-13.3); median age 8.4 months. Mean duration of hospitalization was 4.3 days.

According to the interviews (GINA guidelines) the prevalence of asthma was 18% in our twin sample. The twins did not differ with respect to current asthma; use of inhaled corticosteroid or beta₂-agonist ever; atopic dermatitis ever; F_ENO; baseline lung function; bronchial responsiveness or sensitization (Table 3).

Table 3: Comparison of clinical endpoints at follow-up between monozygotic twin pairs discordant for hospitalization for severe respiratory syncytial virus bronchiolitis

		Hospitalized vs. not-hospitalized		P-value
Asthma	OR (95% CI)	1.21 (0.36-4.00)		1.00 ^a
History of atopic dermatitis	OR (95% CI)	1.14 (0.41-3.14)		1.00 ^a
Use of inhalation steroid ever	OR (95% CI)	1.26 (0.49-3.22)		0.81 ^a
Use of beta2-agonist ever	OR (95% CI)	1.38 (0.55-3.46)		0.64 ^a
logF _E NO (n = 30)	Pbb mean (SD)	2.05 (0.66)	2.17 (0.69)	0.16 ^b
logBaseline FEV ₁ (n = 25)	L mean (SD)	0.42 (0.26)	0.46 (0.23)	0.12 ^b
logMethacholine PD20 (n =24)	μmol mean (SD)	0.74 (1.83)	0.41 (1.71)	0.27 ^b
logsRaw baseline (n = 12)	kPa*s mean (SD)	0.18 (0.26)	0.19 (0.30)	0.79 ^b
sRaw after dry air hyperventilation (n = 5)	% increase (median)	27%	18%	0.40 ^c
Positive skin prick test	OR (95% CI)	0.36 (0.07-2.08)		0.43 ^a

n = number of twin pairs which completed the test

^a Fisher's exact test

^b Paired t-test

^c Wilcoxon's test (paired)

Neonatal lung function prior RSV infection (study III)

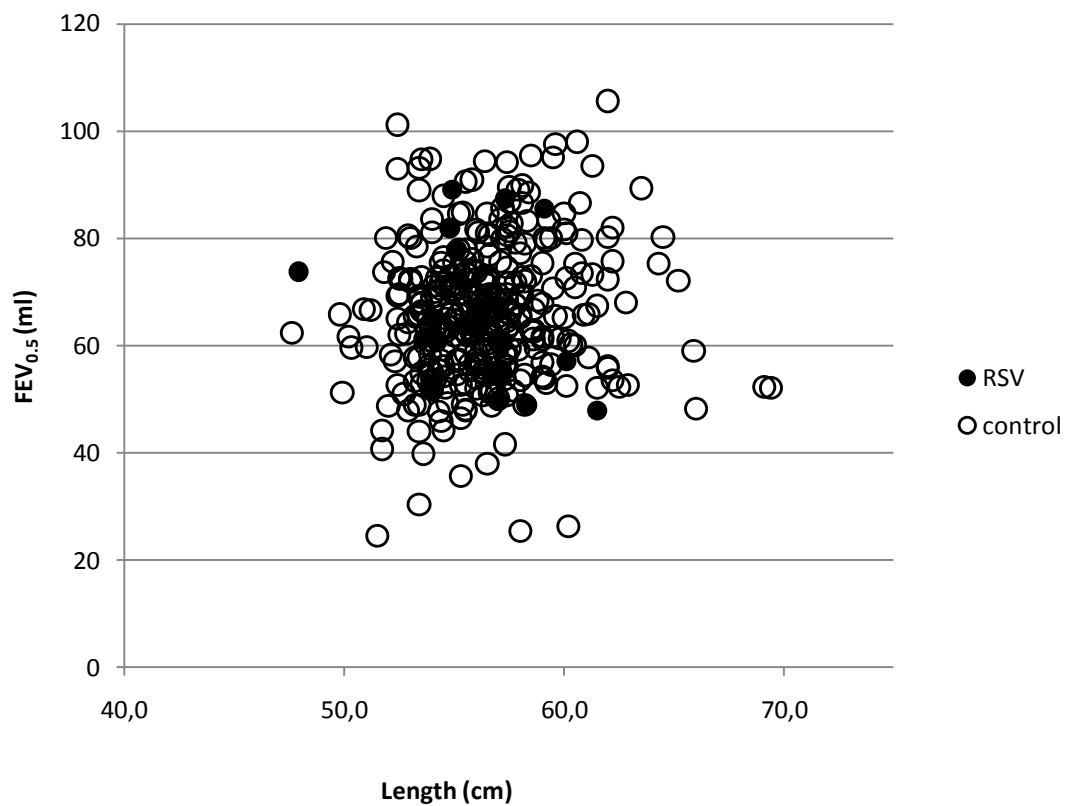
Twenty-two infants developed RSV bronchiolitis before age 2 (16 boys); 17 of the 22 children were under 1 year old when diagnosed with RSV bronchiolitis (mean age 5.7 months). Nineteen of the 22 children were hospitalized with RSV bronchiolitis (mean duration of 4.7 days). Lung function had been completed more than 1 week prior to the bronchiolitis. Baseline characteristics of the lung function measurements are shown in table 4.

Table 4: Baseline characteristics

	Total COPSAC		Control group	RSV bronchiolitis
Baseline lung function (FEV _{0.5} /ml)	404	Number	360	21
		Mean (SD)	66.3 (12.9)	65.5 (13.0)
		Median	65.8	63.6
PD ₁₅ (PTcO ₂ /mmol)	363	Number	322	20
		Mean (SD)	1.81 (12.2)	0.59 (0.88)
		Median	0.33	0.16

Baseline FEV_{0.5} did not differ between infants with RSV bronchiolitis compared with the control group: OR 1.08 [0.09-13.6], p=0.95 (figure 11); z-score -0.06.

Bronchial responsiveness did not differ between infants with RSV bronchiolitis compared with the control group: OR 0.94 [0.74-1.20], p-value 0.64.

**Figure 11: Neonatal baseline lung function versus body length**

Discussion

Twin study

We found no difference within co-habiting MZ twin pairs discordant for hospitalization for RSV bronchiolitis in infancy on the asthma prevalence, baseline lung function, bronchial responsiveness; biomarker of airway inflammation (F_{ENO}) and sensitization 7 years after such severe RSV bronchiolitis.

Limitations and Strengths of the study design

Monozygotic twins were useful for investigating the role of genetic and environmental factors on asthma because of their identical genetic background and similar childhood environmental exposures. However, comparison of MZ twin pairs discordant to hospitalization for RSV bronchiolitis was prone to a number of biases.

We had no evidence to prove that the twin serving as control was actually infected with RSV, but rely on very high virulence of RSV among cohabitating twins previously estimated to 93-96% (115) though this could not be verified. In an optimal study there would have been comparable nasopharyngeal aspirates from the twin pair to quantify RSV virus load (136-138) or a blood sample measuring RSV serum antibodies to detect any difference in association to disease severity (139). Thomsen et al. (2008) reported that in the whole twin cohort the proband-wise concordance rate of hospitalization for RSV-bronchiolitis was significantly higher in MZ (0.66) than in DZ twin pairs (0.53), $p=0.02$ (115).

The decision to hospitalize may have been influenced by local policies such as number of available rooms and the assessment of disease severity varied by different physicians, but this would not influence the fact that only one twin was hospitalized and the other was not.

We do not have positive evidence that the control twin had milder symptoms, but rely on the fact that one was hospitalized while the other was not. Danish children are generally only admitted with RSV infection if they need support with feeding tube, suction of upper airways, mask inhalations or nasal continuous positive airway pressure. Yet, this could be biased from registration practice. Indeed, fifty-four% of the parents answered retrospectively that the hospitalized RSV proband twin suffered more severe

lung symptoms, 19% said it was the control twin and 27% said both twins had comparable symptoms. However, there is a significant risk for recall bias and the MZ pairs could be mixed up since the hospitalization was on average 7 years ago and parents were generally most unsure of the details. Furthermore, it would be impossible to make a subgroup analysis restricted to the twin pairs where the parents' memories and data agreed. The study group would be very small.

It is known that male sex could be a risk factor for RSV hospitalization (140-142). In the whole twin cohort the boys had a higher prevalence of severe RSV infection compared to the girls; odds ratio 1.20; CI [1.07-1.33] as previously presented by Thomsen et al. (2008) (115), but there was a random overrepresentation of females in our study (subgroup).

The discordant MZ twins in our study were born slightly earlier (mean gestational age 35.5 weeks and mean birth weight 2369 grams) than the MZ twins in the overall twin cohort (mean gestational age 36.1 weeks (SD 2.5) and birth weight 2498 grams (SD 549) (115). The differences were small and not significant; therefore it seems that the discordant twins were representative of MZ twins in general.

On the other hand it may bias the result because premature infants as a population have its own risk factors for impaired lung function.

The distinction between possible "wheezers" and "bronchiolitis" may be inaccurate in this as in other studies. The distinction between wheezers and bronchiolitis is normally related to severity of wheeze and concurrent clinical signs of lower airway infection. Our samples included the latter, i.e. first hospitalization for severe wheeze with concomitant signs of lower airway infection.

The highest incidence of hospitalization for RSV-bronchiolitis is often reported in the 0-1-yr-olds (113, 114, 143, 144). However, the reported mean age may be biased from excluding older children from such analyses. For example Sigurs et al. reported mean age of RSV hospitalization to be 3.5 months excluding infants older than 12 months (98, 98). Fjaerli et al. reported admission age median to be 6 months in children under two years old in their study (140). One study included children up to 2 years and

showed the median age of hospitalization was 10 months of age similar to our findings (145).

Most importantly, the power of our study is limited by the low number of 37 paired cases, and the 95% confidential interval is correspondingly wide particularly for the clinical end-point though less so for the objective surrogate markers of asthma such as lung function and $F_{E}NO$. However, our study was based on the complete national database and not power calculations.

Mean age at the clinical examination was 7.6 years. A difference in asthma prevalence later in life cannot be excluded but seems low inasmuch as those studies showing higher asthma prevalence after severe RSV found this mainly in early childhood (96) and most cases of asthma debut before school age.

Interpretation of the study

We studied the direction of the causal relation between severe RSV bronchiolitis and asthma. RSV bronchiolitis has been associated with wheezing, asthma and abnormal pulmonary function in childhood (94-99). One particular cohort study reported an asthma rate of 43% versus 8% and sensitization to common allergens of 45% versus 26% by age 13 years in children with infant hospitalization for RSV bronchiolitis compared with a matched control group (98). A review of pooled data from 10 controlled studies concluded that wheezing (but not recurrent wheezing) is more common after severe RSV bronchiolitis up till 5 years of follow-up (146). Two Finnish studies reported only marginally increase in the prevalence of asthma after RSV bronchiolitis in infancy (145, 147). On the other hand, predisposition to asthma and atopy was associated with increased risk of lower respiratory tract infection and hospitalization for RSV infection (112, 113, 116) and early wheezy symptoms were found to be a strong risk factor for subsequent hospitalisation for RSV (114). Therefore the direction of causality is unknown: Does RSV increase risk of asthma or is asthma constitution increasing the risk of severe response to RSV infection, or are both sharing a common undisclosed environmental exposure.

The model we used adjusted for genetic variation by analyzing long-term outcome in monozygotic twin pairs, in which one infant had been hospitalized for severe lung

symptoms in response to verified RSV bronchiolitis, whereas the twin sibling had not been hospitalized at any time for RSV bronchiolitis. This allowed a comparison of severe versus milder response to RSV infection, genetic factors and environmental exposures during follow-up years being equal between the co-habiting MZ twin proband and control pairs.

A genetic contribution to the high prevalence of asthma in our twin sample compared with the current asthma prevalence in our region (148-150) is suggested from the high prevalence of parental atopy in the present study, which also suggests that the atopic predisposition contribute to susceptibility for severe RSV infection. Likewise, such genetic component was reflected by previous finding of a higher concordance for hospitalization for RSV-bronchiolitis in MZ than DZ twin pairs (115).

Several publications suggest that RSV infection in early infancy stimulates a Th2 response (89-93, 151) and so it is unclear if this association is causal for later development of asthma and atopy. Previous publications have found that severe RSV bronchiolitis was associated with genetic polymorphism (haplotype IL13-IL4) (152-155), which may play an important role in the Th2 response (skew). The same locus have been associated with atopy and asthma in other genetic studies (156-158). These studies together suggest that primary RSV bronchiolitis and atopy share a genetic contribution at the IL13-IL4 locus. Animal studies have demonstrated a potential mechanism by which viral infection severity associate with an increase in Th2 response (159, 160), but outcome have shown to be dependent upon timing of the events. The presence of an established Th1 response have shown to limit the development of airway hyperresponsiveness in an animal study (161). This may explained that repeated or early exposure to respiratory viruses (e.g. large families or daycare attendance) can protect/modulate development away from an allergic type 2 response, which corresponds well to the “hygiene” hypotheses. Delay in immune maturation in infancy has been implicated in the development of atopy with formation of Th2 memory in response to antigen exposure, this may happen in a period of susceptibility where Th1 responses to infections like RSV may be suboptimal (152).

A study with preterm infants (high-risk group) who had received the anti-RSV monoclonal antibody Palivizumab reported a 50% decrease in the incidence of recurrent

wheeze compared with controls (162). This supports the belief that RSV is the cause of respiratory illness in infancy.

A more recent publication based on 8,280 twin pairs showed that a model in which asthma “causes” RSV hospitalization fitted significantly better than a model in which RSV hospitalization “causes” asthma (163). On the other hand Wu and colleagues concluded in another recent paper that they have shown strong evidence for causal relationship of winter viruses and early childhood asthma (figure 8) (118). In the light of these two particular papers Kuehni et al. discussed the causal link between RSV infection and asthma in an editorial (164). The editorial concluded the new data were more in favor of the hypothesis that the association between RSV and asthma is due to shared predisposition rather than to a causal effect of RSV. However, the authors pointed out that the asthma phenotype was poorly defined in both studies. The GINA guidelines have defined three categories: transient early wheezing, persistent early-onset wheezing and late-onset wheezing/asthma (86).

Cohort studies have suggested that the association between RSV infections and later wheeze wanes with time (age) (96, 99). Huge epidemiological studies have power but difficulty getting certain asthma outcomes (e.g. lung function measurements), therefore it is also important to supplement with smaller clinical studies such as this twin study.

We found no differential effect from severity of RSV infection on asthma and allergy 7 years after the infection. This may suggest that some undisclosed environmental factor instead could be responsible for the different severities of RSV infection. A study of the whole twin cohort showed that the severity of RSV infection was determined partly by genetic factors (16%); family environment accounted for 73% and nonshared environment for 11% of the individual susceptibility to develop severe respiratory syncytial virus infection (115). The nature of such exposure is unknown and surprising in monozygotic twins, maybe this study material (subgroup) was unique at some level. Scientists have discussed the theory of epigenetic, which refers to heritable changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence. Monozygotic twin siblings frequently present many phenotypic differences, such as their susceptibility to disease. Recent studies suggest that phenotypic discordance between monozygotic twins is to some extent due to

epigenetic factors. Acute environmental factors are directly associated with epigenetic-dependent disease phenotypes (165-167). Speculations may consider differing co-infections with an agent of less virulence including bacterial colonization (126, 168) or other viral infections. There is increasing evidence that rhinoviruses (RV) are able to cause lower airway infections and to induce wheezing in young children, and it may be as common as RSV as a cause of bronchiolitis (142, 169-171). Human metapneumovirus (hMPV) has also recently been recognised as one of several other viral pathogens that can cause acute bronchiolitis in infants (172, 173), the remaining being mainly parainfluenzavirus and adenovirus. However, the long-term effect of this and other viral agents on lung function and symptoms later in childhood is not yet fully investigated.

Neonatal lung function

Principal findings

We found no association between baseline lung function and bronchial responsiveness in neonates and later development of bronchiolitis with RSV infection compared to the control group.

Limitations and Strengths

We used strict definition of bronchiolitis in accordance with a recent review (127). Our definition of bronchiolitis is consistent with the Anglo-Saxon definition including widespread fine crepitations and sometimes expiratory rhonchi on auscultation in the definition (127, 174-176). In the American literature, the definition sometimes includes all first-time wheezing infants with associated respiratory-tract infection (144, 177, 178). We recognize the lack of evidence for the effect of steroids for bronchiolitis (179-184) but took the pragmatic approach to include their use in our definition of the more acute cases realizing this is a well established clinical practice and indicating a certain clinical severity. Also, we recognize that the diagnosis of acute bronchiolitis is likely to comprise infants with first acute attack of asthma, but this would have biased the risk estimates against the null hypothesis assuming such infants with underlying asthma would have had reduced lung function and bronchial hyperresponsiveness.

The confidence interval of the comparison of baseline lung function includes a wide interval from 0.09 to 13.6 (risk of type 2 error), suggesting the need for an unlikely study size to ever decide prospectively if baseline lung function is lower in infant developing RSV-bronchiolitis. On the other hand comparing bronchial responsiveness showed a narrow confidence interval (-26% to +20%); i.e. suggesting it seems unlikely that bronchial hyperresponsiveness was the distinguishing factor in infants later developing RSV-bronchiolitis.

The strengths of this study were the unique prospective nature of the birth cohort study with lung function assessments before the development of bronchiolitis together with the close clinical follow-up to the research clinic and daily symptom recordings assuring ascertainment of all severe episodes.

Lung function measurements were completed in a large group of asymptomatic infants (404) within a narrow age-range around 1 month after birth. This is the largest study of lung function in neonates under standardized conditions. Baseline lung function was conducted by infant spirometry adapted as the state-of-the-art Raised Volume Rapid Thoracic Compression technique which provides flow-volume measures overall in agreement with ATS/ERS standards (2).

The method of the study differs from the ATS/ERS guidelines in few points, for example it is recommended to deliver an inflation pressure of 30 H₂O cm ~ 2.9 kPa. A previous publication by Loland and Bisgaard commented the safety in the study (123) and recognized that FEV_{0.5} measured in this study may reflect forced flows at lower lung volumes than FEV_{0.5} measured from a 3-kPa inflation pressure. On the other hand the lower compression pressures and obtaining flows at lower lung volumes could give advantages in the success rate for the detection of physiologic change and safety in terms of lower risk for aspiration.

The commonly reported parameters calculated from RVRTC are as follows: FVC, FEV_{0.5}, FEF₅₀, FEF₇₅, FEF₈₅ and FEF₂₅₋₇₅ and in infants younger than 3 months also FEV_{0.4}. However, it is not recommended to report maximal flow at FRC (\dot{V}_{maxFRC}) from RVRTC because it is likely to be highly variable; tidal flow parameters are hardly mentioned in the ATS/ERS recommendations (2). Most of the recommended parameters were measured (FVC, FEF₂₅, FEF₅₀, FEF₇₅ and tidal flow parameters) but not reported in this thesis. The previous analyses found PtcO₂ was the most sensitive parameter for

detection changes in lung function (dose-response curve metacholine challenge), followed by $FEV_{0.5}$, both superior to other indexes of forced spirometry as well as all tidal breathing indexes (10, 133); therefore we only report these two parameters in this study. A shorter time limit as 0.4 s may have improved the sensitivity (185). Values of FEF% have been reported to be more discriminative than FEV(t) in some studies (186). The discrepant finding could be due to the different definition of sensitivity. In our previous study the sensitivity was defined as the ability to detect induced changes in lung function within an individual while in other studies it was defined as the ability to discriminate between disease and healthy infants. An Australian study showed that FEV(t) were more reproducible than flow measurements in the tidal volume range and also found that FEV(t) were significantly lower in wheezy infants with less overlap than flow measurements (187).

The coefficient of variation for $PtcO_2$ and $FEV_{0.5}$ were 4% and 7% respectively based on measurements before and after saline solution inhalation. RVRTC-measurements were automatically registered, and results from $PtcO_2$ were read from the print-outs by an independent person, different from the operators. Volume-time curves were only accepted if the forced expiration proceeded smoothly, with no signs of glottic closure or early inspiration. The software defined FVC as the first plateau on the volume-time curve, and only measurements with FVC appearing after 0.5 seconds, and with $FEV_{0.5}$ being smaller than or equal to FVC were accepted. In addition, FVC was only accepted if correctly defined as the plateau appearing after a full expiration. The curve containing the median value for the $FEV_{0.5}$ was used for the analyses of both volume and flow parameters (133). The choice of “best” trial differs in this study from the ATS/ERS guidelines which recommend choosing the “best” (technically) trial defined as the one with either the highest sum of FVC and $FEV_{0.4/0.5}$ or FEF_{25-75} , provided they are within 10% of the next best loop (2).

We analyzed bronchial responsiveness as a quantitative trait in the RSV and control group rather than dichotomizing the infants into +/- hyperresponsiveness.

All cases of non-RSV bronchiolitis and acute severe wheeze episodes were excluded from the control group, which would tend to favor a difference between our cases and controls. We made a spot check in the control group and found no acute wheeze episodes in their hospital records. 44 children in the control group dropped out before

age 2 years (mean age 11.4 months) in the COPSAC cohort. They could have had RSV bronchiolitis after drop-out of the cohort study without our knowledge. We decided to include them in the control group because in general drop-outs are most likely children who stay healthy, and the families had an option to become active in the cohort again at anytime.

Nineteen of the 22 infants (86%) with RSV bronchiolitis were hospitalized. Danish children are generally only admitted if they need support with feeding tube, suction of upper airways, mask inhalations or nasal continuous positive airway pressure, which ensures a certain severity in their RSV infection in this study.

Confounder adjustment included mothers smoking during 3rd trimester and gender both well known risk factors for bronchiolitis (57, 141, 142, 188-192). Lung function measurement data were calibrated for birth length and lifespan at examination date because these parameters have shown to affect early lung function in the COPSAC cohort (135). We did not confounder adjust for other risk factors such as month of birth, socio-economic status, or siblings.

We found an overweight of boys (73%) in the RSV group in agreement with previous reports (140, 141, 188, 189, 193).

Meaning of the study

Acute RSV bronchiolitis may occur in otherwise healthy infants. It is not known whether viral bronchiolitis is causatively related to asthma or simply identifies infants at risk for subsequent wheezing from an atopic predisposition or pre-existing abnormal lung function (194, 195). Infants with impaired pulmonary function at one month of age was reported to be prone to recurrent wheezy episodes and asthma (58, 100-103, 105, 108, 196). Therefore it has been assumed that acute bronchiolitis or wheeze develop due to pre-morbid abnormal pulmonary lung function consistent with smaller airway size (103, 104, 106, 110, 111). But these are indirect evidence as clinical wheezing illness was used as end-point.

Our study showed no association between early lung function (FEV_{0.5} and bronchial hyperresponsiveness) and subsequent RSV bronchiolitis. There were no difference in pre-morbid lung function and bronchial hyperresponsiveness in infants who later develop RSV bronchiolitis and those without such severe infection. This could mean

that small airways were not the distinguishing feature of later development of RSV bronchiolitis. We recognize that the confidence interval of the comparison of baseline lung function was wide with the risk of type 2 error.

Broughton et al. (2006) studied prospectively premature infants and found those who had symptomatic RSV lower respiratory tract infection had worse lung function (higher resistance using occlusion technique) prior to neonatal unit discharge compared to controls but no difference in the lung volumes (functional residual capacity using helium gas dilution system) (109). It is difficult to compare this study with our result since we used a different lung function technique (forced expiratory maneuvers). Future studies may consider investigating the sensitivity of other lung function tests such as for example whole-body plethysmography measuring airway resistance.

Forced expiratory maneuvers was performed on 155 healthy infants by Jones and colleagues with age range from 3 to 149 weeks (mean 48 weeks) (197). From the results the study group calculated regression equations of the pulmonary functions versus body length. In comparison, our data of FEV_{0.5} was lower (66.3 ml (12.9)), mean length 56 cm; FEV_{0.5} in Jones et al. data with corresponding length was approximately 120-160 ml/s. However, the study was done on cross-sectional data and it does not state clearly how many infants there were in the lower age (length) group. The same applies for two other studies where FEV_{0.5} were obtained in 23 healthy infants (age range 7 weeks to 2 years) (198) and 26 healthy infants (age range 3 to 23 months) (187); the FEV_{0.5} (length 60 cm) were respectively 95.0 ml and 121.2 ml. The two studies had few infants in the lower age group. Studies using forced expiratory maneuvers have shown to discriminate normal infants and wheezy infants or infants with cystic fibrosis (4, 187, 199). Unfortunately, the lung functions of infants with airway disease were not comparable with our data since the infants in these studies were older (age > 3 months).

A cross-sectional study of 37 normal infants found that a family history of asthma had a negative effect on FEV_{0.5} (198). Another study on 63 normal healthy infants found that airway responsiveness in infancy was increased in families with history of asthma or parental smoking (196).

Since our cohort only includes infants with asthmatic mothers the absolute levels of lung function and bronchial responsiveness may not be representative of the general population. However, this does not affect the purpose of comparing lung function of

infants who later develop RSV bronchiolitis and infants who do not develop RSV bronchiolitis. The risk factors for neonatal lung function was studied in the COPSAC cohort in a recent publication (135). The study showed that high body mass index in newborns and mothers smoking were associated with reduced lung function; and parental atopic disease (mother's or father's eczema, urticaria or allergic rhinitis or father's asthma) did not affect the neonatal lung function and bronchial responsiveness. More interestingly, there was a parabola (increasing) development of bronchial responsiveness peaking at 3 months of age (though with few subjects at the later time points), which could coincide with hospitalization for acute viral bronchiolitis.

In our study group of infants less than 2 years of age the mean age of the infants diagnosed with RSV bronchiolitis was 8 months (174). Other studies have limited the group of interest to children less than 12 months of age and accordingly reported even lower age of RSV infection (113, 114, 143, 144, 200).

The incidence of bronchiolitis (5%) was higher than reported in most studies (1-3%) (77-79, 81, 140, 201). This suggests a genetic component in RSV bronchiolitis as all mothers had asthma. Predisposition to asthma and atopy has been associated with increased risk of lower respiratory tract infection and hospitalization for RSV infection (112, 113, 116). Young et al. found 7% of a cohort (253 infants) with the diagnosis of bronchiolitis before 2 years of age (only 2 infants were hospitalized and confirmed for RSV infection); 71% had a family history of atopy (110). A Danish case-control study has also supported that asthmatic disposition and wheezing were strong determinants of subsequent respiratory syncytial virus hospitalization in children <18 months (114); the relative risk of respiratory syncytial virus hospitalization in the offspring was 1.72 for maternal asthma, and 1.23 for paternal asthma.

Although family atopy is a risk factor, the Danish twin cohort study found the severity of respiratory syncytial virus infection was determined partly by genetic factors approximately (16%), and so the environment accounted mostly of the individual susceptibility to develop severe respiratory syncytial virus infection (115, 163).

The RSV group was not characterized with increased bronchial responsiveness in infancy. In order to investigate the association between lung function and bronchiolitis further we could have included all children with bronchiolitis and not only RSV

bronchiolitis. There is an increasing recognition that other viruses (e.g. rhinovirus) are as common as RSV causing bronchiolitis in young children (142, 169-171).

A recent prospective, population-based cohort study examined the associations between hospitalization for RSV infection and invasive pneumococcal disease in Danish children < 2 years. The study found that invasive pneumococcal disease did not increase the risk of RSV hospitalization but recent hospitalization for RSV increased the risk of invasive pneumococcal disease (168). On the other hand a previous study from the COPSAC cohort showed that neonates colonized in the hypopharyngeal region with *S. pneumoniae*, *H. influenza*, or *M. catarrhalis*, or with a combination, were at increased risk for recurrent wheeze and asthma early in life (126). One may consider co-infections with other viral or bacterial infections responsible for different response to RSV infection and the development of asthma (90). This may argue against the “hygiene hypothesis”, where the absence of microbial stimulation postnatal results in a skewed Th-1/Th-2 balance, but the rate of transition from fetal to adult-like Th-1 cells is much slower in those with atopic parents (88). So the Th-1/Th-2 balance results from complex interactions between genetic background and environmental influences during infancy.

Conclusion and future perspectives

Study I

In conclusion, using a biological control we revealed errors in the accuracy of sRaw measurements at some centers despite normal calibration of the mechanical components. This study highlights the need for the development of equipment allowing control of the actual resistance measurements and not only some of its mechanical components. When software versions are upgraded with possible changes in algorithms, this could result in an undiscovered difference in sRaw value, unless the company provides a correction factor. A portable mechanical lung model analogue is needed for quality control of whole-body plethysmograph in preschool children. In addition, quality assurance could be optimized by developing the software to determine the best breath curves from algorithms as flow and respiratory rates or encourage the child (with animated bio-feedback) to breath with optimal respiratory rates and flow. This could be a commercial responsibility or be developed in collaboration with a technical department. Until such equipment becomes available the only option is to use healthy subjects to assure that the absolute value measured is similar to the normative values reported in this and a previous report of healthy young children.

If the measured baseline sRaw value is in the normal range and the clinician still suspects asthma, regardless of equipment type or software version, we recommend to measure bronchial hyperreactivity ($\geq 25\%$ increase in sRaw) (18) or bronchodilator responsiveness with a short-acting beta2-agonist ($\geq 25\%$ decrease in sRaw) (38); these tests can contribute to the asthma diagnose (11). Furthermore, the sRaw method has a good short and long term repeatability between occasions in stable asthmatics as previously presented by Klug et al. (1997) (15, 50), which is useful for the clinician in assessing asthma control for the individual patient.

We have examined the agreement of sRaw values between 6 centers using same equipment and produced a new set of normative data. It is uncertain if the normative values can be applied on other commercial whole-body plethysmographs. Our study showed a variation between the different centers and one can speculate if there exists a true reference interval when measurement of sRaw values seems to depend on the equipment. Preliminary results suggest that there are differences in sRaw values measured between different commercial whole-body plethysmographs. However,

further studies are needed to investigate this possible difference between different types of equipments. Importantly, other centers using normative data for whole-body plethysmographs should first consider proper calibration with biologic controls before applying reference values.

From previous studies it seems that $sRaw_{50\%}$ and $sRaw_{0.2}$ were more robust parameters for asthmatic children. A future study of $sRaw$ accuracy measuring other parameters than $sRaw_{TOT}$ including subjects with airway obstruction (asymptomatic in a stable phase) could be beneficial and may show less variability.

Study II-III

In conclusion, we found no differential effect from severity of RSV infection on the development of asthma and allergy in MZ twin pairs discordant for RSV hospitalization in infancy. This argues against a specific effect of the severe RSV infection in the development of asthma and allergy, and may suggest an undisclosed environmental factor interacting in these genetically similar twins leading to different severity of their response to RSV infection. Furthermore, that atopic predisposition seems to have a major influence on the development of asthma and allergy. Because of the small sample size this study must be considered as a hypothesis generating study.

Further studies are needed in the future to investigate the causal direction of RSV bronchiolitis and asthma. Suggestions for future research: 1) a clinical study with a larger study material of twins discordant for severe RSV infection in collaboration with international study groups with similar twin registries, 2) include dizygotic twin pairs discordant for severe RSV infection, 3) include non-RSV bronchiolitis as well, and 4) search for environmental and genetic markers of disease severity, or investigate the maturation of the immune response and the timing of the infectious exposures during the first years of life, which may be a vulnerable time-window influencing later development of asthma and allergy.

Bronchial hyperresponsiveness in newborns were not predictive of later development of RSV bronchiolitis nor did baseline lung function seem to predict though the confidence limit of comparison was wide. This study suggests that the distinguishing feature is not a mechanical disposition.

We suggest investigating other lung function test methods than RVRTC in infancy for later development of severe RSV infection. But it may be an environmental exposure or genetic disposition (or interaction), which influence the development of severe RSV infection. Preliminary result show significant effect of mothers smoking in 3rd trimester of pregnancy on the development of RSV bronchiolitis in the COPSAC cohort. A risk factor analysis of bronchiolitis and asthma as an outcome in the COPSAC cohort is needed in the future to explore the causal direction between respiratory syncytial virus bronchiolitis and asthma.

Summary

The Ph.D. thesis is based on studies conducted at 6 pediatric departments in following hospitals: Næstved, Gentofte, Kolding, Skejby, Hvidovre and Rigshospitalet.

Study I: Specific airway resistance (sRaw) measured by wholebody plethysmography in preschool children is increasingly used in research and clinical practise. However, there is no available method for calibration of the resistance measure, which raises concern of accuracy.

The primary aim was to determine the agreement of sRaw measurements in 6 centers. Seven healthy young children were brought to each of the 6 centers for sRaw measurements and measured by a center specific investigator as well as an investigator visiting each center.

We found sRaw measurements at two centers were significantly lower in all children compared to the other 4 centers and one center had significantly higher sRaw values than the other centers.

The secondary aim was to expand normative sRaw values for non-asthmatic children in 5 centers. 105 healthy preschool children were recruited locally for sRaw measurements. Normative data was generated and was without significant difference between centers and independent of height, weight, age and gender. Furthermore, there was no effect of the child's history of atopy, parental atopy or smoking. We subsequently pooled these normative data (105 children) with previous data from 121 healthy young children; mean sRaw (SD) 1.27 kPa*s (0.25).

Conclusion: Control using biological standards revealed errors in factory setting and highlights the need for developing methods for verification of resistance measures to assure accuracy. Normative data were subsequently generated. Importantly other centers using such normative data should first consider proper calibration before applying reference values.

Study II-III: Acute respiratory syncytial virus (RSV) bronchiolitis may occur in otherwise healthy infants. It is not known whether viral bronchiolitis is causatively

related to asthma or simply identifies infants at risk for subsequent wheezing from an atopic predisposition or pre-existing abnormal lung function.

First aim was to determine the differential effect within monozygotic twin (MZ) pairs discordant for severe RSV bronchiolitis in infancy on the subsequent development of asthma, pulmonary function and allergy. 37 MZ twin pairs discordant for RSV hospitalization in infancy were compared at the mean age of 7.6 years for lung function and bronchial responsiveness. We found no differences within MZ twin pairs with respect to pulmonary function, fractional exhaled nitric oxide, asthma prevalence, asthma medication use, or sensitization.

Secondary aim was to compare prospectively the baseline lung function and bronchial responsiveness in infants who later develop RSV bronchiolitis with infants who do not develop such severe infection. In a prospective birth cohort study of 411 infants of asthmatic mothers 22 infants developed RSV bronchiolitis. Infants with and without RSV bronchiolitis did not differ significantly in baseline lung function or bronchial responsiveness.

Conclusion: We found no effect of severe RSV bronchiolitis on the development of asthma and abnormal pulmonary function, nor did baseline lung function seem to predict later development of RSV bronchiolitis. This argue against a specific effect of the RSV bronchiolitis in the development of asthma and may suggest a genetic factor or an undisclosed environmental predisposing to severe RSV bronchiolitis, maybe co-infection rather than mechanical difference as the distinguishing feature for the development of RSV bronchiolitis.

Danish summary (Dansk resumé)

Denne ph.d. afhandling er baseret på studier udført på 6 børneafdelinger (Næstved Sygehus; Gentofte Hospital; Kolding Sygehus; Århus Universitetshospital, Skejby; Hvidovre Hospital og Rigshospitalet).

Studie I: Luftvejsmodstand (sRaw) målt på småbørn ved hjælp af helkropspletysmografi bliver i stigende grad brugt i forskningen samt i klinikken. Aktuelt findes dog ikke en kalibreringsmetode til selve sRaw målingen, hvilket medfører en usikkerhed på nøjagtigheden.

Det primære formål var at bestemme overensstemmelsen af sRaw målinger på 6 børneafdelinger. Syv raske småbørn besøgte hvert af de 6 center og fik lavet sRaw målinger af en centerspecifik observatør og en gennemgående observatør som var med på hvert center.

Vi fandt at to centre havde signifikant lavere sRaw målinger i forhold til de andre centre og et center havde signifikant højere målinger end de andre centre.

Det andet formål var at udvide sRaw normalmateriale ved målinger på 5 centre hos ikke-astmatiske børn. 105 raske småbørn blev rekrutteret lokalt til indsamling af sRaw målinger. Der var ingen signifikant forskel mellem centrene, og sRaw værdierne var uafhængig af højde, vægt, alder og køn. Allergi eller eksem hos børnene, forældres atopi eller rygning havde ingen betydning på sRaw værdierne. Derefter lagde vi normalværdierne (105 børn) sammen med tidligere data fra 121 raske småbørn med følgende resultat: gennemsnit sRaw (SD) 1,27 kPa*s (0,25).

Konklusion: Vi brugte børn til kvalitetskontrol af sRaw målinger, hvilket afslørede fejl i fabriksindstilling. Dette understreger behovet for udvikling af metoder til at verificere luftvejsmodstandsmålingers nøjagtighed. Andre centre bør overveje kvalitetskontrol før brug af disse referenceværdier.

Studie II-III: Ellers raske spædbørn kan få akut respiratorisk syncytial virus (RSV) bronchiolitis. Man ved ikke om viral bronchiolitis er årsagsrelateret til udvikling af astma eller blot er et udtryk for at spædbørn med risiko for hvæsen er på baggrund af deres atopisk disposition eller medfødt dårlig lungefunktion.

Det ene formål var at finde forskel på senere udvikling af astma, lungfunktion og allergi hos monozygotiske (MZ) tvillingepar diskordante for svær RSV bronchiolitis. 37 MZ tvillingepar diskordante for RSV hospitalisering i spædbarnsalderen blev sammenlignet mht. lungfunktion (gennemsnitsalder 7,6 år), nitrogenoxid niveau i udåndingsluft (FeNO), astma prævalens, brug af astma medicin samt allergitest.

Det andet formål var at sammenligne lungfunktion og bronkial hyperreaktivitet på spædbørn som senere får RSV bronchiolitis med spædbørn som ikke udvikler en sådan svær infektion. I et prospektiv fødselskohorte studie med 411 spædbørn født af mødre med astma, fik 22 spædbørn RSV bronchiolitis. Spædbørn som udviklede RSV bronchiolitis adskilte sig ikke signifikant med hensyn til lungfunktion eller bronkial hyperreaktivitet i forhold til kontrolgruppen.

Konklusion: RSV bronchiolitis havde ingen effekt på udviklingen af astma eller dårlig lungfunktion. Tidlig lungfunktionsmåling kunne heller ikke forudsige senere udvikling af RSV bronchiolitis. Det argumenterer imod RSV bronchiolitis som en specifik effekt på udviklingen af astma. Det kunne være en genetisk faktor eller en ukendt miljøfaktor som prædisponerer til svær RSV bronchiolitis, måske en co-infektion og ikke en mekanisk/anatomisk forskel som det væsentlige for udvikling af RSV bronchiolitis.

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Appendix

Papers I-III

Paper I

CHEST[®]

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Accuracy of Whole-Body Plethysmography Requires Biological Calibration

Pornniva Poorisrisak, Carsten Vrang, Jorn Molgaard Henriksen, Bent Klug, Birgitte Hanel and Hans Bisgaard

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Accuracy of Whole-Body Plethysmography Requires Biological Calibration*

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Background: Specific airway resistance (sRaw) measured by whole-body plethysmography in young children is increasingly used in research and clinical practice. The method is precise and feasible. However, there is no available method for calibration of the resistance measure, which raises concern of accuracy. Our aim was to determine the agreement of sRaw measurements in six centers and expand normative sRaw values for nonasthmatic children including these centers.

Method: Identical hardware with different software versions was used at the six centers. Measurements followed a standard operating procedure: (1) seven healthy young children were brought to each of the six centers for sRaw measurements; and (2) 105 healthy preschool children (52 boys; mean age, 5.1 years; interquartile range, 4.3 to 6.0) were recruited locally for sRaw measurements.

Results: (1) The sRaw of the seven-children study group was significantly lower at two centers compared with the other four centers, and one center had significantly higher sRaw than all the other centers ($p < 0.05$). Error in the factory settings of the software was subsequently discovered in one of the deviating centers. (2) Normative data (105 preschool children) were generated and were without significant difference between centers and independent of height, weight, age, and gender. We subsequently pooled these normative data (105 children) with our previous data from 121 healthy young children (overall mean sRaw, 1.27; SD, 0.25).

Conclusion: Control using biological standards revealed errors in the factory setting and highlights the need for developing methods for verification of resistance measures to assure accuracy. Normative data were subsequently generated. Importantly, other centers using such normative data should first consider proper calibration before applying reference values.

(CHEST 2009; 135:1476–1480)

Key words: lung function tests; multicenter study; quality control; specific airway resistance; whole-body plethysmography

Abbreviations: BTPS = body temperature and pressure, saturated; P = pressure; sRaw = specific airway resistance; \dot{V} = airflow

We have introduced and documented whole-body plethysmography for measurement of specific airway resistance (sRaw) in young children during the recent decade.^{1–5} sRaw assesses the airway resistance from measurements of the pressure changes driving the airflow (\dot{V}) during tidal breathing. These measurements require no active cooperation and are therefore feasible in children from 2 years of age. sRaw is now increasingly used in both research and clinical practice.

We previously documented the precision (interobserver variability)⁴ of sRaw measurements, but the accuracy of the method has not been reported. Using reference values generated by other centers is par-

ticularly vulnerable to the accuracy of the methods used.² Attempts have been made to develop a mechanical infant lung model analog for quality control

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of a whole-body infant plethysmograph,^{6,7} but it turned out to be difficult because of the small pressure and flow changes and is not readily available. Therefore, it is of concern that the accuracy of sRaw measurements cannot be verified. Flow and box leak are checked routinely, but the composite resistance measure is generated by algorithms buried in the software with settings often inaccessible to the end user. Thus, errors in

software or mechanics could go unnoticed with a potential impact on clinical evaluation.

The aim was to study center agreement by comparing sRaw measurements in young children among six centers in Denmark currently using the same equipment. First, we used seven healthy young children as a biological standard and brought them to each center for sRaw measurements. Second, we recruited healthy young children at five of the centers to compare and expand normative data.

MATERIALS AND METHODS

The study was approved by the local ethics committee as a quality assurance project and approved by the Danish Data Protection Agency. Parents gave written informed consent.

Design

Seven healthy young children were recruited for the measurements at six participating centers. The children were measured at each center by a center-specific observer as well as an observer visiting each center (Dr. Poorisrisak). The two observers were blinded to each other's measurements. Measurements in the individual children were finished within a period of 3 months. The order of center visits was randomized.

Healthy preschool children were recruited by random selection through the Central Person Registry from the local catchment area of the five centers. Children included were born at term, with no history of asthma-related symptoms, other chronic lung symptoms, or use of asthma treatment. If the child had a lower respiratory tract infection within the week before the appointment, the measurement was rescheduled. The children attended their local center where duplicate measurements were done by a local observer.

Principle of Measurement

Measurements were conducted in a constant volume whole-body plethysmograph (Master Screen Body; Erich Jaeger GmbH; Würzburg, Germany). A transducer measured pressure changes

in this sealed box, and a pneumotachograph simultaneously measured the flow swing at the mouth.

sRaw was calculated as the ratio between the pressure (P) generated by thoracic and abdominal movements during tidal breathing and the resulting \dot{V} .

$$sRaw = \Delta P / \Delta \dot{V}$$

where ΔP is the change in P and $\Delta \dot{V}$ is the change in \dot{V} , in comparison with resistance in ohms.

Flow and volume measurements were corrected to body temperature and pressure, saturated (BTPS) with water vapor conditions, as follows:

$$sRaw = (\Delta P / \Delta \dot{V}) \times (P_{amb} - PH_2O)$$

where P_{amb} is ambient pressure and PH_2O is pressure of water vapor at body temperature. The equipment was calibrated daily for ambient conditions (room temperature, atmospheric pressure, and humidity), box calibration (leak test result should be between 4 and 7 s and test for internal pressure, which should result in a correction factor of < 3%), and volume calibration (piston was pulled regularly 10 times with a 3-L piston and automatically accepted or rejected by the software). All the centers used identical hardware, but software versions differed between centers (JLAB, versions 4.51, 4.53 with different subversions, 4.65, and 4.67; available at <http://www.viasyshealthcare.com>).

Procedure of Measurement

The same procedure was followed by all observers. The children were seated alone in the box with the door closed. The child's breathing aimed for a frequency of 30 to 45 breaths/min.^{8,9}

The children used a face mask with a large cushion, which ensured a good seal and stabilized the cheeks and chin. A built-in flexible tube ensured that the mouth remained open to avoid nasal breathing.

"Loops" on the screen showed the relation between pressure (or volume) [x-axis] and flow (y-axis) [ie, the pressure driving the air flow in and out of the lungs]. sRaw was estimated from the inclination of these loops using the line between points of maximum pressure (or sRawTOT).

Technically acceptable loops were chosen as those that were "closed" in the middle. "Open" loops normally indicated insufficient BTPS correction. The loops assumed a straight line with a tendency to form an S shape and to be symmetrical around the inclination.

BTPS correction was done automatically by the software when the result was analyzed. sRaw from one run was calculated as the median value of at least five technically satisfactory loops with similar configuration and inclination.¹⁻³

Statistical Analysis

We used analysis of variance with unbalanced block design (SAS Proc GLM) to analyze differences due to center, child, center-specific observer, accompanying observer (Ponrtiva Poorisrisak), and age of the child. We included age in the analysis of center agreement because of the small number of children. Younger children could theoretically have a higher variation of sRaw values throughout the many visits. Our data were powered to detect a difference of 0.078 in expected log (sRaw) between two prespecified centers. If centers are not prespecified, our data were powered to detect a difference of 0.117 using Bonferroni correction. For the normative data, we used a mixed model with repeated measurements using log-transformed sRaw values ad-

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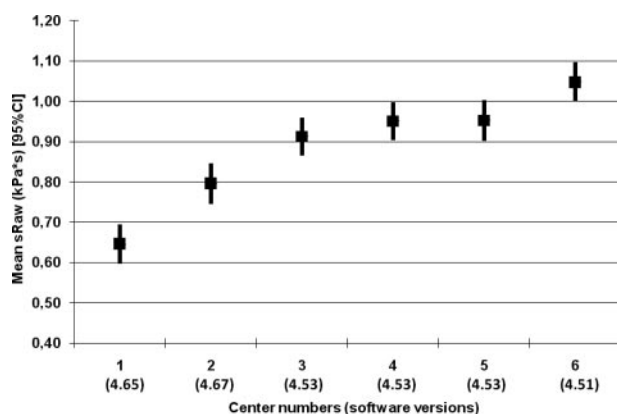


FIGURE 1. Center agreement study: least squares mean for sRaw for six centers with 95% confidence interval (CI) [software versions are indicated in parentheses].

justed for center number. A comparison between data from center 3 with previous reported normative values was done using a two-sample *t* test for means with log-transformed sRaw values. The calculations were done with a statistical software package (SAS, version 9.1; SAS Institute; Cary, NC).

RESULTS

All seven children completed measurements at each of the six centers. The children were between 4.9 and 6.6 years old (three boys). None of the children had a history of asthma or allergy. Three children had had atopic dermatitis, three had parental atopy, and none had smoking parents.

Lung function measurements differed significantly between centers (Fig 1). sRaw at centers 1 and 2 were significantly lower in all children compared with the other four centers, and center 6 had significantly higher sRaw values than the other centers. Mean sRaw for all six centers was 0.88 kPa/s

(SD, 0.23). The within-subject SD was 0.01, and the between-center SD for each child was 0.02. Observer and age of the child did not significantly affect the measurements ($p > 0.5$). For the individual results of the seven children for all six centers, see supplementary Fig 1 online.

A technician from the company (Cardinal Health) was sent to identify the problems in the deviating centers (centers 1, 2, and 6). This revealed an incorrect setting of the “ASC (automatischer schleifen-computer) Compensation” at center 1. “Time delay for compensation” was set to 20 ms and should have been 50, which resulted in 19–32% lower values. This was a factory setting not accessible to the operator. The technician found no reason for the deviating measurements at the other centers (numbers 2 and 6). It was not possible to reanalyze the data because the software saved the sRaw values after the primary calculation of sRaw.

Subsequently, 105 preschool children (52 boys) were measured in five of the centers (Table 1) with a mean age of 5.1 years (interquartile range, 4.3 to 6.0). One child was of Latin American descent and two of Arabic descent. Centers 1 through 5 provided data for the healthy cohort. The center numbers in the biological control study represent the same center numbers in the normative study.

Mean sRaw was 1.21 kPa/s (SD, 0.33) independent of height (Fig 2), weight, age, and gender (Table 1) [$p > 0.05$ for all estimates]; within-subject SD was 0.07. There was no significant effect of center. Furthermore, there was no effect of the child’s history of atopy, parental atopy, or smoking ($p > 0.05$ for all estimates).

Center 3 used the exact same equipment (hardware and software) as in our previous report on normative data.³ A comparison was made to ensure that time (10 years between the two studies) did not

Table 1—Normative Study: Clinical Characteristics of the Children From the Five Centers*

Characteristics	Center No.						Estimate (95% CI)	p Value
	1	2	3	4	5	Total		
Children, no.	21	28	29	16	11	105		
sRaw†	1.30 (0.32)	1.09 (0.24)	1.26 (0.31)	1.28 (0.46)	1.10 (0.23)	1.21 (0.33)		
Age, † yr	5.39 (1.16)	5.24 (1.04)	5.07 (1.18)	4.53 (1.19)	5.39 (1.03)	5.13 (1.15)	0.98 (0.93–1.03)	0.47
Male gender‡	11 (52)	15 (54)	16 (55)	6 (38)	4 (36)	52 (49.5)	1.05 (0.98–1.13)	0.16
Weight, † kg	22.0 (4.29)	20.0 (4.84)	20.4 (4.32)	18.9 (4.24)	19.4 (2.88)	20.3 (4.39)	1.01 (0.99–1.03)	0.26
Height, † cm	115.2 (8.95)	110.2 (9.90)	112.3 (9.79)	106.2 (8.27)	111.2 (8.28)	111.3 (9.61)	0.99 (0.99–1.01)	0.52
Rhinitis‡	4 (19)	0 (0)	4 (14)	1 (6.3)	1 (9.0)	10 (9.5)	0.91 (0.79–1.04)	0.16
Dermatitis‡	2 (9.5)	10 (36)	5 (17)	4 (25)	1 (9.0)	22 (20.9)	1.05 (0.96–1.15)	0.29
Parental‡ atopy	12 (57)	13 (46)	21 (75)	11 (69)	5 (45)	62 (59.6)	0.99 (0.92–1.07)	0.85
Smoking‡	4 (19)	8 (29)	4 (14)	3 (19)	4 (36)	23 (22)	1.02 (0.94–1.11)	0.64

*CI = confidence interval.

†Values are given as the mean (SD).

‡Values are given as No. (%).

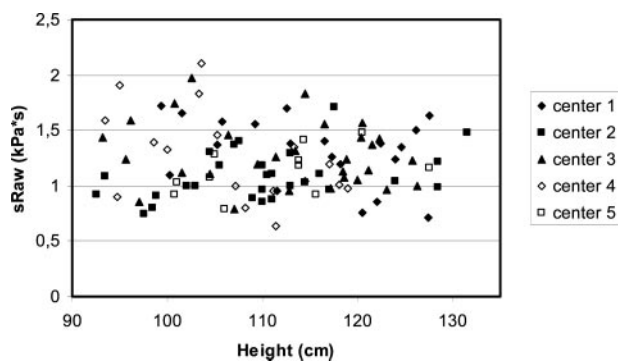


FIGURE 2. Normative study: healthy sRaw data against height for five centers.

have an effect on sRaw measurements before we pooled the data. We previously reported normative values from a population of 121 children, 2 to 5 years of age; the mean sRaw was 1.31 (SD, 0.20). The center 3 result was as follows: mean sRaw, 1.26; SD, 0.31. We compared the two data sets with a two-sample *t* test for means with log-transformed sRaw values (*p* value 0.20). There was no significant difference between these previous data and the current normative data. Therefore, we pooled the previous data (121 children) and the current normative data (105 children) [mean sRaw, 1.27; SD, 0.25] (Fig 3).

DISCUSSION

Between-Center Variation

sRaw offers a method for clinical monitoring and research during the critical period of growth and development early in life. The method is feasible from the age of 2 years, and the precision is high.¹⁻³ However, the present study showed that the accuracy of sRaw measurements in young children was flawed due to errors in the factory setting in one

center. It is the key message of our study that center effects were seen and could only be explained by differences in the software hidden from the end user. After correcting the factory settings at the deviating center, there were no longer differences between the centers, and normative values were generated in this multicenter setting. The problem was not discovered by the standard calibration of flow, box leak, and internal box pressure. Current calibration only assesses flow measured by the pneumotachograph, leak from the box, and pressure transducer. The available calibration does not assess the final resistance measure, which is generated by algorithms buried in the software with settings often inaccessible to the end user. Thus, errors in software or mechanics may go unnoticed with a potential impact on clinical evaluation and flawed accuracy as illustrated in our study. A mechanical infant lung model analog has previously been developed for quality control of infant whole-body plethysmographs,⁷ but a model testing for preschool children is not available to the end user. This study suggests the need for development of methods for control of the actual resistance measure for young children and not only the flow and box leakage. Without such a proof of accuracy, normative values generated at other centers may not be applicable. Until a mechanical standard becomes available, the biological standard (healthy subjects) is the only possible substitute.

We used a standardized protocol including standard calibration of flow, box leakage, and internal box pressure in six Danish centers at secondary and tertiary referral hospital departments. The six centers included in the study of accuracy were spread over the country, which prevented measurements on the same day. Therefore, the day-to-day variability reduced the sensitivity by which we could identify outliers among the centers. The children were not trained before entering the study. The visit order was randomized to ensure a possible difference between the first and second visit did not bias the center variation.

In the current study, the within-subject SD on the same day and center was 0.01, and the within-subject SD between centers was 0.02. In our previous study, the precision (repeatability) of sRaw measurements 9 days (mean) apart in young children with asthma (asymptomatic during the study period) was found to have an intraclass coefficient of 0.87 (within-subject SD 0.03) for baseline measurements between occasions.¹⁰ The higher within-subject SD could be explained by the asthma status of children in the previous study.

The current study was designed to find a possible center effect. We were able to account for any possible observer bias by having a center-specific

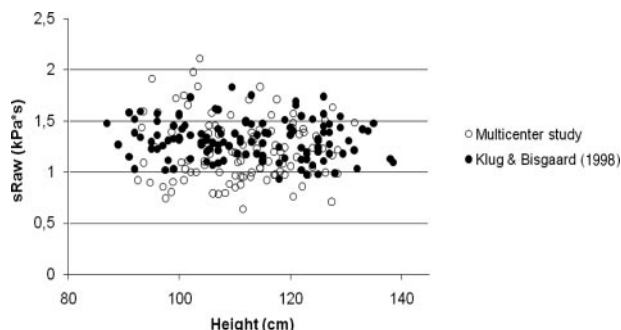


FIGURE 3. Normative data from the multicenter study (five centers) and the previous study by Klug and Bisgaard.³

observer as well as a common observer visiting every center. The order of the biological control for the local and traveling observer was not randomized, but we found no effect on the measurements of the investigator who traveled between the centers ($p > 0.5$).

Normative Data

In the second part of the study, a center effect could not be found though, probably because center 1 was corrected and center 6 did not participate. Atopy and smoking did not significantly differ between centers (Table 1). The high incidence of parental atopy could be a selection bias, but we did not find a statistical difference between atopic disposed and nondisposed children.

We previously reported normative values from a population of 121 children 2 to 5 years of age³; the mean sRaw was 1.31 (SD, 0.20). The previous study differs from the current in several of the following aspects: (1) measurements were made at one center; (2) children exposed to tobacco smoke and anyone with a history of eczema or doctor-diagnosed atopy in first-degree relatives were excluded from the study; and (3) the study included more 2- and 3-year-old children. Many of these measurements had an accompanying adult in the whole-body plethysmograph. The current data included only children who had performed a lung function measurement alone. There was no difference between sRaw measurements in the previous normative and the current normative data for the same center using the very same equipment. Therefore, we decided to pool the two sets of normative data, showing the normal sRaw in young children to be 1.27 kPa/s (SD, 0.25 kPa/s) independent of age, height, and gender (Fig 3).

In conclusion, using a biological control we revealed errors in the accuracy of sRaw measurements at some centers despite normal calibration of the mechanical components. This study highlights the need for the development of equipment allowing control of the actual resistance measurements and

not only some of its mechanical components. Until such equipment becomes available, the only option is to use healthy subjects to assure that the absolute values measured are similar to the normative values reported in this and a previous report of healthy young children.

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Accuracy of Whole-Body Plethysmography Requires Biological Calibration

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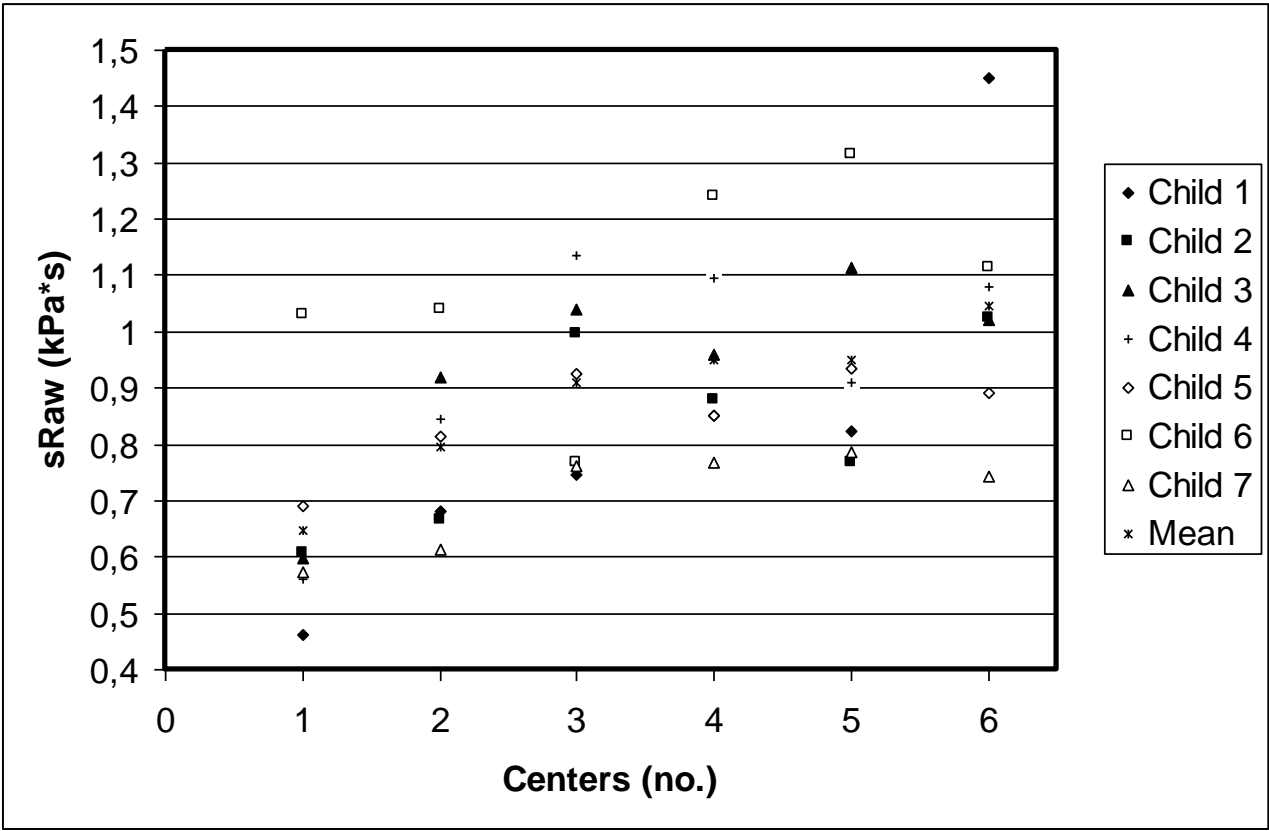
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Figure 1 Online:
Center agreement study: Each child's measurement at the different centres



Paper II

1 Title: Causal Direction between Respiratory Syncytial Virus Bronchiolitis and Asthma
2 Studied in Monozygotic Twins

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22

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25

26 Short running title: Twin study: Causal Direction between Respiratory Syncytial Virus
27 Bronchiolitis and Asthma
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ABSTRACT

Background: Respiratory syncytial virus bronchiolitis has been associated with later development of asthma, wheezing, abnormal pulmonary function and sensitization.

Objective: To determine the differential effect within monozygotic twin pairs discordant for severe respiratory syncytial virus bronchiolitis in infancy on the subsequent development of asthma, pulmonary function and allergy.

Methods: 37 monozygotic twin pairs discordant for respiratory syncytial virus hospitalization in infancy (mean age 10.6 months) were compared at the mean age of 7.6 years for lung function, bronchial responsiveness, fractional of exhaled nitric oxide, asthma diagnosis, use of asthma medication, and skin prick test to common inhalant allergens.

Results: There were no differences within monozygotic twin pairs discordant for respiratory syncytial virus hospitalization in infancy with respect to pulmonary function, fractional exhaled nitric oxide, asthma prevalence, asthma medication use, or sensitization ($p > 0.1$ for all comparisons).

Conclusion: We found no differential effect from severity of RSV infection on the development of asthma and allergy in MZ twin pairs discordant for RSV hospitalization in infancy. This argues against a specific effect of the severe RSV infection in the development of asthma and allergy, and may suggest an undisclosed environmental factor interacting in these genetically similar twins leading to different severity of their response to RSV infection. Because of the small sample size this study must be considered as a hypothesis generating study.

Word count for abstract: 220 words

INTRODUCTION

Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract disease and hospitalization in infants and young children (1-4), and therefore a major cause of healthcare utilization (5).

Severe RSV bronchiolitis has been associated with later development of abnormal pulmonary function, wheezing, asthma and allergic sensitization (6-11), but it is unclear whether severe RSV bronchiolitis causes wheezing, or genetic predisposition or other environmental risk factors increase the propensity to such exaggerated response to RSV.

The aim of this study was to compare the long-term outcome of asthma, allergy and pulmonary function in monozygotic (MZ) twin pairs discordant for hospitalization with verified RSV bronchiolitis in infancy as a surrogate marker of the RSV disease severity. Any differential long-term effect from RSV disease severity in these genetically identical twins would suggest a causal role of RSV.

METHODS

The study was approved by the ethics committee of Copenhagen (KA-20060022) and by the Danish Data Protection Agency (J.nr. 2005-41-5163 and J.nr. 2005-2311-0121). The children were enrolled after written consent was obtained from the parents or guardians.

Registries used for recruitment of the study population

The Danish Civil Registration System registers every Danish citizen by a unique personal identification number, providing a key for linking register information (12).

The Danish Twin Registry contains complete ascertainment of all live-born twins since 1968. Zygosity information in the registry is determined from questions on similarity and mistaken

identity in a postal questionnaire (13, 14) and confirmed by DNA analysis with 10 highly polymorphic marker in our study population.

The Danish National Patient Registry (DNPR) records all hospitalizations based on the 10th revision of the "International Statistical Classification of Diseases and Related Health Problems" (ICD10).

A research database was established between January 1996 and May 2003 recording RSV test from all hospitalizations in Denmark (15).

Study population

The target population was identified by linking the personal identification number to 1) the twin status, 2) living address and 3) hospitalizations due to the diagnoses RSV pneumonia (J12.1), RSV bronchitis (J20.5), RSV bronchiolitis (J21.0), and other diseases caused by RSV (B97.4) during the period 1/1-1994 to 31/12-2003. The registry assures that the control twin had never been hospitalized with RSV infection. Hospital records were retrieved to verify respiratory symptoms compatible with RSV bronchiolitis (severe cough, positive X-ray of thorax, use of β 2-agonist, crackles or wheeze by auscultation of lungs) (16, 17) and verified with an enzyme-linked immunosorbent assay or immunofluorescence assay test for RSV.

Clinical examination

Twins and their parents were summoned to the Danish Pediatric Asthma Center two centers in East- and West- Denmark for clinical examination including interviews on the child's asthma according to the GINA guidelines (18); information about medical history and objective assessments. The interviews and clinical examinations were done by one physician (PP).

Lung function test

Children of 7 years and above used a spirometer to measure Forced Expiratory Volume in the first second (FEV₁) (Vitalograph Spirotrac) according to the criteria of the American Thoracic Society (19).

Children less than 7 years were tested using the whole body plethysmography to measure specific airway resistance (sRaw) as previously detailed (20, 21). We used the MasterScreen Body Unit Software JLAB (E. JAEGER GmbH, Wuerzburg, Germany).

Bronchial responsiveness

Children of 7 years and above were tested for responsiveness to methacholine chloride with FEV₁ measurements before and 3 minutes after each dose. Methacholine was delivered in successively increasing doses according to a protocol previously validated for school children (22) by an automatic, inhalation synchronized, dosimetric jet-nebulizer (Spira Elektro 2; Respiratory Care Center, Hämeenlinna, Finland). PD20 value (μmol) was calculated by linear interpolation between the dose points bracketing the 20% fall in FEV₁ (23).

Children less than 7 years were tested with dry-air hyperventilation as previously described and validated in this age-group (24, 25).

Fractional exhaled nitric oxide

Fractional exhaled nitric oxide (F_ENO) was measured by the online single breath method according to European Respiratory Society/American Thoracic Society task force (26) using the NIOX equipment (Nitric Oxide Monitoring System; Aerocrine, Sweden).

Sensitization

Skin prick test was done with standard inhalation allergens: birch, mugwort, grass, horse, dog, cat, dust mite and mould (Soluprick, SQ ALK-Abelló A/S) (27).

Statistical analysis

SAS version 9.1 was used for statistical analyses. The continuous data were log-transformed and presented as geometric means in the table. Odds ratio were calculated for the dichotomous data with 95% confidence intervals. Data were analyzed with paired t-test for continuous outcome measures, Fisher's exact test for dichotomous outcomes and Wilcoxon's (paired) test for dry-air hyperventilation outcome. All hypotheses tests were 2-sided and used a significance level of 0.05.

RESULTS

During the period 1/1 1994 to 31/12 2003 12,349 twin pairs were born in Denmark. The proband-wise concordance rate of hospitalization for RSV-bronchiolitis was significantly higher in MZ (0.66) than in DZ twin pairs (0.53), $p=0.02$ (28). Fifty-seven MZ twin pairs (26 males) were discordant for RSV hospitalization (figure 1). Nine pairs were unavailable due to death or address protection. Five pairs were excluded based on their hospital records because the RSV infection was found incidentally during hospitalization for other reasons (i.e. elective surgery) not associated with lower respiratory symptoms. Forty-three pairs were invited of which 37 pairs accepted to participate (14 males; mean age 7.6 years; interquartile range: 5.9-9.2). Twenty-two pairs were examined in Copenhagen and 15 twin pairs in Aarhus.

Fifty-four% of parents answered retrospectively that the hospitalized RSV proband twin suffered more severe lung symptoms, 19% said it was the control twin and 27% said both twins had comparable symptoms.

Mean gestational age was 35.5 weeks. There was no significant difference between RSV hospitalized and non-hospitalized twins with respect to birth weight (mean 2369 g), neonatal treatment for lung complications (Continuous Positive Airway Pressure, surfactant or pneumothorax) and birth order (22/37 probands were first born).

Forty-nine % of the twin pairs had parental atopic predispositions. The average age when hospitalized for severe RSV bronchiolitis was 10.6 months (interquartile range: 5.1-13.3); median age 8.4 months. Mean duration of hospitalization was 4.3 days.

According to the interviews (GINA guidelines) the prevalence of asthma was 18% in our twin sample. The twins did not differ with respect to current asthma; use of inhaled corticosteroid or beta₂-agonist ever; atopic dermatitis ever; F_ENO; baseline lung function; bronchial responsiveness or sensitization (Table 1, table 1 online).

DISCUSSION

We found no difference within co-habiting MZ twin pairs discordant for hospitalization for RSV bronchiolitis in infancy on the asthma prevalence, baseline lung function, bronchial responsiveness; biomarker of airway inflammation (F_ENO) and sensitization 7 years after such severe RSV bronchiolitis. Though a number of criticisms may be raised including the small study population, it is noteworthy that no trends suggested a differential effect from severe RSV infection. Therefore a strong effect from this virus seems unlikely.

Limitations and Strengths of the study design

Monozygotic twins are useful for investigating the role of genetic and environmental factors on asthma because of their identical genetic background and similar childhood environmental exposures (29). However, comparison of MZ twin pairs discordant to hospitalization for RSV bronchiolitis is prone to a number of biases.

164 The underlying assumption was that RSV sequelae were proportional to the severity of the
165 RSV infection. We have no evidence to prove the twin serving as control was actually
166 infected with RSV, but rely on very high virulence of RSV among cohabitating twins
167 previously estimated at 93-96% (28) though this could not be verified.

168 We do not have positive evidence that this twin had milder symptoms, but rely on the fact that
169 one was hospitalized while the other was not. Danish children are generally only admitted if
170 they need support with feeding tube, suction of upper airways, mask inhalations or nasal
171 continuous positive airway pressure. Yet, this could be biased from registration practice.
172 Indeed, 19% of parents recalled the control twin being most severely affected. However, there
173 is a significant risk for recall bias and the MZ pairs could be mixed up since the
174 hospitalization was on average 7 years ago and parents were generally most unsure of the
175 details. We could have restricted the analysis to the 20 pairs who recalled the proband twin to
176 have been the most severely affected, but this was limited by the low numbers in the resulting
177 analysis. Instead we relied on the Danish registration practice and included all 37 pairs.
178 The distinction between possible "wheezers" and "bronchiolitis" may be inaccurate in this as
179 in other studies. The distinction between wheezers and bronchiolitis is normally related to
180 severity of wheeze and concurrent clinical signs of lower airway infection. Our samples
181 included the latter, i.e. first hospitalization for severe wheeze with concomitant signs of lower
182 airway infection.

183 It is known that male sex could be a risk factor for RSV hospitalization (30, 31). In the whole
184 twin cohort there were more males than females in all outcomes (28), but there was a random
185 overrepresentation of females in our study.

The discordant MZ twins in our study were born slightly earlier (mean gestational age 35.5 weeks and mean birth weight 2369 grams) than the MZ twins in the overall twin cohort (mean gestational age 36.1 weeks (SD 2.5) and birth weight 2498 grams (SD 549) (28). The differences were small and not significant; therefore it seems that the discordant twins were representative of MZ twins in general.

The highest incidence of hospitalization for RSV-bronchiolitis is often reported in the 0-1-year-olds (32-35). However, the reported mean age may be biased from excluding older children from such analyses. Fx Sigurs et al. reported mean age of RSV hospitalization to be 3.5 months excluding infants older than 12 months (10). Fjaerli et al. reported admission age median to be 6 months in children under two years old in their study (31). One study included children up to 2 years and showed the median age of hospitalization was 10 months of age similar to our findings (36).

Most importantly, the power of our study is limited by the low number of 37 paired cases, and the 95% confidential interval is correspondingly wide particularly for the clinical end-point though less so for the objective surrogate markers of asthma such as lung function and F_ENO. However, our study was based on the complete national database and not power calculations.

Mean age at the clinical examination was 7.6 years. A difference in asthma prevalence later in life cannot be excluded but seems low inasmuch as those studies showing higher asthma prevalence after severe RSV found this mainly in early childhood (8) and most cases of asthma debut before school age.

Interpretation of the study

We studied the direction of the causal relation between severe RSV bronchiolitis and asthma. RSV bronchiolitis has been associated with wheezing, asthma and abnormal pulmonary

function in childhood (6-10). One particular cohort study reported an asthma rate of 43% versus 8% and sensitization to common allergens of 45% versus 26% by age 13 years in children with infant hospitalization for RSV bronchiolitis compared with a matched control group (10). A review of pooled data from 10 controlled studies concluded that wheezing (but not recurrent wheezing) is more common after severe RSV bronchiolitis up till 5 years of follow-up (37). Two Finnish studies reported only marginally increase in the prevalence of asthma after RSV bronchiolitis in infancy (36, 38). On the other hand, predisposition to asthma and atopy was associated with increased risk of lower respiratory tract infection and hospitalization for RSV infection (33, 39, 40) and early wheezy symptoms were found to be a strong risk factor for subsequent hospitalisation for RSV (32). Therefore the direction of causality is unknown: Does RSV increase risk of asthma or is asthma constitution increasing the risk of severe response to RSV infection, or are both sharing a common undisclosed environmental exposure.

The model we used adjusted for genetic variation by analyzing long-term outcome in monozygotic twin pairs, in which one infant had been hospitalized for severe lung symptoms in response to verified RSV bronchiolitis, whereas the twin sibling had not been hospitalized at any time for RSV bronchiolitis. This allowed a comparison of severe versus milder response to RSV infection, genetic factors and environmental exposures during follow-up years being equal between the co-habiting MZ twin proband and control pairs.

Our recent publication based on 8,280 twin pairs showed that a model in which asthma “causes” RSV hospitalization fitted significantly better than a model in which RSV hospitalization “causes” asthma (41). On the other hand Wu and colleagues concluded in another recent paper that they have shown strong evidence for causal relationship of winter

viruses and early childhood asthma (42). In the light of these two particular papers Kuehni et al discussed the causal link between RSV infection and asthma in an editorial (43). The editorial concluded the new data were more in favor of the hypothesis that the association between RSV and asthma is due to shared predisposition rather than to a causal effect of RSV. However, the authors pointed out that the asthma phenotype was poorly defined in both studies.

A genetic contribution to asthma and severe RSV-bronchiolitis is suggested from the high prevalence of asthma in our twin sample compared with the current asthma prevalence in our region (44-47) as well as the high prevalence of parental atopic predisposition in the present study. Likewise, such genetic component was reflected by our previous finding of a higher concordance for hospitalization for RSV-bronchiolitis in MZ than DZ twin pairs (28).

Several publications suggest that RSV infection in early infancy stimulates a Th2 response (48-53). Previous publications have found that severe RSV bronchiolitis was associated with genetic polymorphism (haplotype IL13-IL4) (54-57), which may play an important role in the Th2 response (skew). The same locus have been associated with atopy and asthma in other genetic studies (58-60). These studies together suggest that primary RSV bronchiolitis and atopy share a genetic contribution at the IL13-IL4 locus.

We found no differential effect from severity of RSV infection on the asthma and allergy 7 years after infection. This may suggest that some undisclosed environmental factor instead could be responsible for the different severities RSV infection. The nature of such exposure is unknown and surprising in monozygotic twins. Studies suggest that phenotypic discordance between monozygotic twins is to some extent due to epigenetic factors. Acute environmental factors are directly associated with epigenetic-dependent disease phenotype (61-63).

Speculations may consider differing co-infections with an agent of less virulence including bacterial colonization (64) or other viral infections. There is increasing evidence that rhinoviruses (RV) are able to cause lower airway infections and to induce wheezing in young children, and it may be as common as RSV as a cause of bronchiolitis (65-68). However, the long-term effect of this and other viral agents on lung function and symptoms later in childhood is not yet fully investigated.

In conclusion, we found no differential effect from severity of RSV infection on the development of asthma and allergy in MZ twin pairs discordant for RSV hospitalization in infancy. This argues against a specific effect of the severe RSV infection in the development of asthma and allergy, and may suggest an undisclosed environmental factor interacting in these genetically similar twins leading to different severity of their response to RSV infection. Because of the small sample size this study must be considered as a hypothesis generating study.

Abbreviations:

RSV – Respiratory Syncytial Virus

MZ – Monozygotic

FEV₁ - Forced Expiratory Volume at first second

PD20 – Dose of methacholine producing a 20 percent fall in FEV₁

sRaw – Specific Airway Resistance

F_ENO - Fractional Exhaled Nitric Oxide

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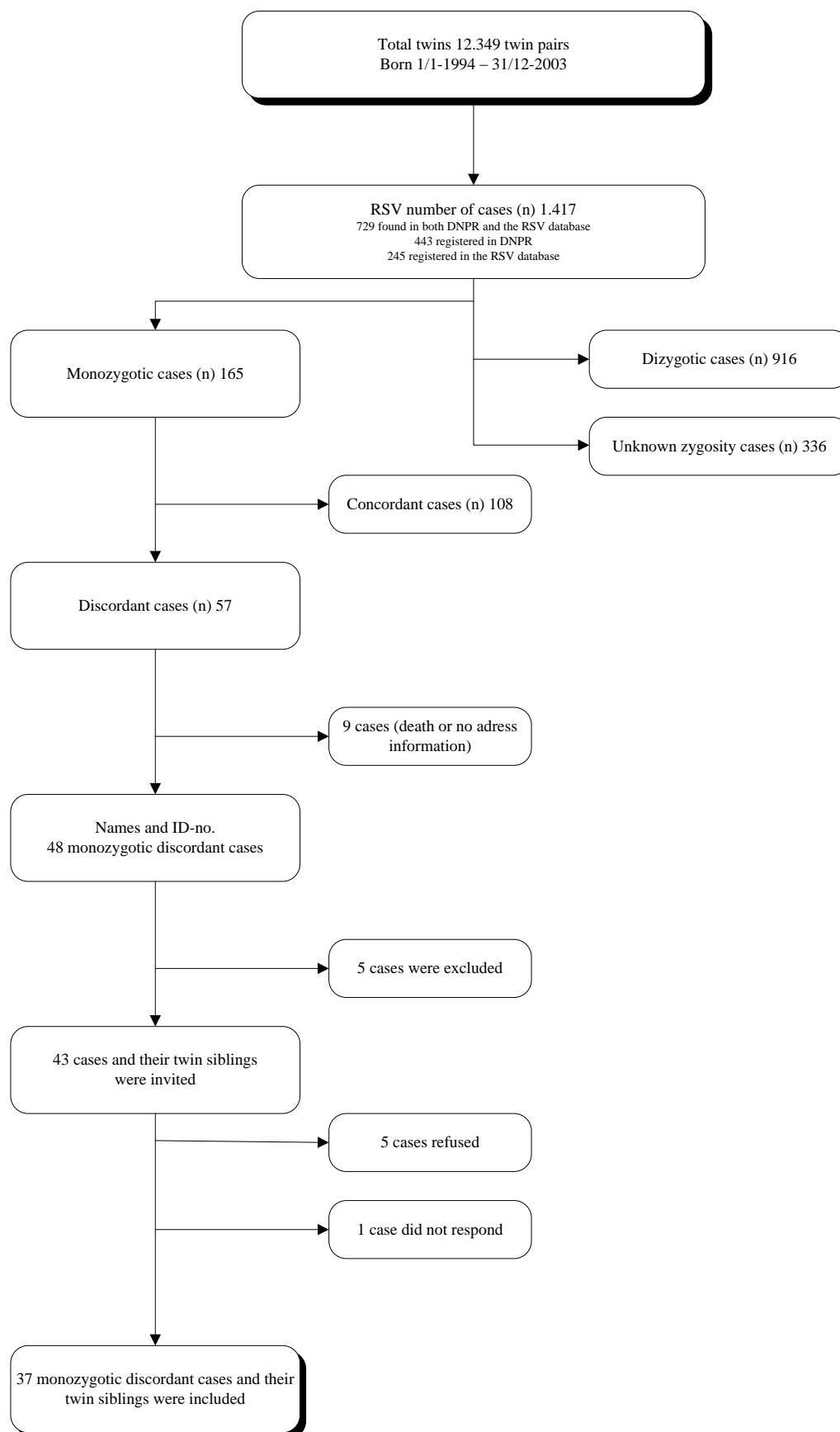
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488 Table 1: Comparison of clinical endpoints at follow-up between monozygotic twin pairs
489 discordant for hospitalization for severe respiratory syncytial virus bronchiolitis.

		Hospitalized vs. not-hospitalized		P-value
Asthma	OR (95% CI)	1.21 (0.36-4.00)		1.00 ^a
History of atopic dermatitis	OR (95% CI)	1.14 (0.41-3.14)		1.00 ^a
Use of inhalation steroid ever	OR (95% CI)	1.26 (0.49-3.22)		0.81 ^a
Use of beta2-agonist ever	OR (95% CI)	1.38 (0.55-3.46)		0.64 ^a
logF _E NO (n = 30)	Pbb mean (SD)	2.05 (0.66)	2.17 (0.69)	0.16 ^b
logBaseline FEV ₁ (n = 25)	L mean (SD)	0.42 (0.26)	0.46 (0.23)	0.12 ^b
logMethacholine PD20 (n =24)	μmol mean (SD)	0.74 (1.83)	0.41 (1.71)	0.27 ^b
logsRaw baseline (n = 12)	kPa*s mean (SD)	0.18 (0.26)	0.19 (0.30)	0.79 ^b
sRaw after dry air hyperventilation (n = 5)	% increase (median)	27%	18%	0.40 ^c
Positive skin prick test	OR (95% CI)	0.36 (0.07-2.08)		0.43 ^a

490 Abbreviations:

491 n = number of twin pairs which completed the test

492 OR – Odds Ratio

493 CI – Confidence Interval

494 SD – Standard Deviation

495 RSV – Respiratory Syncytial Virus

496 MZ – Monozygotic

497 FEV₁ - Forced Expiratory Volume at first second

498 PD20 – Dose of methacholine producing a 20 percent fall in FEV₁

499 sRaw – Specific Airway Resistance

500 F_ENO - Fractional Exhaled Nitric Oxide

501

502 ^a Fisher's exact test

503 ^b Paired t-test

504 ^c Wilcoxon's test (paired)

505 Tabel 1 online: Prevalence of atopic outcomes in the proband and control twin group.
506

	Hospitalized twin	Non-hospitalized twin
Asthma	19 %	16 %
History of atopic dermatitis	30 %	27 %
Use of inhalation steroid ever	41 %	35 %
Use of beta2-agonist ever	51 %	43 %
Positive skin prick test	6 %	14 %

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Paper III

Neonatal Lung Function and Bronchial Responsiveness Prior to Respiratory Syncytial Virus Bronchiolitis

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Abbreviations: CI = Confidence Interval; COPSAC = COPENhagen Studies on Asthma in Childhood; ELISA = Enzyme-Linked ImmunoSorbent Assay; FEV_{0.5} = Forced Expiratory Volume at 0.5 seconds; PD₁₅ = Provocative Dose of methacholine producing a 15 percent fall in PtcO₂; OR = odds ratio; PtcO₂ = Transcutaneous Oxygen Pressure; RSV = Respiratory Syncytial Virus; RVRTC = Raised Volume Rapid Thoraco-abdominal Compression; SD = Standard Deviation

Keywords: infants; lung function measurements; respiratory syncytial virus; bronchiolitis

None of the authors have conflicts of interest to disclose.

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Abstract

Background: Two-three percent of infants react with acute bronchiolitis in association with Respiratory Syncytial Virus (RSV) infection. It has been suggested that such exaggerated response to a common infection is due to pre-existing abnormal pulmonary function.

Aim: We aimed to compare prospectively the baseline lung function and bronchial responsiveness in newborns that later develop RSV bronchiolitis with those who do not develop such severe infection within the first 2 years of life.

Method: Spirometry was measured at one month of life by the infant spirometry and bronchial responsiveness to increasing doses of metacholine was determined in a prospective birth cohort study of 411 infants of asthmatic mothers.

Results: 22 infants in the cohort developed RSV bronchiolitis before age 2 (mean age 8 months). Children with and without RSV bronchiolitis did not differ significantly in baseline lung function ($\log FEV_{0.5}$), odds ratio (OR) estimate [CI 95%] 1.08 [0.09-13.6]; $p=0.5$ or bronchial responsiveness to metacholine ($\log PD_{15}TcO_2$) OR [CI 95%] 0.94 [0.74-1.20]; $p=0.64$.

Conclusion: Neither baseline lung function nor bronchial hyperresponsiveness in newborns were predictive of later development of RSV bronchiolitis (though the confidence limit of this comparison was wide). Our findings suggest an environmental exposure or genetic disposition rather than congenital mechanical difference as the distinguishing feature in children who develop RSV bronchiolitis.

Word count for abstract: 211

Introduction

Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract disease in infants (1-4). Two-three percent of infants react to RSV infection with bronchiolitis before age 1 (5-7). Infants with impaired pulmonary function seem more prone to recurrent wheezing episodes (8-16). Therefore it has been speculated that pre-existing abnormal airway resistance and/or bronchial hyper-responsiveness could account for the development of bronchiolitis in response to RSV infection (17-19).

“Copenhagen Prospective Study on Asthma in Childhood” (COPSAC) is a prospective clinical study of a birth-cohort, which included measurements of baseline lung function and bronchial responsiveness to methacholine at 1 month by infant spirometry (20). The close clinical follow-up allowed prospective identification of infants who developed RSV bronchiolitis.

This report aims to compare the preexisting baseline lung function and bronchial responsiveness in infants who later developed RSV bronchiolitis with those who did not develop bronchiolitis.

Materials and methods

411 infants born at term (203 boys) of mothers with physician-diagnosed asthma were enrolled at the age of 1 month in the prospective birth cohort study Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) (21-23). Both parents gave written informed consent. The study was approved by the Local Ethics Committee ((KF)01-227/97), and the Danish Data Protection Agency (2008-41-1754).

Lung function measurement in neonates

Lung function was assessed during sedation by infant spirometry at 1 month applying the raised volume rapid thoracic compression technique (RVRTC). The method was performed in agreement with ATS/ERS standards (24). An inflatable “squeeze”-jacket was wrapped around the infant's chest and abdomen. A subsequent inflation of the jacket provided the passive expiration and flow was measured using a pneumotachograph with an aircushion facemask (20, 25, 26).

Baseline flow was measured as the Forced Expiratory Volume at the first 0.5 second ($FEV_{0.5}$) (20, 25).

Bronchial responsiveness to methacholine was assessed by $FEV_{0.5}$ as well as continuous measurements of trans-cutaneous oxygen ($PTcO_2$) (TCM3; Radiometer; Copenhagen, Denmark). The methacholine was administered in quadrupling dose-steps by a dosimeter attached to a nebulizer (SPI-

RA 08 TSM 133; Respiratory Care Center; Hämeenlinna, Finland) as previously detailed (25). The result was assessed by PTcO₂ in accordance with previous sensitivity analyses (25, 27) and the provocative dose causing a 15% drop in PTcO₂ was estimated from the dose response curves fitted with a logistic function.

Bronchiolitis

Bronchiolitis was defined as an acute respiratory illness before the age of 2 years based on symptoms of coryza progressing over a few days to cough, tachypnea, chest retraction and wide spread crackles, wheezes or both (28) diagnosed by the COPSAC clinical research unit doctor or admission acutely to the local hospital for such symptoms.

RSV bronchiolitis

RSV bronchiolitis was defined as bronchiolitis with a positive ELISA test for RSV infection within 10 days of the acute episode.

Acute severe wheezy exacerbations

Acute severe wheezy exacerbations was defined as an acute obstructive respiratory illness diagnosed by the COPSAC clinical research unit doctor and judged to be a severity requiring treatment with oral or high-dose inhaled corticosteroids (>800 micg budesonide/day) or hospitalizations caused by airway symptoms where an anti-asthmatic drug was prescribed (pseudocroup episodes were excluded).

Control group

Children who developed neither bronchiolitis nor acute wheezy exacerbations before age 2 were considered as controls.

Statistics

Lung function measurement data (FEV_{0.5} and PD15) were calibrated with lifespan and birth length in accordance with previous lung function analyses of the newborn (29) calculated by a generalized linear model. Lifespan at examination date was calculated as the sum of estimated gestational age in weeks and weeks since birth. The lung function measurements were log transformed and we used a multivariable logistic regression model to calculate adjusted odds ratios and 95% confidence intervals using SAS version 9.1. Outcome variable was RSV bronchiolitis compared to control group. Confounder adjustment included mothers smoking during 3rd trimester (dichotomized) and gender.

Results

Twenty-two infants developed RSV bronchiolitis before age 2 (16 boys); mean age 8 months (interquartile range 3-12); 17 of the 22 children were under 1 year old when diagnosed with RSV bronchiolitis (mean age 5.7 months). Nineteen of the 22 children were hospitalized with RSV bronchiolitis (mean duration of 4.7 days). Lung function had been completed more than 1 week prior to the bronchiolitis. Baseline characteristics of the lung function measurements are shown in table 1.

The control group consisted of 366 children, excluding the 22 with RSV bronchiolitis and 23 with acute severe wheezy exacerbations from the main cohort of 411 (figure 1).

Baseline FEV_{0.5} did not differ between infants with RSV bronchiolitis compared with the control group; OR 1.08 [0.09-13.6], p=0.95 (z-score -0.06).

Bronchial responsiveness did not differ between infants with RSV bronchiolitis compared with the control group; OR 0.94 [0.74-1.20], p-value 0.64.

Discussion

Principal findings

We found no association between baseline lung function and bronchial responsiveness in neonates and later development of bronchiolitis with RSV infection compared to the control group.

Limitations and Strengths

We used strict definition of bronchiolitis in accordance with a recent review (28). Our definition of bronchiolitis is consistent with the Anglo-Saxon definition including widespread fine crepitations and sometimes expiratory rhonchi on auscultation in the definition (28, 30-32). In the American literature, the definition sometimes includes all first-time wheezing infants with associated respiratory-tract infection (33-35). We recognize the lack of evidence for the effect of steroids for bronchiolitis (36-40) but took the pragmatic approach to include their use in our definition of the more acute cases realizing this is a well established clinical practice and indicating a certain clinical severity. Also, we recognize that the diagnosis of acute bronchiolitis is likely to comprise infants with first acute attack of asthma, but this would have biased the risk estimates against the null hypothesis assuming such infants with underlying asthma would have had reduced lung function and bronchial hyperresponsiveness.

The confidence interval of the comparison of baseline lung function includes a wide interval from 0.09 to 13.6 (risk of type 2 error), suggesting the need for an unlikely study size to ever decide

prospectively if baseline lung function is lower in infant developing RSV-bronchiolitis. On the other hand comparing bronchial responsiveness showed a narrow confidence interval (-26% to +20%); i.e. suggesting it seems unlikely that bronchial hyperresponsiveness was the distinguishing factor in infants later developing RSV-bronchiolitis.

The strengths of this study were the unique prospective nature of the birth cohort study with lung function assessments before the development of bronchiolitis together with the close clinical follow-up to the research clinic and daily symptom recordings assuring ascertainment of all severe episodes.

Lung function measurements were completed in a large group of asymptomatic infants (404) within a narrow age-range around 1 month after birth. This is the largest study of lung function in neonates under standardized conditions. Baseline lung function was conducted by infant spirometry adapted as the state-of-the-art Raised Volume Rapid Thoracic Compression technique which provides flow-volume measures. The previous analyses found PtcO₂ was the most sensitive parameter for detection changes in lung function (dose-response curve metacholine challenge), followed by FEV_{0.5}, both superior to other indexes of forced spirometry as well as all tidal breathing indexes (25, 27). We analyzed bronchial responsiveness as a quantitative trait in the RSV and control group rather than dichotomizing the infants into +/- hyperresponsiveness.

All cases of non-RSV bronchiolitis and acute severe wheeze episodes were excluded from the control group, which would tend to favor a difference between our cases and controls.

Nineteen of the 22 infants (86%) with RSV bronchiolitis were hospitalized. Danish children are generally only admitted if they need support with feeding tube, suction of upper airways, mask inhalations or nasal continuous positive airway pressure, which ensures a certain severity in their RSV infection in our study.

Confounder adjustment included mothers smoking during 3rd trimester and gender both well known risk factors for bronchiolitis (41-47). We found an overweight of boys (73%) in the RSV group in agreement with previous reports (41-43, 48, 49). Lung function measurement data were calibrated for birth length and lifespan at examination date because these parameters have shown to affect early lung function in the COPSAC cohort (29).

Meaning of the study

Acute RSV bronchiolitis may occur in otherwise healthy infants. Infants with bronchiolitis are at significant risk for subsequent recurrent wheezing and childhood asthma (11, 50-53).

It is not known whether viral bronchiolitis is causatively related to asthma or simply identifies infants at risk for subsequent wheezing from an atopic predisposition or pre-existing abnormal lung function (54, 55). Infants with impaired pulmonary function at one month of age was reported to be prone to recurrent wheezy episodes and asthma (8-11, 13, 14, 56, 57). Therefore it has been assumed that acute bronchiolitis or wheeze develop due to pre-morbid abnormal pulmonary lung function consistent with smaller airway size (11, 12, 15, 18, 19). But these are indirect evidence as clinical wheezing illness was used as end-point

This is the first study to look specifically at neonatal lung function before RSV bronchiolitis. Our study showed no association between early lung function ($FEV_{0.5}$ and bronchial hyperresponsiveness) and subsequent RSV bronchiolitis. This could mean that small airways were not the distinguishing feature of later development of RSV bronchiolitis. We recognize that the confidence interval of the comparison of baseline lung function was wide with the risk of type 2 error.

Broughton et al. (2006) studied prospectively premature infants and found those who had symptomatic RSV lower respiratory tract infection had worse lung function (higher resistance) prior to neonatal unit discharge compared to controls but no difference in the lung volumes (functional residual capacity) (17). It is difficult to compare this study with our result since we used a different lung function technique. Future studies may consider using other lung function tests such as for example whole-body plethysmography measuring airway resistance.

Studies using forced expiratory maneuvers have shown to discriminate normal infants and wheezy infants or infants with cystic fibrosis (58-60). These studies were done on older children (age > 3 months) and therefore not comparable with our data.

A cross-sectional study of 37 normal infants found that a family history of asthma had a negative effect on $FEV_{0.5}$ (61). Another study on 63 normal healthy infants found that airway responsiveness in infancy was increased in families with history of asthma or parental smoking (56). Since our cohort only includes infants with asthmatic mothers the absolute levels of lung function and bronchial responsiveness may not be representative of the general population. However, this does not affect the purpose of comparing lung function of infants who later develop RSV bronchiolitis and infants

who do not develop RSV bronchiolitis. The risk factors for neonatal lung function was studied in the COPSAC cohort in a recent publication (29) and showed that high body mass index in newborns and mothers smoking were associated with reduced lung function, also that parental atopic disease (mother's or father's eczema, urticaria or allergic rhinitis or father's asthma) did not affect the neonatal lung function and bronchial responsiveness.

In our study group of infants less than 2 years of age the mean age of the infants diagnosed with RSV bronchiolitis was 8 months (30), which is higher than reported by other study groups. Other studies have limited the group of interest to children less than 12 months of age and accordingly reported even lower age of RSV infection (35, 62-65).

The incidence of bronchiolitis (5%) was higher reported in most studies (1-3%) (5-7, 48, 66, 67). This suggests a genetic component in RSV bronchiolitis as all mothers had asthma. Predisposition to asthma and atopy has been associated with increased risk of lower respiratory tract infection and hospitalization for RSV infection (64, 68, 69). Young et al (1995) found 7% of a cohort (253 infants) with the diagnosis of bronchiolitis before 2 years of age (only 2 infants were hospitalized and confirmed for RSV infection); 71% had a family history of atopy (18). A twin cohort study showed that the severity of RSV infection was determined partly by genetic factors (16%); family environment accounted for 73% and nonshared environment for 11% of the individual susceptibility to develop severe respiratory syncytial virus infection (70, 71). A Danish case-control study has also supported that asthmatic disposition and wheezing were strong determinants of subsequent respiratory syncytial virus hospitalization in children <18 months (63); the relative risk of respiratory syncytial virus hospitalization in the offspring was 1.72 for maternal asthma, and 1.23 for paternal asthma.

The RSV group was not characterized with increased bronchial responsiveness in infancy. In order to investigate the association between lung function and bronchiolitis further we could have included all children with bronchiolitis and not only RSV bronchiolitis. There is an increasing recognition that other viruses (e.g. rhinovirus) are as common as RSV causing bronchiolitis in young children (72-75).

A recent prospective, population-based cohort study examined the associations between hospitalization for RSV infection and invasive pneumococcal disease in Danish children < 2 years. The study found that invasive pneumococcal disease did not increase the risk of RSV hospitalization but recent hospitalization for RSV increased the risk of invasive pneumococcal disease (76). On the other

hand a previous study from the COPSAC cohort showed that neonates colonized in the hypopharyngeal region with *S. pneumonia*, *H. influenza*, or *M. catarrhalis*, or with a combination, were at increased risk for recurrent wheeze and asthma early in life (23). Future studies may consider co-infections with other viral or bacterial infections responsible for different response to RSV infection and the development of asthma (77).

Conclusions

Bronchial hyperresponsiveness in newborns were not predictive of later development of RSV bronchiolitis nor did baseline lung function seem to predict though the confidence limit of comparison was wide. This study suggests that the distinguishing feature is not a mechanical but maybe an environmental exposure or genetic disposition.

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Figure 1: Flow chart

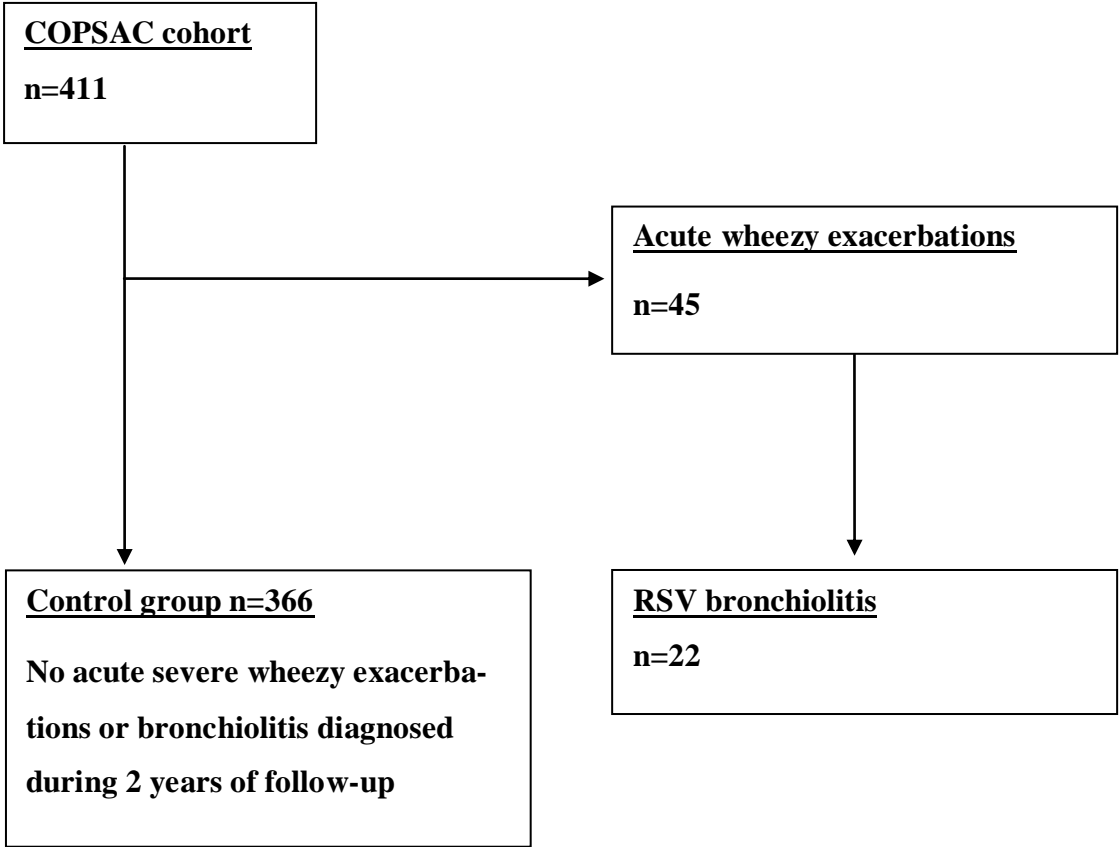


Table 1: Baseline characteristics

	Total COPSAC		Control group	RSV bronchiolitis
Baseline	404	Number	360	21
lung function (FEV _{0.5} /ml)		Mean (SD)	66.3 (12.9)	65.5 (13.0)
		Median	65.8	63.6
PD ₁₅	363	Number	322	20
(PTcO ₂ /mmol)		Mean (SD)	1.81 (12.2)	0.59 (0.88)
		Median	0.33	0.16