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ADVANCES IN PEDIATRIC RESEARCH

The Argentina Premature Asthma and Respiratory Team (APART): objectives, design, and recruitment results of a prospective cohort study of viruses and wheezing in very low birth weight infants

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Abstract

Background: Asthma and wheezing account for a substantial disease burden around the world. Very low birth weight (VLBW, <1500 grams) infants are at an increased risk for the development of severe acute respiratory illness (ARI) and recurrent wheeze/asthma. The role of respiratory viruses in asthma predisposition in premature infants is not well understood. Preliminary evidence suggests that infection with human rhinovirus (RV) early in life may contribute to greater burden of asthma later in life.

Methods: A prospective cohort study of premature VLBW infants from Buenos Aires, Argentina, was enrolled year-round during a three-year period in the neonatal intensive care unit and followed during every ARI and with monthly well visits during the first year of life. Longitudinal follow-up up until age five years is ongoing.

Results: This report describes the objectives, design, and recruitment results of this prospective cohort. Two hundred and five patients were enrolled from August 2011 through January 2014, and follow-up is ongoing. A total of 319 ARI episodes were observed from August 2011 to July 2014, and 910 well visits occurred during this time period.

Conclusions: The Argentina Premature Asthma and Respiratory Team (APART) is a unique cohort consisting of over 200 patients and over 1200 specimens who have been and will continue to be followed intensively from NICU discharge to capture baseline risk factors and every ARI, with interceding well visits during the first year of life, as well as longitudinal follow-up to age 5 years for asthma and atopy outcomes.

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Introduction

Premature infants, especially very low birth weight (VLBW) premature infants, are at high risk for severe acute respiratory illness episodes (ARI) and asthma [1-3]. Eleven percent of all live births globally were premature in 2010 [4]. Underdeveloped lungs and conditions such as bronchopulmonary dysplasia (BPD) lead to breathing problems later in life in these infants [5, 6]. Research over the last several years has suggested that respiratory viruses like human rhinovirus (RV) and respiratory syncytial virus (RSV) are associated with development of asthma and wheezing in term infants [7-14], especially if wheezing is present during the initial respiratory illness [15]. However, this relationship has not been well studied in premature infants.

The burden and severity of ARI may be elevated in premature infants compared to babies born at a later gestational age (GA). Infection with respiratory viruses is most frequent in younger premature infants [16]. Respiratory viruses in very premature infants (<33 weeks GA) lead to longer hospital stays, more mechanical ventilation and an increased likelihood of developing bronchopulmonary dysplasia compared to those without a detectable virus [17].

Premature infants also have increased susceptibility to acute wheezing during ARI. Fourteen percent of premature infants develop bronchiolitis and have longer hospital stays and more severe disease associated with that bronchiolitis compared to term infants [16, 18-21] In premature infants less than 29 weeks GA, almost half of the infants will be admitted to the hospital for respiratory distress at some point [22]. Moreover, the rate of severe ARI is high in infants who live in developing countries, with up to 25% having severe ARI during the first year of life [23, 24]. In a previous study by our group, 55% of VLBW infants in Argentina tested positive for RV during a respiratory illness, and 40% of the infants with bronchiolitis were RV positive [2].

Asthma affects over 20% of premature infants [25-27]. Recent evidence suggests that RV may play a role in the development of recurrent wheeze and asthma [8, 21]; however, this link has not been examined in VLBW infants. The association of RV with bronchiolitis and asthma exacerbations suggests that RV may be associated with the inception of asthma. This study was designed to prospectively examine, test, and follow ARI episodes in VLBW premature infants and subsequent development of asthma and recurrent wheeze.

The primary goals of the study are to: 1) characterize the impact of RV in the epidemiology of ARI occurring among VLBW premature infants during the first year of life, 2) define the role of RV in recurrent wheezing and asthma development in the same population of children, and 3) elucidate pathogenic mechanisms associated with the development of ARI and asthma in this population. This study utilizes a unique prospective enrollment and follow-up method that allows us to conduct intensive investigations of both well and sick visits, to perform viral and cytokine testing during ARIs, and to determine how these factors contribute to acute and recurent wheezing and asthma as these children age. The study design will allow investigators to understand RV and other respiratory viral infections, their impact on burden of acute and chronic disease in these vulnerable infants, and modifiable risk factors within the host and environment. This report describes the design and methods used to recruit and sample this study cohort.

Methods

Study objectives and design

The Argentina Premature Asthma and Respiratory Team (APART) study is a prospective cohort of VLBW premature infants in Buenos Aires, Argentina. This multicenter cohort study enrolled premature infants from the Pediatric Hospital de J.P.Garrahan (Hospital Garrahan) and the Hospital Materno Infantil Ramón Sarda (Hospital Sarda) High Risk Clinics in Buenos Aires, Argentina.

Infants were enrolled from August 2011 through April 2014, and follow-up is ongoing. The components and timeline of the subject visits are outlined in Table 1. The project has been conducted in association with the Department of Pediatrics, Division of Pulmonary, Allergy, and Immunology of Vanderbilt University, USA and Fundación INFANT, Argentina.

Table 1. Timeline of sample collection

Item	Enrolment	≤ 1 year well visits	≤ 1 year ARI visits	≥ 1 Year	Yearly age 2-5	≥ 5 years
Approximate year	2011-2014	2011-2015	2011-2015		2013-2016	2016-2019
Consent	X					
Enrolment survey	X					
Monthly well exam		X				
Bi-monthly phone call		X				
ISSAC survey						X
Bonner survey [28]					X	
Acute ARI exam			X			
Nasal aspirate	X		X			
Nasal swab		X				
Urine	X					
Blood				X		
Saliva				X		
Annual phone call					X	
Spirometry						X

ARI – Acute Respiratory Illness; ISAAC – International Study of Asthma and Allergies in Children

The Internal Review boards from Vanderbilt University, Fundación INFANT, Hospital Garrahan, and Hospital Sarda have approved this study. Investigators sought and obtained ethics approval from all participating institutions, as well as signed informed consent from all participants' parents.

Study population and patient recruitment

Two hundred and five infants were enrolled year-round in the Neonatal Intensive Care Unit (NICU) from August 1, 2011 through January 31, 2014. Families of premature infants who were followed at Hospital Garrahan or Hospital Sarda clinics and met inclusion criteria were invited to participate in the study. Infants were enrolled in follow-up clinics at Hospitals Garrahan or Sarda directly after discharge from the NICU. Inclusion criteria were: birth <37 weeks GA and weight <1500 grams, patients within 6 months of corrected gestational age, and living within 70-kilometer radius of the hospitals. Exclusion criteria included: children with life expectancy <6 months, immunodeficiency or bleeding disorders, or children with orofacial malformations that would make it difficult to collect an adequate sample. Parents provided written informed consent for their child's participation in the study, and were compensated with travel money and meals for the child and siblings. VLBW infants were enrolled either before discharge in the NICU or shortly thereafter in the follow-up clinics. Recruitment was performed Monday through Friday by the two key study neonatologists (TP and LB).

Enrollment visit and examination

During enrollment, the father, mother or guardian of the VLBW infant provided informed consent for the study. Subjects and their families could opt out or refuse to give samples at any time. The neonatologists also administered a survey during this initial visit that documented information on demographics; detailed family history of asthma, allergic rhinitis, urticaria and atopic diseases including family chronic or intermittent use of salbutamol or corticosteroids; home exposures including pets and detailed smoking exposure; number of siblings and day-care attendance; maternal diet and medications used during pregnancy; prenatal and neonatal medical history; previous medical history if the subject was enrolled as an outpatient; requirement for

supplemental oxygen, medication administration, feeding methods, and comorbid conditions. During the enrollment visit subjects underwent a clinical exam including pulmonary assessment, room-air pulse oximetry, and documentation of any acute medical symptoms. A nasal aspirate and urine sample were collected at enrollment. Families received verbal instructions for recognizing ARI and respiratory signs and symptoms in special workshops at the time of infant discharge from the NICU or study enrollment. The telephone number of one of the neonatologists was given to each participating family, and parents/guardians were asked to call study personnel at any time if their child developed upper or lower respiratory symptoms, changes in baseline respiratory status, or were hospitalized. The study neonatologists conducted a visit during each ARI episode for clinical assessment and nasal aspirate collection.

Cohort follow-up

Infant specimen collection included a nasal aspirate, nasal swabs, urine, blood, and saliva during the first year of life. Nasal swabs were collected at monthly well visits; nasal aspirates were collected at enrollment and all ARI episodes. Urine was collected at enrollment. Table 2 describes the methods of specimen collection.

Healthy visit follow-up during the first year

Subjects were extensively followed from enrollment to the end of the first year of corrected gestational age. Families were contacted by telephone every two weeks during the first year of life by the study neonatologist with a standard survey to inquire about changes in respiratory status. Subjects were examined monthly in well visit clinics during the first year of life at either Hospital Garrahan or Hospital Sarda. Surveys were completed during these visits to document the preceding month of respiratory condition, requirement of supplemental oxygen, medication administration, infant feeding method, vaccine schedule, and palivizumab schedule. Clinical assessments were conducted to determine pulmonary status and room-air pulse oximetry. Nasal swabs were also collected during monthly visits during the first year of life to determine asymptomatic infection with respiratory viruses.

Acute respiratory illness follow-up during the first year

When a child developed an ARI, a neonatologist saw them for a sick visit. Subjects were examined and a pulmonary assessment was conducted. Surveys were administered to collect information about duration of symptoms before the visit, clinical assessment, baseline and actual pulmonary scores, respiratory severity score, hospitalization status, and treatment received. Nasal aspirates were collected at each sick visit to establish the presence of respiratory viruses, cytokines, and chemokines. In a very few cases the nasal aspirate could not be collected because of clinical conditions or by the parent's request. In such cases, a nasal swab was collected and cytokines could not be tested in these specimens.

In the event a child was hospitalized, the study neonatologist visited once to conduct a clinical assessment, complete the survey, and collect a nasal aspirate during that hospitalization. The study pediatrician then maintained phone contact until hospital discharge took place.

Annual follow-up

After the subjects reach one year of corrected GA, it is expected that they will be followed annually until 5 years of age. In a subset of participating infants, blood and saliva samples will be collected after 12 months of age. It is expected that subjects will be followed every year with annual visits, phone calls, and surveys. The development of recurrent wheezing (3 or more episodes of wheezing) or atopy (allergic rhinitis, asthma, or eczema) will be ascertained as these subjects age. Spirometry with bronchodilation will be performed during a clinic visit when children reach 5 years of age. Participating parents or guardians were also asked to call the study neonatologists if a healthcare provider diagnosed their child with asthma at any time during the 5-year period.

Table 2. Description of sample collections

Specimen	Collection technique
Nasal aspirate	The sample was obtained by a K30 or 33 probe with an occlusion extension connected with an aspiration system. Five cc of saline solution were used and 1 cc was put into each of 5 tubes. Tubes were immediately placed in the freezer at – 80°C.
Nasal swab	The child laid in the supine position, and the swab was introduced into one nostril, remaining motionless for 5 seconds and then withdrawing gently while rotating. Samples of both nostrils were obtained with the same swab. Swabs were then placed in a tube containing universal transport medium (16x100mm size tube with 3ml UTM™ medium). Tubes were immediately frozen at -80°C.
Urine	For males, the sample was collected by wrapping a collection band around the penis. For females, a piece of sterile cotton placed on her genital area and then urine was aspirated with a syringe and then transferred to a sterile tube. Tubes were immediately frozen at -80°C.
Blood	Five mL of blood was drawn and centrifuged at 3000 rpms for 10 minutes. Serum was alloquated in 1ml increments into sterile tubes and frozen at -80°C.
Saliva	Saliva was collected in an Oragene Discover ORG-500 and OGR-575 kit. If the child was able, they deposited the saliva through the funnel. For infants, sterile sponges were placed near the salivary glands under the tongue, being careful not to rub too much along the gums or cheek and placed into the Oragene collection tube. Children were instructed to not eat or drink for 30 minutes prior to saliva collection. Samples were stored at room temperature.
Spirometry	This will be performed in children at least 5 years of age by a trained technician. To assess bronchodilator response, 200 ug of albuterol will be administered using a metered dose inhaler and a pediatric chamber. Spirometry will be performed before and 20 minutes after administration of albuterol and a positive response will be considered with an increase of >12% of predicted forced expiratory volume (FEV1). Forced vital capacity (FVC, FEV1 and $FEF_{22-75\%}$) and flow-volume curves will be measured according to the American Thoracic Society guidelines.

Asthma and atopy surveys

Modified versions of both the International Study of Asthma and Allergies in Children (ISAAC) and Bonner [28] (Supplemental document) surveys were used to assess wheezing, asthma, atopy and allergy symptoms. Surveys were translated into Spanish and read to the families so that the children and their parents would be able to understand the questions.

Laboratory analysis of specimens

Once a year, specimens were shipped to Vanderbilt University in the USA for testing. Total nucleic acids were extracted from nasal aspirates and swabs using the Roche Magna Pure system (#3038505001). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was conducted for eight viruses: RV, RSV, metapneumovirus (MPV), parainfluenza virus (PIV) 1-3, and influenza A and B. Conventional RT-PCR was then conducted on RV-positive specimens to amplify the VP4/VP2 region as described [29]. Samples with positive bands after conventional RT-PCR were then either directly sequenced after ExoSap-IT purification (Affymetrix #78205) or cloned into a plasmid using the pGEM-T Easy Vector (Promega #A1360) kit and sequenced.

Cytokine testing was conducted using real-time RT-PCR for mRNA (Applied Biosystems #4331182) and enzyme-linked immunosorbent assay (ELISA) (eBioscience 'Ready Set Go!' kits) to examine protein levels. Manufacturer instructions were followed, using 100 µl of nasal aspirate sample directly on the ELISA wells for each sample. Standard curves were run with each plate.

Data and statistical analysis

All data was collected in paper form and then recorded in an electronic database shared by the three centers and processed by the RedCAP program. For future data analysis, logistic regression models, generalized linear equations, chi-square tests, and Wilcoxon rank sum tests will be used to compare odds ratios, adjust for demographic variables, and compare viral loads and cytokine expression, depending on specific questions that are addressed.

Results

Two hundred and forty four families were approached to join the study. Of the 205 infants initially enrolled (84%), 71 were from Hospital Garrahan and 134 from Hospital Sarda. Over the course of the study, three infants died, two from sudden infant death syndrome and the other infant from respiratory complications in the hospital. Fourteen additional infants were lost to follow-up. The remaining 187 subjects are currently enrolled in the study and continue to be followed.

During the three years of completed follow-up (August 2011 to July 2014), there were 319 ARI episodes among the subjects. Nasal swabs were collected from 910 well episodes, and 587 bimonthly phone calls were made. Eighty initial urine samples were collected from the subjects, and collection is ongoing from those who did not provide urine at enrollment.

Our first enrollment was in August 2011; on average (mean), there are 680 (standard error: 18.0) days of follow-up (1.86 years) for the patients (median = 612 days). The first patients enrolled have approximately 1140 days (3.12 years) of follow-up. The final patients enrolled (from January of 2014) have approximately 216 days of follow-up as of the date of manuscript submission.

Discussion

Eleven percent of infants are born prematurely [4]. Premature infants are at an increased risk of respiratory disease due to both incomplete lung development [5, 6], increased risk and severity of viral infections [16, 30, 31], and this may be associated with recurrent wheezing and asthma [1-3]. This prospective cohort study was established to determine the role respiratory viruses play in the development of recurrent wheezing and asthma in VLBW infants. The study takes place in a developing country where the burden of respiratory disease is high and where our group initially examined a cohort of VLBW infants among this same population. Because there are two analogous cohorts of VLBW children enrolled in Buenos Aires in 2003 and 2011, there is a unique ability to compare the populations. Though we are not sampling all of Buenos Aires, 16.8% of VLBW infants born in Buenos Aires were born at Hospital Sarda in 2012 [32].

Recruitment of VLBW infants was successful, with 84% of the families who were approached providing consent for enrollment. Due to the combination of bimonthly phone calls and having the parents call our study's neonatologists directly when the infants developed any ARI symptoms, we likely captured the majority of ARI episodes in these children. It was unexpectedly difficult to collect urine and blood from the children due to lack of parental agreement. We postulate that this may have related to concerns of what the specimens may be used for, even though we provided families with information and consent forms.

Conclusions

This prospective cohort will allow us to examine many different aspects of the interaction between respiratory viruses, the immune system, and development of asthma or recurrent wheezing in VLBW infants. Future reports using this data will allow researchers to examine host and viral determinants of disease at baseline, during acute ARIs, and with or without wheezing. The burden of RV and other specific respiratory viruses during the first year of life in VLBW infants will be assessed, as will viral association with recurrent wheezing/asthma up to five years of age, and if the immune system profiles of those children with asthma differed when they were very young infants. It is our hope this cohort will allow the field to better understand the role respiratory viruses play in wheezing and asthma in premature infants. Future reports from this cohort will help define the complicated relationship between premature infant respiratory viral infections.

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