Washington University in St. Louis
SCHOOL OF MEDICINE

The Edward Mallinckrodt Department of Pediatrics
Division of Allergy, Immunology & Pulmonary Medicine

APW-RSV-II:

Azithromycin to Prevent Wheezing following severe RSV bronchiolitis-II

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Support: **NHLBI**

Abbreviations

AE: Adverse Event

APW-RSV: Azithromycin to Prevent Wheezing following severe RSV bronchiolitis

BPD: Bronchopulmonary Dysplasia

CDAD: Clostridium difficile associated diarrhea

CF: Cystic fibrosis

DPB: Diffuse Panbronchiolitis

DSMB: Data Safety Monitoring Board

FDR: False discovery rate

HR: Hazard rate

HRPO: Human Research Protection Office

NHLBI: National Heart, Lung, Blood Institute

RBEL: RSV Bronchiolitis Early in Life

RW: Recurrent wheezing

SAE: Severe Adverse Event

SLCH: St. Louis Children's Hospital

SUSAR: Suspected Unexpected Serious Adverse Reaction

WGS: Whole genome sequencing

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I. TRIAL SUMMARY

The **A**zithromycin to **P**revent **W**heezing following severe **RSV** bronchiolitis II (APW-RSV II) clinical trial is designed to address a significant clinical problem: the development of recurrent wheezing (RW) and asthma following severe RSV bronchiolitis.

The APW-RSV II clinical trial is a double blind, placebo-controlled, parallel-group, randomized trial, including otherwise healthy up to 200 participants, ages 1 month (30 days) -18 months, who are hospitalized due to RSV bronchiolitis. The study includes active treatment phase with azithromycin or placebo for 2 weeks, and an observational phase for up to 48 months.

The main objective of the APW-RSV II clinical trial is to evaluate if the addition of azithromycin to routine bronchiolitis care, among infants hospitalized with RSV bronchiolitis, reduces the occurrence of RW during the preschool years. The secondary objective of the APW-RSV II clinical trial is to examine how azithromycin therapy changes the upper airway microbiome composition, and to determine if these changes are related to the reduction in the occurrence of post-RSV RW.

Study participants will be enrolled during 3 consecutive RSV seasons beginning in Fall 2016. Study participants will be randomized to receive PO azithromycin 10 mg/kg/day for 7 days followed by 5mg/kg/day for additional 7 days, or matched placebo. The primary clinical outcome is the occurrence of a third episode of wheezing. The duration of follow up is 18-48 months, which is determined based on the year in which the participants is recruited: first year recruits will be followed for up to 48 months, while the 3rd year recruits will be followed for at least 18 months. In addition, we will characterize microbiome composition in nasal wash samples obtained before and after the study treatments, and will be correlated microbiome composition parameters with the outcome of RW.

This trial may identify the first effective intervention to apply during severe RSV bronchiolitis with the goal of preventing post-RSV RW and ultimately asthma.

II. BACKGROUND AND RATIONALE

A. INTRODUCTION: OVERVIEW OF THE CLINICAL PROBLEM AND THE SUGGESTED INTERVENTION

This project is designed to establish that azithromycin therapy, an **easily implemented intervention, could have a high impact on pediatric health affecting a highly relevant clinical outcome**. If our preliminary data will be confirmed, this will shift the focus in the treatment of severe RSV bronchiolitis from supportive care to long-term prevention of post-RSV RW, potentially representing the first successful post-RSV asthma prevention modality.

Asthma is the most common chronic disease in childhood that carries substantial morbidity

Approximately 7 million children under 18 years of age have a lifetime diagnosis of asthma (current asthma prevalence 9.5%). Childhood asthma exerts a significant societal burden, accounting for 5.6% of all hospitalizations in children[1], more than 539,000 ED visits, over 155,000 hospitalizations (more than any other chronic condition in children), 167 deaths in 2005, over 12.8 million missed school days annually[2], and has a total annual expenditure exceeding $70 billion[3]. **Asthma prevention is highly desirable, as prolonged treatment with asthma controller medications does not influence the long-term outcomes of the disease[4].**

Early life viral respiratory infections, particularly Respiratory Syncytial Virus (RSV) bronchiolitis, have a major role in childhood asthma inception

RSV and rhinovirus are the most common viral causes of preschool wheezing episodes, and both play major roles in asthma inception[5-8]. However, the mechanisms underlying these relationships likely differ between these 2 viruses, and a causative role has been recently demonstrated between early life severe RSV infection and future RW (described below)[9]. Although most children infected with RSV experience mild disease that does not require hospitalization, RSV bronchiolitis is the leading cause of hospitalizations in infants younger than one year in the US during the winter[10-12]. The occurrence of early in life severe RSV illness confers substantial risk for subsequent wheezing and asthma development[13-17], and there is a gradient of increasing risk of asthma based upon bronchiolitis severity (as reflected by level of heath care utilization), with the greatest risk of asthma following bronchiolitis hospitalization[18, 19].

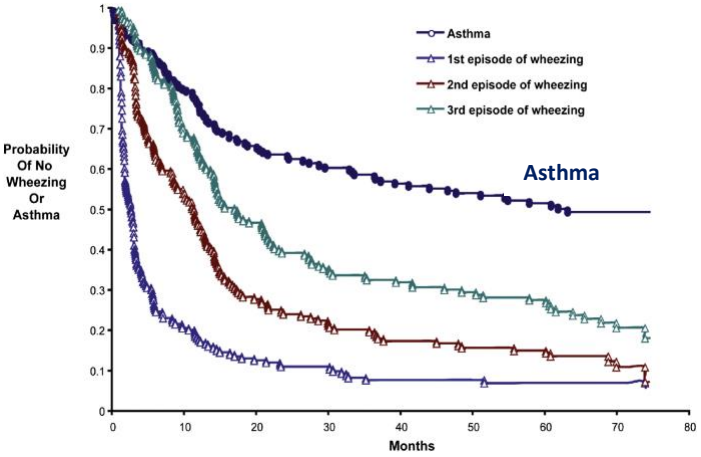


Figure 1. The occurrence of recurrent wheezing and asthma following hospitalization for RSV bronchiolitis as determined in our RBEL cohort. The outcome of 3 episode of wheezing is an antecedent to asthma.

We have demonstrated in the **RSV Bronchiolitis in Early Life (RBEL-I) study,** a prospective cohort of 206 infants hospitalized for RSV bronchiolitis, that 48% of participants had a physician diagnosis of asthma, and that 75% of participants experienced recurrent (at least 3) wheezing episodes by their 7th birthday (**Figure 1**)[17]. As hospitalized infants have the greatest risk for post-RSV wheezing episodes and asthma, they represent an attractive target population in which to explore intervention strategies for the prevention of post-RSV RW and asthma.

Prevention of RW and asthma following RSV bronchiolitis will have a profound impact on child health

Up to 13% of new childhood asthma cases are attributable to RSV bronchiolitis and thus could be averted by the prevention of severe RSV bronchiolitis[20]. Primary prevention of RSV bronchiolitis would be an ideal strategy, as confirmed by a recent placebo-controlled trial demonstrating that prophylactic administration of the anti-RSV monoclonal antibody palivizumab to late pre-term infants led to a nearly 50% reduction in RW during the 1st year of life[9]. These results are highly informative, support the causative role of RSV infection in the pathway of asthma development, and provide encouragement that post-RSV asthma may be preventable. However, the high expense of this specific therapy and its need to be given to a broad population prior to acquisition of RSV infection limits its widespread use, as does the need for parenteral administration. **Therefore, there is a need for a simple, inexpensive, and non-toxic intervention capable of modifying the outcomes of RW and asthma in children who are already hospitalized for RSV bronchiolitis.**

Numerous asthma-related therapies have been unsuccessfully tried in attempts to prevent post-RSV wheezing and asthma[5]

The lack of efficacy of treatments typically used for asthma, such as inhaled corticosteroids (ICS)[21-23], systemic corticosteroids[24-26], and montelukast[27, 28] on the occurrence of post-bronchiolitis wheezing, may be related to the minimal effect that these medications have on non-eosinophilic (i.e., neutrophilic) airway inflammation[29], the

dominant pattern seen during viral bronchiolitis[30, 31]. Therefore, a medication with anti-neutrophilic properties may be effective in attenuating post-RSV wheezing.

Azithromycin, a macrolide antibiotic, is a potentially attractive intervention for the prevention of post-RSV wheezing

Macrolides provide clinical benefits in inflammatory airway diseases with a dominant neutrophilic airway inflammation component, such as cystic fibrosis and diffuse panbronchiolitis, likely through anti-inflammatory activities[32, 33]. Azithromycin also has anti-neutrophilic activities in-vitro[32], and within the airway in patients with refractory neutrophilic asthma[34]. A small study with substantive limitations, which was followed by a major critics, has resulted in inconclusive conclusions about whether macrolides reduce post-RSV wheeze[35,36,37]. Moreover, a single dose of azithromycin during viral bronchiolitis (~50% due to RSV) did not reduce the rate of re-admission over the following 6 months, but no data were reported regarding the effect of the intervention on the occurrence of subsequent wheeze[38]. As high quality data were not available regarding the potential benefits of macrolides to prevent post-RSV wheezing, and based on our positive findings in a mouse model of viral bronchiolitis[39], we performed the **Azithromycin to Prevent Wheezing following RSV Bronchitis (APW-RSV) trial.** This proof-of-concept (POC) trial in 40 infants hospitalized with RSV bronchiolitis revealed that azithromycin treatment for 2 weeks, when added to routine bronchiolitis care, significantly reduced the probability of having RW over the following year (**Figure 2**)[40]. Based on these preliminary data, we now propose a longer confirmatory clinical trial to investigate the effect of azithromycin on post-RSV recurrent (≥3 episodes) wheeze during the preschool years.

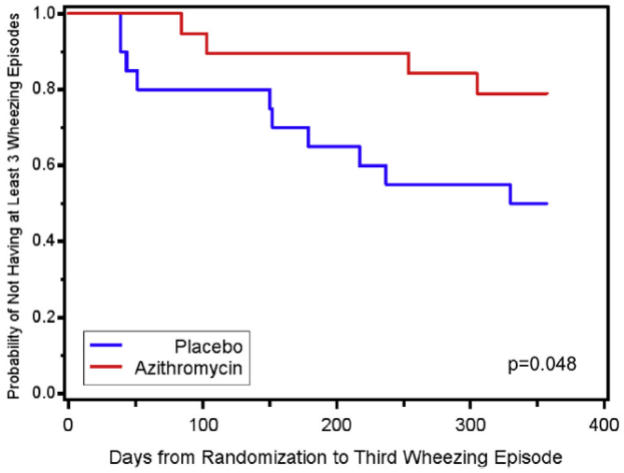


Figure 2. The addition of azithromycin therapy to routine bronchiolitis care among participants of the APW-RSV pilot trial (n=40) significantly reduced the probability of developing post-RSV recurrent wheeze.

Recent evidence suggests that the airway microbiome has an important role in asthma inception[41-43]

In addition to the long-established association between early-life viral respiratory infections and subsequent asthma[5-8], bacterial microbes that comprise the airway microbiome have recently emerged as significant contributors to asthma inception. Asymptomatic colonization of the hypopharynx with encapsulated bacteria, including *Moraxella catarrhalis,* was associated with higher risk of developing bronchiolitis or pneumonia[44], and persistent wheezing and asthma[43] during the preschool years. A *post-hoc* analysis of children hospitalized during their first viral induced wheezing episode showed that 60% had positive nasopharyngeal cultures for *S. pneumoniae*, *H. influenzae*, or *M. catarrhalis,* and these children had longer hospitalizations and were more likely to develop RW[45]. Moreover, there is emerging evidence linking the airway microbiome during viral respiratory infections and the development of RW[46]. Our preliminary data suggest that azithromycin treatment during RSV bronchiolitis modifies the upper-airway microbiome composition, including a reduction in *Moraxella* abundance, which in turn was associated with lower odds of subsequent RW.

Based on these observations describing the role of the microbiome in asthma inception, our preliminary data, and our local expertise with sequencing and analyzing the human microbiome at The Genome Institute at Washington

University, we aim to investigate the relationships between azithromycin treatment, the changes in upper airway microbiome composition, and the development of RW. This aim will explore a currently unstudied pathway that may mediate the beneficial effects of azithromycin. **Understanding how azithromycin affects the microbiome, and how these changes relate to the development of RW, may enable us to develop additional microbiome-directed therapies to prevent asthma following RSV bronchiolitis.** These therapies may include: oral supplementation with prebiotic nutrients, specific live microbial species (probiotics), microbe-derived products (bacterial extracts, immune stimulants), or elimination of harmful bacteria using targeted antibiotics. Approaches using the microbiome as therapeutic agents and/or targets have already been successfully adopted in other conditions, most notably demonstrated by the efficacy of duodenal infusion of donor feces in the management of recurrent *Clostridium difficile* infections[47].

B. REVIEW OF CLINICAL TRIALS RELEVANT TO THIS PROTOCOL

B.1. Macrolide antibiotic (azithromycin)

Macrolides are bacteriostatic antibiotics that reversibly bind to 50S ribosomal subunit of susceptible microorganism and inhibit RNA-dependent protein synthesis. Over the past 30 years, macrolide antibiotics have been used to treat chronic inflammatory airway diseases based on their presumptive immunomodulatory activity activities[32, 33 48].

B.2. Previous trials using macrolides in the setting of severe RSV bronchiolitis

The APW-RSV proof-of-concept trial, clinical outcomes

As noted above, we performed a randomized, double-masked, placebo-controlled, proof-of-concept trial (the **APW-RSV trial**) in 40 otherwise healthy infants hospitalized in RSV bronchiolitis who were treated with azithromycin or placebo for 14 days, in addition to routine bronchiolitis care. **Azithromycin-treated participants had a reduced probability of having RW (p=0.048; Figure 2), and fewer days with respiratory symptoms over the subsequent year in comparison to placebo-treated participants (36.7 vs. 70.1 days; p=0.01).** In addition, azithromycin resulted in a greater decline in nasal lavage IL-8 at the end of the treatment period, (p=0.03; **Figure 3**), supporting the anti-neutrophilic effects noted in our previous studies in the mouse model[39]. Azithromycin therapy, compared to placebo, was not associated with higher rate of adverse reactions, and the therapy was well tolerated (see the RISK/PROTECTION AGAINST RISK Sections for more details).

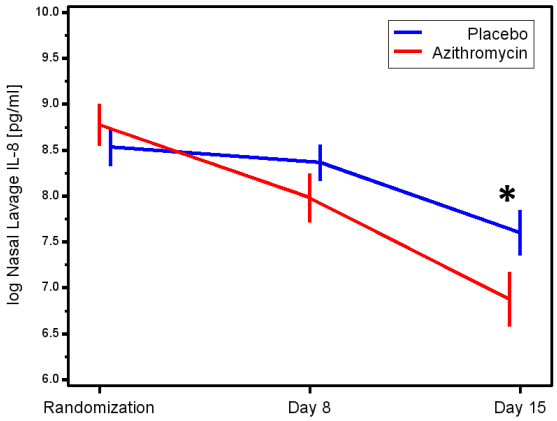


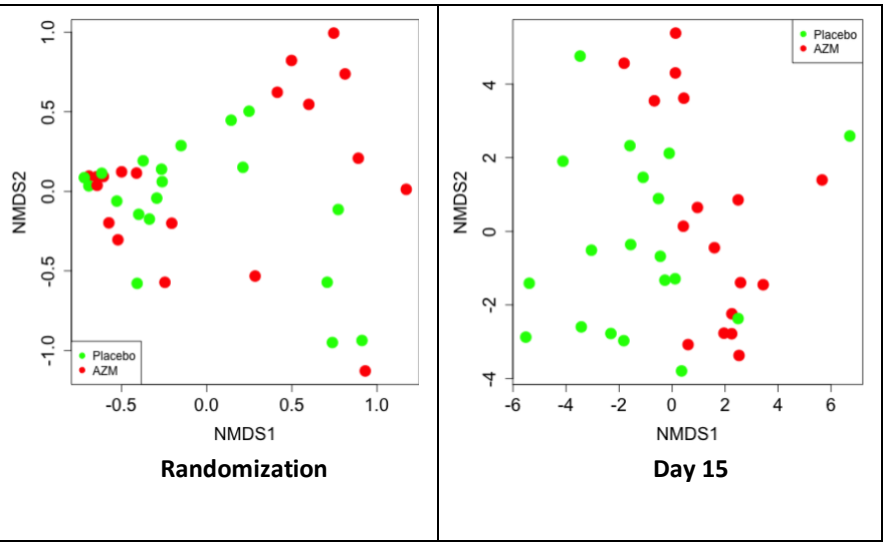
Figure 3. azithromycin treatment among participants of the APW-RSV clinical trial reduced nasal lavage IL-8, a marker of neutrophilic inflammation; *p=0.03.

The APW-RSV proof-of-concept trial, microbiome outcomes

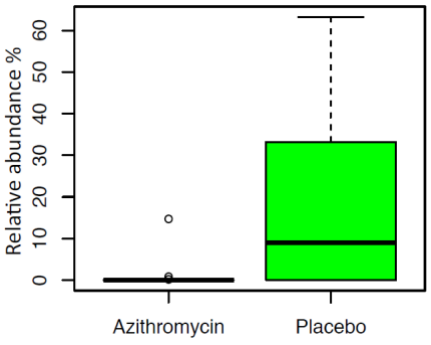
We investigated whether azithromycin alters the airway microbial composition and whether these changes could be associated with RW by sequencing 16S rRNA genes in the nasal wash from 39 participants of the APW-RSV pilot trial. We compared the upper airway bacterial communities at randomization and at the end of treatment (2 weeks later). As illustrated by Nonmetric Multi-dimensional Scaling (NMDS) based on the Bray-Curtis dissimilarity (**Figure 4 left**) and confirmed by permutational multivariate ANOVA (PERMANOVA), samples from azithromycin and placebo groups harbored similar bacterial communities at randomization. However, after two weeks of treatment, samples from the azithromycin group formed a distinct cluster (**Figure 4 right**), and the community structures of the 2 groups became significantly different. Longitudinal comparison revealed altered bacterial communities in both groups after two weeks of treatment. However, azithromycin and Placebo group's microbiome changed with different patterns: decreased *Moraxella* (p=0.003, q=0.03 (q-value is an estimate of the False Discovery Rate)) in the azithromycin group, and increased *Cornyebacterium* (p=0.008, q=0.09), *Dolosigranulum* (p=0.006, q=0.07), and decreased streptococcus in the placebo group (p=0.001, q=0.004). These preliminary findings suggest azithromycin has a significant impact on the composition of the upper airway microbiome during RSV bronchiolitis.

Figure 4. Nonmetric Multi-dimensional Scaling (NMDS) showing the airway microbiome composition before (left) and after (right) the study treatments (each dot represents one sample; The closer the dots are to each other, the more similar is their microbiome composition).

No difference was noted in airway community structure between the 2 groups at randomization (PERMANOVA p=0.71); however microbiome clusters differ at the end of the study treatment (PERMANOVA p=0.001), indicating that the azithromycin treatment changed the airway microbiome composition.



Differential feature analysis by Metastats, an analysis performs to identify the genera that contribute to the difference between two bacterial communities[49], showed that the *Moraxella* genus was the bacteria taxon that statistically distinguished (p=0.0002, q=0.009) the azithromycin and placebo groups at the end of study treatments (**Figure 5**).



of *Moraxella* at the end of the study treatments.

Lower *Moraxella* abundance at the end of study treatments was significantly associated with lower odds of experiencing subsequent RW (OR=0.86; 95% CI 0.75-0.99, p=0.03) suggesting that *Moraxella* may have a potential role in the development of post-RSV wheezing, and highlights the potential efficacy of the intervention to reduce the abundance of this taxon and probably other taxa, and hence to modify the clinical outcome of RW. This finding may be highly relevant to asthma inception, as asymptomatic upper airway colonization with *Moraxella catarrhalis* at the age of one month was associated with the development of RW and asthma during the preschool years[43]. However, the exact role of *Moraxella* in asthma development still deserves more investigation, as a recent study suggested that infants with a *Moraxella* predominant airway microbiome are less likely to develop mild respiratory infections[50].

Other clinical trials that have utilized macrolides in the setting of severe RSV bronchiolitis

Clinical trials that have been conducted prior to our APW-RSV pilot trial did not result with solid conclusions regarding the potential utility of macrolides to prevent post-RSV wheezing. One of these studies was a small study with substantive limitations, which was followed by a major critics, that has resulted in inconclusive conclusions about whether macrolides reduce post-RSV wheeze[35,36,37]. Another study revealed that a single dose of azithromycin during viral bronchiolitis (~50% due to RSV) did not reduce the rate of re-admission over the following 6 months, but no data were reported regarding the effect of the intervention on the occurrence of subsequent wheeze[38]. Based on the limitations of these studies, and in order to prove the feasibility of preventing RW after severe bronchiolitis, we conducted the APW-RSV trial that suggested the feasibility of post-RSV RW prevention using azithromycin.

B.3. Azithromycin, as an anti-inflammatory therapy, for the prevention of Bronchopulmonary Dysplasia (BPD) among extremely low birth weight pre-term infants

To our best knowledge, only 2 studies (in addition for the RSV studies discussed above) have evaluated the potential non-antibacterial effects of azithromycin in young infants. The first study was a pilot double blind placebo-control study evaluating the effectiveness and safety of prophylactic azithromycin, based on its anti-inflammatory properties, in reducing the incidence and severity of BPD in extremely low birth weight (≤ 1000 grams) pre-term infants [51]. Pre-term infants (mean gestational age was 25 wks) were treated with azithromycin (n=19) or placebo (n=16). The treatment group received azithromycin 10 mg/kg/day for 7 days followed by 5mg/kg/day for the duration of the study (up to 6 weeks of therapy). Azithromycin treatment did not affect the incidence of BPD; however, post-natal systemic corticosteroid use was significantly less in the azithromycin group and the duration of mechanical ventilation was significantly shorter in treatment survivors. More relevant to our study: the treatment was safe and well tolerated, and there were no side-effects that could be attributed to the azithromycin therapy during or after the study [51]. In a subsequent study[52], Ballard et al used the same methods and dosing regimen in 211 neonates with birth weight <1250 g. They reported on decreased incidences of BPD in neonates that were colonized with Ureaplasma. More importantly, this study re-demonstrated the tolerability and safety of azithromycin therapy in this population[52]. **We adopted this azithromycin-dosing regimen, albeit for a shorter duration, based its excellent safety profile among these young and critically ill infants.**

B.4. Macrolides have beneficial effects in airway diseases such as cystic fibrosis (CF) and Diffuse Panbronchiolitis (DPB)

Clinical trials in CF have documented significant improvement in lung function[53] and quality of life parameters (e.g., weight gain) along with fewer exacerbations when using chronic long-term (duration of 6 months) azithromycin treatment [53-56]. Patients with CF are often colonized with *Pseudomonas aeruginosa*, an organism known to be resistant to the antimicrobial activity of macrolides. An initial meta-analysis that investigated the proposed anti-inflammatory effects in CF suggested that azithromycin improves lung function of CF patients, mainly in the subgroup of patients colonized with *Pseudomonas aeruginosa*[57] and to a lesser degree in patients not colonized with this organism. More recent meta-analysis (n=654) confirmed these beneficial effects, as it concluded that long-term use of azithromycin improves lung function, especially for *Pseudomonas* aeruginosa-colonized CF patients, and that the treatment was not associated with an increase in adverse events[58]. Therefore, it seems that the beneficial effects of macrolides in CF are distinct from their antibacterial effect.

Diffuse panbronchiolitis (DPB) is a chronic inflammatory disease of the respiratory bronchioles characterized by colonization with *Haemophilus influenzae* and/or *Streptococcus pneumoniae,* often with a change to *P. aeruginosa* over time. In the early 1980's, studies done in Japan revealed that long-term, low-dosage erythromycin improved

symptoms and increased 10-year survival from 12% to greater than 90% even in patients colonized with mucoid strains of *P. aeruginosa* [59-61]. Similar to the findings in CF, these beneficial effects do not appear to be mediated by the anti-bacterial activity of erythromycin. The precise mechanism of action of macrolides in CF and DPB is unknown but thought to be due to an influence of macrolides on *P. aeruginosa* biofilms [55] and to additional anti-inflammatory effects.

B.5. Macrolide for asthma

Numerous trials have examined the potential efficacy of macrolides in asthma with variable results, including several studies demonstrating beneficial effects.[34, 56, 62-73]. The above studies differ in their study designs, study populations, treatment protocols, and outcome measures, making generalization of the findings difficult. There is a long-standing debate whether these beneficial effects of macrolide in asthma are related to the antimicrobial activity of the macrolide against *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* (organisms which are known to promote asthma exacerbation and potentially contribute to asthma severity and/or persistence), or whether these agents have distinct additional anti-inflammatory effects. In order to review only studies that are relevant to this protocol, we will focus on studies that measured serum and/or airway cytokines as an outcome as markers for potential anti-inflammatory properties of macrolides, or on pediatric studies.

Fonseca-Aten et al[74] investigated whether clarithromycin started within 72 hours of the onset of acute wheezing episodes can affect inflammatory mediators' concentration in 28 children, 4-17 years old, with a history of RW or asthma (self-reported by the patient or caregiver). Nasopharyngeal concentrations of TNF-α, IL-1β, and IL-10 were significantly and persistently lower in children treated with clarithromycin compared with placebo. On the other hand, the clarithromycin had no effect on clinical symptoms (dyspnea, chough, wheeze retraction, fever or clinical score) 3-5 days after initiation of treatment. Negative clinical findings could be potentially related to the heterogeneity of patients' characteristics (age, underlying airway disorder), delay in initiation of treatment, or insufficient statistical power. Kraft et al. evaluated the role of clarithromycin in stable adult asthma patients with moderate – severe disease, with and without evidence of airway *Mycoplasma pneumonia* or *Chlamydia pneumonia.*[68] In this study, clarithromycin treatment for 6 weeks improved FEV_1 only in the sub –group of patients with evidence of infection. On the other hand, significant reductions were noted in bronchial alveolar lavage IL-12 and TNF-α mRNA expression that were not dependent on the bacteriologic status of the patient.

Finally, a recent clinical trial that included 606 preschool children with history of RW reveled that the use of azithromycin early during an apparent respiratory-tract-illness reduced the likelihood of developing severe lower respiratory-tract-illness[75].

C. SELECTION OF INTERVENTION FOR THIS TRIAL

In this trial, we will use azithromycin, a member of the macrolide antibiotic family, which was effective, safe, and well tolerated in our APW-RSV pilot trial. Three different forms of macrolides are FDA-approved for use in children in the US: erythromycin, clarithromycin, and azithromycin. All have been demonstrated to have anti-inflammatory and/or immunomodulatory effects (see section B). Erythromycin treatment is inconvenient since it needs to be given 3-4 times daily; therefore might result in poor compliance. In addition, it has significant gastrointestinal side effects that would limit its use in a pediatric trial. Clarithromycin has an inhibitory effect on the P450 enzyme system that metabolizes several drugs, including corticosteroids[76]. In addition, clarithromycin is administered twice daily and this might contribute to suboptimal adherence.

Azithromycin is a macrolide antibiotic which does not have the many of limitations discussed above. Azithromycin is a macrolide antibiotic approved for use in children 6 months of age and above for pneumonia, sinusitis, and

otitis media, and 2 years and above for tonsillitis or pharyngitis. In contrast to older macrolides, azithromycin does not interfere with the cytochrome P-450 complex liver enzyme systems that are responsible for metabolizing medications[77]. In addition, azithromycin is administered once daily and this might contribute to increased adherence.

Azithromycin is one of the most commonly prescribed medications in the USA. An FDA report, which evaluated azithromycin safety, suggests that ~28.3 million pediatric patients (0 to 16 years old) received a prescription for azithromycin from October 2005 to September 2009; ~4.3 million of them were in the age of 0 to 1 year olds [78] . Severe adverse events (AE) since market approval (June 2005) were very rare and were estimated as 0.46 events per one million. Please see section P (RISKS/PROTECTION AGAINST RISKS) for a more detailed description of azithromycin's safety profile in children.

Azithromycin therapy in our APW-RSV pilot trial[40] was well tolerated. Gastrointestinal adverse events (diarrhea, vomiting, or abdominal pain) during the active treatment phase were recorded in 7 children treated with azithromycin and in 8 children treated with placebo, and none were severe enough to warrant discontinuation of study medication.

From all of possible macrolides, we would expect the greatest tolerability and adherence to azithromycin during the study than with the other available macrolides.

The inclusion of a placebo arm is ethical since currently there is no effective and easily available therapy for the acute RSV bronchiolitis, or for the prevention of post-RSV RW, and the outcome of post-RSV RW is very common:

1. The current American Academy of Pediatrics Guidelines for the Management of Viral Bronchiolitis emphasizes the role of supportive therapy as no intervention has been proven effective to change the course of the acute bronchiolitis[79].
2. No current safe intervention has been proven effective in preventing post-RSV wheezing or asthma. In our RBEL study, 48% of participants had a physician diagnosis of asthma and 75% of participants experienced recurrent (at least 3) wheezing episodes by their 7th birthday (Figure 1)[17] . The goal of the APW-RSV II is to prevent these post-RSV wheezing episodes.

D. RATIONALE FOR SELECTED STUDY POPULATION

The target study population is infants who experience severe RSV bronchiolitis (defined as an episode that requires hospitalization). Severe RSV bronchiolitis is a major independent risk factor for RW episodes and asthma[17], [13-16, 80]. Recent data from our institution suggest that by the age of 7 years old up to 80% of the hospitalized children experience recurrent (≥3) wheezing episodes, and approximately 50% of these have a physician diagnosis of asthma[17].

Study population will include infants 1 month (30 days) -18 months of age admitted to St. Louis Children's Hospital (SLCH) for the first episode of lower respiratory tract symptoms[28] with confirmed RSV infection. We focus hospitalized infants since these have the highest risk to develop RW and asthma[18, 19]. In order to apply the intervention with azithromycin in the first days of the acute bronchiolitis, when this medication has the greatest potential to limit inflammatory damage to the airway, infants will be eligible to be enrolled in the study if the duration of respiratory symptoms until admission is 5 days or less, and if they can be randomized within 7 days from initiation of symptoms. We will exclude children with history of prematurity (gestational age < 36 weeks) or any chronic lung disease since these will increase the risk of RW and might affect our clinical outcome. Children

with history of any other chronic diseases (CNS, lung, cardiac, renal, GI, (including significant GERD), hepatic disease, hematologic, endocrine or immune disease) will be excluded for safety reasons. Children who had any previous treatment with inhaled and/or oral corticosteroids (for respiratory conditions) will be excluded. For more detailed description of inclusion and exclusion criteria, please refer to section V below.

E. SELECTION OF STUDY MEDICATION, DOSAGE, AND DURATION

E.1. Dosing strategy

We will utilize the dosing regimen that was effective and well tolerated in our APW-RSV pilot trial. Eligible participants will be randomized 1:1 to either the azithromycin or placebo group based on a blocked randomization allocation sequence that will be generated by the study statistician. Randomization will be stratified by prior use of open-label non-macrolide antibiotic over the past 2 weeks prior to randomization. As in our APW-RSV trial, the active treatment group will receive azithromycin orally 10 mg/kg once daily for 7 days followed by 5mg/kg once daily for additional 7 days. Azithromycin and placebo will be packaged at the SLCH investigational pharmacy, which performed this function for the APW-RSV pilot study and has confirmed feasibility and costs of developing a matching placebo for this trial. Adherence to study medication will be monitored based on measurements of medication bottle weights before and after treatment and based on medication diary that will be completed daily by the parents. Upon discharge, parents will be supplied with the medication and will continue daily treatment and documentation of treatment at home. As azithromycin suspension is stable for 10 days only, a second bottle of study medication will be shipped to the parents by a courier. Participants will be seen at the clinical research unit on day 15. At that time we will monitor treatment adherence as documented in the diary, and by weighing the medication bottles.

E.2. Rationale for azithromycin dosing regimen

The optimal dose and duration of azithromycin treatment needed to provide an anti-inflammatory effect is not established. The dosing regimen used in our APW-RSV pilot trial: provided significant clinical benefit (**Figure 2**), was effective in exerting anti-inflammatory effects evident by reduced airway IL-8 levels (**Figure 3)**, and was not associated with higher rates of treatment-related adverse reactions compared to placebo. This treatment regimen was based upon previous studies in animal models of viral bronchiolitis that revealed immunologic events up to 21 days after infection that can lead to Th2-prone phenotype[81]. As azithromycin has a very long half-life in the lung tissue[82], 14 days of treatment with azithromycin should result in at least 23 days with effective anti-microbial concentrations in the lung tissue[82], and in at least 35 days of measurable quantity in airway macrophages[83], which will provide coverage during the recovery from the bronchiolitis. We also aim to minimize potential drug adverse reaction by using a dosing regimen that was safe in our APW-RSV trial (n=40), **and had an excellent safety profile in a vulnerable population of very-low birth-weight pre-term infants (≤ 1250 grams; n=263), once given for up to 6 weeks[51, 52]** (see the RISKS/PROTECTION AGAINST RISKS Section for additional details). We shortened treatment duration to 2 weeks based on the considerations described above. As in our APW-RSV pilot trial, we obtained an FDA Investigational New Drug (IND) for this study. Rates of study medication related the investigators, the study DSMB and the IRB would monitor adverse reactions.

F. PRIMARY OUTCOME MEASURES AND RATIONALE

F.1. Primary outcomes:

1. Clinical trial primary outcome: The time until the occurrence of a third episode of post-RSV wheezing measured over a follow up duration of 18-48 months, quantified by a Cox proportional hazards regression model, and compared between the groups.

   Time frame for primary outcome assessment: For the purpose of this study, wheezing detected during the initial RSV bronchiolitis will not be included in the count of post-RSV wheezing. The time frame for measurement of post-RSV wheezing (and other long-term outcomes) starts at the end of the treatment period (2 weeks after randomization) and ends at end of the follow-up period. Therefore, a wheezing episode that occurs at the first 2 weeks of the study, does not "count" toward the outcome of RW.

2. Microbiome study outcome: Upper airway microbiome community structure measured at the end of study treatments, and its relationship with the occurrence of post-RSV RW (≥ 3 episodes).

F.2. Rationale for primary outcome selection

Our long-term goal is asthma prevention. The goal of this proposal is to identify of a strategy capable of preventing RW, a surrogate and relevant marker for future asthma. The occurrence of recurrent episodes of wheezing is commonly used as an intermediate outcome for future asthma in studies involving young children, and is supported by the finding that children who experience 3 or more wheezing episodes in early life are at least 4 times more likely to develop asthma by the age of 7 yrs, compared to children who have less frequent wheezing episodes[84].

In our RSV Bronchiolitis Early in Life (RBEL) observational cohort[17], which includes participants with the same characteristics as we propose to recruit in this project, **the outcome of the occurrence of 3 or more wheezing episodes (i.e., RW) was a robust intermediate surrogate marker for asthma diagnosis at the age of 7 yrs: OR=7.31 (95% CI: 1.24-43.3).**

As reflected in **Figure 1**, most children hospitalized for RSV bronchiolitis develop 1-2 additional wheezing episodes. Given the high prevalence of 1-2 wheezing episodes in this population, and since a substantial proportion of such children do not go on to develop asthma, this outcome is not an appropriate surrogate marker for future asthma. In contrast, there is substantially greater specificity for 3 or more wheezing episodes and subsequent asthma, and thus the outcome of 3 or more wheezing episodes is a better intermediate marker to a subsequent asthma diagnosis than the less specific outcomes of one or two wheezing episodes.

A diagnosis of asthma during the preschool years is difficult to establish, as early childhood wheezing is common, and is associated with multiple wheezing trajectories with variable long-term asthma diagnosis[85-88]. Approximately one-third of children who wheeze once before the age of 3 years will develop asthma by school age[89, 90]. In addition, the diagnosis of asthma in young children is further complicated by the lack of objective lung function measurements and definitive biomarkers. In order to overcome the objective obstacle of diagnosing asthma during the preschool years, and since the occurrence of 3 or more episodes of wheezing is an established surrogate marker for future asthma, many studies that have investigated the inception of asthma in early childhood have used a comparable rationale to select this outcome of RW as the primary outcome. Examples include: the NIAID-funded Urban Environment and Childhood Asthma (URECA) birth cohort study[91, 92], and the NHLBI-funded Maternal Vitamin D Supplementation to Prevent Childhood Asthma (VDAART): a clinical trial for the prevention of childhood asthma (ClinicalTrials.gov Identifier:NCT00920621).

In addition to serving as a predictor of subsequent asthma, demonstration of a significant reduction in post-RSV wheezing is a very important goal by itself. These wheezing episodes carry a significant burden on affected individuals, the health system, and society since preschool children with episodic wheeze experience high morbidity and health care utilization during these acute exacerbations. Preschool children with episodic wheeze require 50% more ambulatory visits, almost twice as many emergency room visits, and 3 times more

hospitalization relative to school age children with establish asthma[2]. Therefore, the reduction in post-RSV RW would be of great clinical value irrespective of eventual asthma development.

In summary, the occurrence of RW (3 or more wheezing episodes) is a relevant intermediate outcome and an antecedent to a diagnosis of childhood asthma. This outcome can be reliably measured during this proposed study; and based on its strengths, it has been previously utilized in other NIH-supported studies that have investigated the inception of asthma during the preschool years.

G. RESEARCH QUESTIONS

1. Does treatment of infants hospitalized for severe RSV bronchiolitis with azithromycin modify the natural history of post-RSV RW by reducing the occurrence of post-RSV RW?
2. Does treatment of infants hospitalized for severe RSV bronchiolitis with azithromycin modify the upper airway microbiome community structure, and are these modifications related to the occurrence of post-RSV RW?

III. HYPOTHESES TO BE TESTED BY THE APW-RSV II TRIAL

A. PRIMARY HYPOTHESES

In infants hospitalized with RSV bronchiolitis, the addition of azithromycin therapy (compared to placebo) to routine bronchiolitis care:

1. Reduces the likelihood of developing post-RSV RW (≥ 3 episodes) measured over a follow-up period of 18-48 months.
2. Modifies the airway bacterial microbiome community structure, and these changes are related to the occurrence of post-RSV RW:
    a. More specifically, based on our preliminary data we will evaluate if azithromycin therapy results in reduction of *Moraxella* abundance and whether lower *Moraxella* abundance is associated with reduced odds to develop RW.

B. SECONDARY AND EXPLORATORY HYPOTHESES:

In infants hospitalized with RSV bronchiolitis, azithromycin therapy (compared to placebo) will result in:

1. Decreased nasal lavage levels of IL-8, neutrophil elastase, and matrix metallopeptidase-9 (MMP-9) measured on day 15 after randomization.
2. Not different rates of upper airway colonization with macrolide-resistant bacteria measured at the end of study treatments (day 15 after randomization) and at the 6-months clinic visit.
3. Not different incidence of treatment-related adverse reactions.
4. Reduced likelihood and longer time to develop 4th wheezing episode.
5. Reduced likelihood and longer time to develop physician asthma diagnosis.

6. Fewer annualized days with: a) wheezing, b) any respiratory symptoms, c) use of rescue albuterol, d) antibiotic use, e) oral corticosteroid use, f) parental absence from work and infant absence from day care, and g) nights with awakening due to respiratory symptoms.
7. Lower rates of: a) oral corticosteroid use, b) antibiotic courses, c) ED and urgent care visits, d) hospitalizations for respiratory symptoms, f) upper respiratory infections.
8. Smaller proportion of children: a) who were prescribed asthma controllers medications: ICS and/or montelukast, b) who developed RW, c) have physician asthma diagnosis.
9. Lower cumulative number of subsequent wheezing episodes.
10. Lower mean total IgE level and eosinophil counts measured at yearly follow up visits.
11. Smaller proportion of children with at least one positive specific IgE (SigE) to inhalant allergens measured at the first and last yearly follow up visit.
12. Modifications of the viral and/or the fungal components of the airway microbiome, which are related to the occurrence of post-RSV RW.

IV. STUDY PROTOCOL OVERVIEW AND DESIGN

The APW-RSV II trial is double blind, placebo-controlled, parallel-group, randomized trial, including otherwise healthy up to 200 participants, ages 1 month (30 days) -18 months, who are hospitalized due to RSV bronchiolitis. (i.e., severe RSV bronchiolitis). Participants will be recruited over 3 consecutive RSV seasons. The study will include treatment phase of 2 weeks (azithromycin or a matched placebo) and observation phase (18-48 months depending on the season of recruitment) during which we will assess the clinical outcomes.

The SLCH hospital census list will be reviewed daily (during the RSV season) for all potential admissions with RSV bronchiolitis. Eligible patients whose parent(s) provide written informed consent to participate in the study will be randomized. There will be no run-in period for this study, since we would like to apply intervention relatively early in the course of the acute bronchiolitis. Infants will be randomized to receive PO azithromycin 10 mg/kg/day for 7 days followed by 5mg/kg/day for additional one week or matched placebo.

The clinical primary outcome is the occurrence of a third episode of wheezing measured over a follow up duration of 18-48 months, compared between the azithromycin and placebo treated children. For the microbiome investigations we will assess the upper airway microbiome community structure at the end of study treatments, compared between the azithromycin and placebo treated children, and its relationship with the occurrence of post-RSV RW (≥ 3 episodes). Clinical outcomes will be measured in clinic visits and by phone interviews, which will be conducted every 2 months.

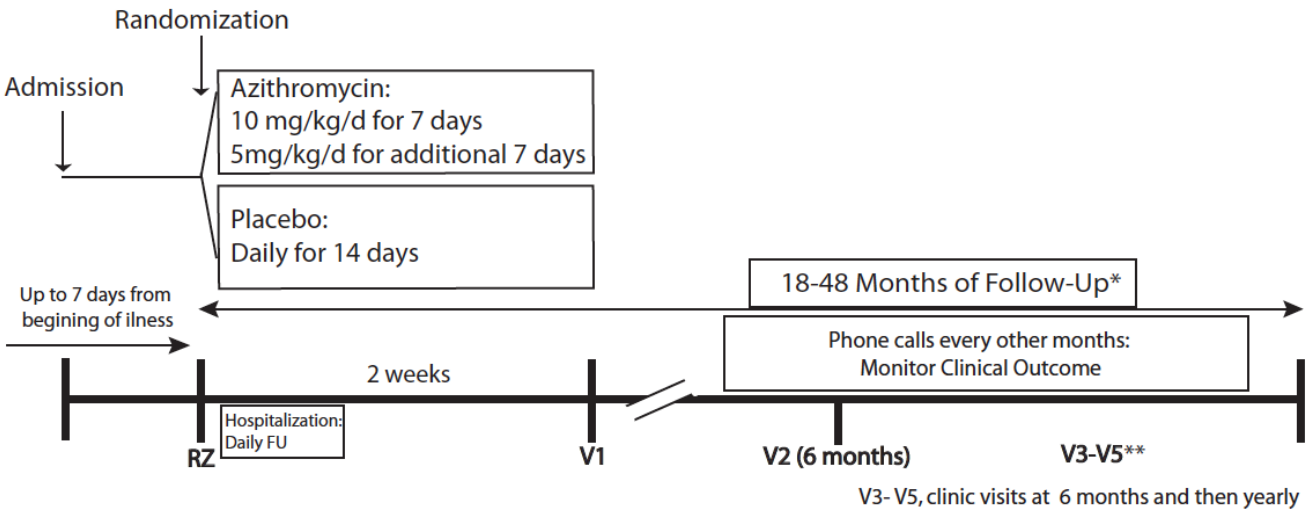


Figure 6: The APW-RSV II trial design

*Variable durations of follow-up periods are necessary, as patients will be recruited over 3 consecutive RSV seasons (see also Figure 7: study timeline); Therefore, not all patients will perfom V4 and V5. ** V3-V5 will be conducted yearly.

V. PROTOCOL

A. STUDY GROUPS

We will randomize up to 200 children, 1 month (30 days) -18 months of age, who meet all inclusion criteria and do not have any of the exclusion criteria. Children will be randomized in a 1:1 manner to one of the 2 treatment arms (azithromycin or placebo).

PATIENT IDENTIFICATION AND ENROLLMENT: Recruitment and enrollment will be conducted over 3 consecutive RSV seasons: starting with the 2016-2017 RSV season (usually November- April) and concluding during the 2018-2019 RSV season. The SLCH census list will be reviewed daily (during the RSV season) for all potential admissions with RSV bronchiolitis. Furthermore, all positive RSV FA/PCR from the virology lab at SLCH will be investigated to assess eligibility for this study. Based on these lists, we will pre-screen the medical records for study inclusion/exclusion criteria. If there are no apparent exclusions, the study coordinator will approach the child, after obtaining permission from the physician in charge of the child's care, to determine eligibility.

Estimates of annual enrolment for each RSV season were based on the institution's (SLCH) historical data. Over seven years in two prior studies (the RBEL cohorts), the average enrolment rate at SLCH was 65-70 subjects per RSV season. The range of newly enrolled subjects during the same period varied between 35 and 100.

B. INCLUSION CRITERIA

1. Age: 1 month (30 days) -18 months.
2. Hospitalization in SLCH for the first episode of RSV bronchiolitis.

a) *Documentation of RSV infection*: Confirmed RSV infection by positive nasal swab results (PCR assay and/or direct antigen detection) from the SLCH virology lab, or from an other CLIA approved laboratory (if the patient is being transferred to SLCH from other institution during this illness). If no viral testing was performed prior to admission, this may be done by the APW-RSV II research team.

b) *Bronchiolitis definition*: At least two of the following symptoms/signs of bronchiolitis: respiratory rate greater than 40 breaths/minute; cough; wheezing; audible rales, crackles, and/or rhonchi; paradoxical chest movements (retractions)[28].

3. Duration of significant* respiratory symptoms from onset of symptoms of the current illness to admission is 120 hours (5 days) or less. Time of admission will be defined by the time the child was seen in the ED for the visit that led to hospitalization.
4. Randomization can be performed within 168 hours (7 days) from onset of significant* respiratory symptoms: I,e, a child who admitted after 5 days of symptoms, has to be randomized within the first 2 days of hospitalization.
5. Willingness to provide informed consent by the child's parent or guardian.

*Significant respiratory symptoms: lower respiratory tract symptoms, such as: wheezing, significant cough, retractions.

C. EXCLUSION CRITERIA

1. Prematurity (gestational age < 36 weeks).
2. Presence or history of other significant disease (CNS, lung, cardiac, renal, GI, hepatic disease, hematologic, endocrine or immune disease). Children with atopic dermatitis and/or food allergy will not be excluded from the study.
3. Clinically significant gastroesophageal reflux currently treated with a daily anti-reflux medication (anti- H2 or PPI).
4. The child has significant developmental delay/failure to thrive, defined as weight < 3% for age and gender.
5. History of previous (before the current episode) wheeze or previous (before the current episode) treatment with albuterol.
6. History of previous treatment with corticosteroid (systemic or inhaled) for respiratory issues. This criterion was included to identify children who may wheezed in the past.
   a. Prior treatment with systemic corticosteroids for non-respiratory issues (e.g., eczema, food allergy) will not exclude the child from participation in this study.
   b. Although corticosteroids are not recommended by the American Academy of Pediatrics as a therapy for RSV bronchiolitis, it might be that some RSV patients will be treated with corticosteroids. Such a therapy during the current acute RSV bronchiolitis (but not before this illness) will not exclude the child from participation in this study.
7. Treatment (past of present) with montelukast.
8. Treatment with any macrolide antibiotic (azithromycin, clarithromycin or erythromycin) over the past 4 weeks or current treatment with any macrolide antibiotic. Current or prior treatment with

non-macrolide antibiotic is not an exclusion criterion (unless this antibiotic is included in the list of medications that may cause QT interval prolongation- see #16 below). The use of topical antibiotics, including topical macrolides (e.g., ear/eye drops and eye ointments) is NOT an exclusion criteria.

9. Chronic treatment with any daily medication other than vitamins or nutritional supplements. Although routine vitamin D supplement (400 IU per day) is not an exclusion criterion, high dose vitamin D supplements are not allowed. Other allowed medications include: topical medications (e.g., topical corticosteroids), allergy (anti-h1) medications, ear/eye drops.
10. Participation in another clinical trial.
11. Participant currently requires invasive mechanical ventilation (intubation) or non-invasive mechanical ventilation (CPAP, BiPAP) due to RSV bronchiolitis. Participant who required invasive ventilation, and now weaned from the ventilator, may be enrolled if he/she meets all other inclusion criteria. Also, participant who required CPAP/BiPAP therapy may be enrolled if he/she no longer needs CPAP/BiPAP therapy, and if meets all other inclusion criteria. A need for O_2 or high flow nasal cannula is NOT an exclusion criterion.
12. Evidence that the family may be unreliable or non-adherent, or has definitive plans to move from the clinical center area before trial completion.
13. Contraindication of use of azithromycin or any other macrolide antibiotics such as history of allergic reaction (or other adverse reaction) to these antibiotics.
14. Diagnosis of asthma.
15. The child's sibling is a participant in the APW-RSV II study (we will allow enrolment of participants but not at the same RSV season).

Pre AND Post randomization exclusion criteria:

16. During study intervention (the first 14 days of the study) subjects shall not receive other types of antibiotics, proton pump inhibitor, or any other drugs which may cause QT interval prolongation. Medications that are not known to cause QT interval prolongation are allowed. Please see **Appendix 1** for a comprehensive list of medications that may cause QT interval prolongation.

    Medications included in **Appendix 1** that may be commonly used in young children are:

    a. Pantoprazole (Protonix ®): a proton pump inhibitor.
    b. Famotidine (Pepcid ®): anti-H2 medication.
    c. Macrolide antibiotics (azithromycin, clarithromycin, and erythromycin); quinolone antibiotics (ciprofloxacin, levofloxacin); metronidazole (Flagyl).
    d. Fluconazole (Diflucan ®): anti-fungal.
    e. Hydroxyzine (Atarax ®): anti-H1 medication.
    f. Diphenhydramine (Benadryl®)
    g. Ondansetron (Zofran®): anti-emetic.
    h. Dexmedetomidine (Precedex®): sedative used in the ICU setting.

- If the child is using one of the medications included in **Appendix 1** at the time of enrollment, he/she will not be enrolled in the study.
- If the child is prescribed with **macrolides,** after enrollment but during the active treatment phase of the study (first 14 days), the study medication use will be discontinued and the child's participation in the study will be terminated.

- If the child is prescribed with one of the medications included in **Appendix 1, with the exception of macrolides,** after enrollment but during the active treatment phase of the study (first 14 days), the study medication use will be discontinued and the child will continue to be followed per study protocol.
- If the child is prescribed with one of the medications included in **Appendix 1** after the active treatment phase of the study (after the first 14 days), no action is required.

D. STUDY TREATMENTS, AND OTHER TREATMENTS

Study treatments:

Eligible participants will be randomized to azithromycin or placebo groups. The active treatment group will receive azithromycin orally 10 mg/kg once daily for 7 days followed by 5mg/kg once daily for additional 7 days. Both the azithromycin and the matched placebo will be supplied by the investigational pharmacy in the SLCH. As azithromycin suspension is stable for up to 10 days, the family will be initially provided with the study medication for the first week of treatment; Suspension for the second week of therapy: The study medication powder will be provided to the parents in the study medication bottle. Before initiation of the second week therapy, the parents will add sterile water to the study medication bottle based on our instruction (premeasured bottle of water will be provided to the family).

Other treatments:

General: the child's clinical care will be directed by the participant's treating physician. The investigators will not prescribe any other treatments other than study medications (azithromycin or placebo). These treatments will be recorded and will be included in our analyses (see Analysis Plan Section).

Bronchiolitis clinical care: during the hospitalization, infants will be treated according to a predefined care-path, the SLCH Bronchiolitis Pathway, which is based on the American Academy of Pediatrics guidelines for treatment of bronchiolitis[93]. The SLCH Bronchiolitis Pathway emphasizes supportive care, and strongly discourages use of systemic corticosteroids and antibiotics. All decisions regarding medical treatment during the hospitalization, other than those related to study participation will be made by the child's primary attending physician.

Other treatments (including antibiotics, inhaled corticosteroids, systemic corticosteroids, montelukast and albuterol) may be prescribed by the SLCH physician and/or the child's primary care physician by their clinical judgment, and will be recorded by the study staff in the child's research file. These will be acknowledged in the analysis plan.

Treatment with open-label macrolide antibiotics will not be allowed during the active treatment phase of this study for safety concerns. If macrolide treatment will become mandatory during the active treatment phase of this study, then participation in the study will be terminated for the child and open label macrolide could be prescribed.

Therapy for future wheezing episodes: any therapy for such episodes, such as albuterol, oral/inhaled steroids, montelukast, or antibiotics will be prescribed by the primary care physician per this/her clinical judgment.

E. VISIT SPECIFIC PROCEDURES

1. OVERVIEW OF STUDY VISITS:

Overall, there are 5 types of scheduled study visits or contacts as follows:

1. Enrollment and Randomization visit (RZ) during the acute hospitalization.
2. Daily follow up during the acute hospitalization (HSP1, HSP2 etc…).
3. At least 2 phone calls during the 2 weeks of active treatment phase of the study (MED 1, MED2). These phone calls will be conducted at least once a week during the 2 weeks in which participants will be treated with the study medications. The goals of these calls are: to monitor mediation adherence and potential medications related adverse reactions, to answer families questions regarding study medications, and to schedule the first clinic visit (V1)
4. Clinic visit at the end of study treatment: 14 days following randomization (V1).
5. Follow up telephone calls to obtain clinical outcome (P1-P24): the first phone call will be conducted 6 weeks after the first clinic visit ((V1), 8 weeks from randomization), and then every 2 month. The maximal number of phone calls that a participant may have is 24 calls (if a call has to be done in-lieu of a yearly clinic visit- see next section (page 23, #6) for clarification). This maximal number of calls could be conducted only for participants who is recruited early during the first RSV season (2016-2017) and is followed for the maximal duration of follow-up (48 months).
6. Yearly clinic visits (V2-V5): the first yearly visit will be conducted 6 months after randomization, and then every 12 months. Participants recruited early during the first RSV season (2016-2017) may have up to 4 yearly visits (V2-V5). Participants recruited late during the last RSV season (2018-2019) may have only one yearly visit (V2)- see **Figure 7, and Table 1** for study time-line details.
7. Extended Observation Period by follow up telephone calls. During the extended observation period, we will ask the same questions that have been asked at each follow up telephone call throughout the observational period that started immediately after the initial treatment period. These additional follow up telephone calls will occur approximately every four months starting from the participants final phone call from their initial enrollment observation period. If a participant was enrolled into the study during year one recruitment, we will follow that participant up to four years. If the participant was enrolled during year two recruitment we will follow that participant up to three years and if the participant was enrolled during the final year of recruitment we will follow that participant up to two years. These phone calls will occur until the child reaches the age of seven.

2. SPECIFICS OF STUDY PROCEDURES:

Visit	RZ	V1	PC1	PC2	V2 (PC3)	PC4	PC5	PC6	PC7	PC8	V3 (PC9)	PC10	PC11	PC12	PC13	PC14	V4 (PC15)	PC16	PC17	PC18	PC19	PC20	V5 (PC21)	PC22	PC23	PC24
Month	0	0.5	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
History	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Physical exam [a]	√	√			√						√						√						√			
Safety / adverse events	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Adherence monitoring	√	√																								
Nasal wash:	√	√			√						√[b]						√[c]									
• Microbiome	√	√			√																					
• Inflammatory markers	√	√																								
• Viral	√																									
• Antibiotics resistance [b]	√	√			√																					
• Bio-banking	√	√			√						√[b]						√[c]									
Nasal brush	√	√			√																					
Blood:	√	√									√[b]						√[c]									
• CBC & differential	√																									
• Eosinophil count											√[b]						√[c]									
• Serum IgE		√									√[b]						√[c]									
• Serum Specific- IgE											√[b]						√[c]									
• Genetics bio-banking		√																								
• Serum bio-banking	√	√									√[b]						√[c]									
• Serum Covid-19											√[b]						√[c]						√			
Stool collection	√	√			√						√[b]						√[c]									
Urine collection	√	√																								

Table 1. Study visit schedule, and sample collection.

The table presents the maximal number of visits and calls for a participant enrolled early during the first RSV season (2016-2017) who will have up to 48 months of follow-up. As a result of the variable duration of follow-up not all participants will have the V4-V5 visits, or phone calls (see also **Figure 7**: study timeline).

RZ: Enrollment and randomization visit, V: clinic visit, PC: Phone-call.

See Section O (SPECIAL STUDY PROCEDURES AND TECHNIQUES) for the specifics of studies that will be performed on the biological samples.

[a] Complete physical exam during the randomization visit, then brief physical exams during the following clinic visits. [b] Nasal wash, blood, and stool will be obtained at V3 visit only for the year three recruits as they won't be in the study long enough for a V4 and V5 visits. [c] Nasal wash, blood, and stool will be obtained at V4 visit for the year one and year two recruits. If for some reason the year one recruits are not able to provide the designated samples at V4 visit, we may obtain these samples at their V5 visit with parent permission. The samples that are listed to be obtained at V4 will be obtained at V5 ONLY if they were not obtained at V4 (applicable to participants who will be recruited at the 1st year of the study). In any case, each participant will contribute only one set of samples at the V3-V5 visits.

DNA sample will be obtained from blood sample, unless the parent doesn't agree to blood draw: in this case DNA sample will be obtained by a buccal swab.

If blood is to be obtained at enrollment or a scheduled study visit, but parent opts out of blood draw at that time, this blood may be drawn at a future visit with parent permission.

1. Enrollment and Randomization visit (RZ) during the acute hospitalization:

a. Eligibility determined based upon inclusion and exclusion criteria.

b. Informed consent obtained.

c. Release of medical data obtained.

d. Provide an information brochure about the study (will include study ID, contact phone number of the investigators, instruction to avoid any macrolide antibiotic during the 2 weeks of study therapies). A copy of this brochure will be send to the primary care physician.

e. Detailed family history and environmental allergy questionnaires obtained.

f. Medical history obtained.

g. Study physical examination including height and weight performed. This physical exam is performed to assure the child can participate in the study. **This exam has different goals than the physical exam performed by the child's attending physician as a standard of care, and cannot replace the attending's exam.**

h. Document the administration over the past 24 hrs of: supplemental oxygen, IV fluids, albuterol or other bronchodilators, antibiotics.

i. Calculate bronchiolitis severity score[40] .

j. Provide education about study medications, and discuss the need to avoid any macrolide antibiotic during the active phase of the study.

k. Provide and teach Study Medication Diary (This paper diary monitor for study medications adherence and potential side effects) completion.

l. Obtain baseline blood, stool, nasal wash and nasal brush samples, and urine (if able to obtain while child is hospitalized).

m. Dispense first batch of study medications and dosing syringes. Teach study medication administration.

n. Coordinate study medication delivery with the nursing staff of the inpatient unit.

o. Schedule the clinic follow up visit (V1).

p. Obtain contact information (including e-mail if available) and preferred time for follow up telephone calls (PCs).

q. Provide/arrange for parent's compensation.

r. If a Respiratory Pathogen Multiplex PCR testing has been performed in SLCH, document the presence of other respiratory viruses (if not done, this will be performed at the end of the RSV season on a nasal wash sample).

2. Daily follow up during the acute hospitalization (HSP1, HSP2 etc…):

a. Review Study Medication Diary card.

b. Review study medication treatment and adherence.

c. Remind date of clinic follow up visit (V1) and the need to bring study medication bottle to the visit. Dispense materials for collection of stool sample before the V1 visit.

d. At the end of hospitalization document the following: supplemental oxygen (days), IV fluids (days), albuterol or other bronchodilators, hypertonic saline inhalations, antibiotics, oral steroids, inhaled steroids, montelukast, length of hospitalization (days).

e. Schedule the date for the MED1 phone call and the date/time for the delivery of the second bottle of study medications (for the 2nd week 2 of the study treatment).

3. A weekly phone call during the 2 weeks of active treatment phase of the study (MED 1, MED2):

a. Review Study Medication Diary card (monitor medications adherence and potential adverse reactions).

b. Confirm date/time for delivery of second bottle of study medication (during week one only).

c. Schedule the date for the MED2 phone call (during week one only).

d. Remind date of follow up visit (V1) and the need to bring study medication bottle to the visit. Remind the procedures for collection of stool sample before V1 visit.

e. Answer families questions regarding study medications and study visits.

4. Clinic visit: V1: 14 days after randomization (day 15 with a window of ± 3 days):

If the family absolutely cannot attend the clinic visit, a phone call will be conducted in lieu of the clinic visit to collect clinical outcomes.

a. Brief history (including drug adverse reactions) and partial physical exam aiming to exclude (or document) the presence of wheezing on lung exam.

b. Review and collect Study Medication Diary card.

c. Document adherence to study medications by the Study Medication Diary. Weigh study medication bottles.

d. Obtain blood, nasal wash and brush samples, and urine (if able to obtain while child is at V1).

e. Collect stool sample that was obtained before the visit. If a stool sample could not be collected before/during the visit, it will be shipped to the investigators by a courier.

f. Review any unanticipated change in medical status-if needed, document this change in the AE or SAE form and report accordingly.

g. Review contact information and schedule PC1

h. Provide parent's compensation.

5. Follow up telephone calls (PC): 2 months after enrolment, and then every 2 months (these phone calls have a window of ± 3 weeks):

a. Monitor for the following since the last visit/ telephone call*:

1. Symptoms of wheezing (with and without a cold), use of rescue albuterol, use of oral corticosteroids, ED /unscheduled office visits, hospitalizations for respiratory symptoms, parental absence from work due to child's respiratory illnesses.
2. Physician diagnosis of asthma
3. Use of antibiotics, inhaled steroids, montelukast.
4. Change in pet exposure, day care status, and cigarette smoke exposure.
5. Any unanticipated change in medical status-if needed, document this change in the AE or SAE form and report accordingly.
6. Review contact information and obtain as many options for additional contact options.
7. Schedule the next PC.
8. Mail parent's compensation.

*For PC1 only: time frame for measurements of clinical outcomes is starting at the end of study treatments (2 weeks from randomization=V1).

6. Yearly clinic visits (V2-V5): The first yearly visit will be conducted 6 months after randomization, and then every 12 months (these yearly visits have a time window of ± 1 month):

Participants recruited early during the first RSV season (2016-2017) may have up to 4 yearly visits (V2-V5). Participants recruited late during the last RSV season (2018-2019) may have only one yearly visits (V2). If any of these visits would be conducted within the time frame of any of the PC phone calls, then the information from this visit will be recorded in lieu of the phone call data. However, if the family absolutely cannot attend the yearly clinic visit (e.g., left the St. Louis metro area), a phone call will be conducted in lieu of the clinic visits to collect clinical outcomes.

a. Partial physical exam (including weight and height).
b. Monitor for the following since the last telephone call:
    1. Symptoms of wheezing (with and without cold), use of rescue albuterol, use of oral corticosteroids, ED /unscheduled office visits, hospitalizations for respiratory symptoms, parental absence from work due to child's respiratory illnesses.
    2. Physician diagnosis of asthma
    3. Use of antibiotics, inhaled steroids, montelukast.
    4. Change in pets exposure, day care status, cigarette smoke exposure.
    5. Any unanticipated change in medical status-if needed, document this change in the AE or SAE form and report accordingly.
c. Obtain nasal wash sample (V2 and V4 or last visit (V3) for year 3 recruits).
    1. If the COVID-19 virus is still circulating in the St. Louis region at the time of the child's last nasal wash collection (V4 or last visit (V3) for year 3 recruits), we may not obtain this nasal wash sample to prevent undue risk of contamination to the child of contracting COVID-19.

d. Obtain nasal brush sample (V2 only).

e. Collect stool sample that was obtained at home (V2 and V4 or last visit (V3) for year 3 recruits).

f. Obtain blood for (V4, or last study visit (V3); for participants enrolled in year 3 who will not have V4):

1. Specific IgEs (inhalant allergens- see #7 at the list of secondary outcomes below): Specific IgEs will be performed at V4 or at the last study visit (V3) for year 3 recruits. Serum from the other yearly visits will be saved and may be used for allergy testing at a later time.

2. Eosinophil Count, Total IgE: V4 or last visit (V3) for year 3 recruits.

3. We may obtain COVID-19 antibody testing with parent permission: V5, V4 or last visit (V3) for year 3 recruits.

g. Review contact information and obtain as many options for additional contact options.

h. Schedule the next PC.

i. Provide parent's compensation.

7. Extended Observation Follow up telephone calls (PC): Extended observation calls will start following the participants final phone call from their initial enrollment observation period and will occur every 4 months (these phone calls have a window of ± 6 weeks):

a. Monitor for the following since the last visit/ telephone call*:

1. Symptoms of wheezing (with and without a cold), use of rescue albuterol, use of oral corticosteroids, ED/unscheduled office visits, hospitalizations for respiratory symptoms, parental absence from work due to child's respiratory illnesses.

2. Physician diagnosis of asthma

3. Use of antibiotics, inhaled steroids, montelukast.

4. Change in pet exposure, day care status, and cigarette smoke exposure.

5. Any unanticipated change in medical status-if needed, document this change in the SAE form and report accordingly.

6. Review contact information and obtain as many options for additional contact options.

7. Schedule the next PC.

8. Mail parent's compensation.

F. OUTCOME VARIABLES

1. PRIMARY OUTCOME MEASURES

a. Clinical trial outcome: The occurrence of a third episode of post-RSV wheezing measured over a follow up duration of 18-48 months, quantified by a Cox proportional hazards regression model, and compared between the groups.

Primary outcome assessment: Episode of wheezing will be detected by the question "Has your child's chest sounded wheezy or whistling (with and without a cold)? This question was adopted from the International Study of Asthma and Allergies in Childhood (ISAAC) study[94], and was previously utilized in our pilot APW-RSV study[40] and in the RBEL study[17]. Data collection will be conducted in a standardized way, during study visits and phone calls, utilizing Data Collection Forms that have been modified from our APW-RSV study[40] and in the RBEL study[17].

Definition of a new distinct wheezing event vs. continuation of previous wheezing symptoms: In order to define a new wheezing episode a child should not wheeze for at least 7 days before the new event. If the time frame from the previous wheezing episode (last time that the child wheezed) to the onset of wheezing symptoms is shorter than 7 days, these wheezing symptoms would be attributed to the previous wheezing episode and will not be counted as a new event. This approach was utilized in our previous pilot APW-RSV study[40].

Time frame for primary outcome assessment: For the purpose of this study, wheezing detected during the initial RSV bronchiolitis will not be included in the count of post-RSV wheezing. The time frame for measurement of post-RSV wheezing (and other long-term outcomes) starts at the end of the treatment period (2 weeks after randomization) and ends at end of the follow-up period. Therefore, a wheezing episode that occurs at the first 2 weeks of the study, does not "count" toward the outcome of RW.

b. Microbiome study outcome: Upper airway microbiome community structure measured at the end of study treatments, and its relationship with the occurrence of post-RSV RW (≥ 3 episodes).

See the analysis plan for details on the analytic approach of the microbiome studies.

2. **SECONDARY OUTCOME VARIABLES MEASURED DURING THE FOLLOW-UP PERIOD (18-48 MONTHS):**

a. Time to physician asthma diagnosis.
b. Time to asthma diagnosis OR to the third episode of wheezing.
c. Annualized number of days with: any respiratory symptoms (wheezing, cough, or shortness of breath), or albuterol use.
d. Rate of oral corticosteroid courses use.
e. Rate of antibiotic courses use.
f. Rates of drug related side effects and severe adverse reactions.

Secondary clinical outcomes (including the outcomes listed in #3 below) will be assessed by clinic visits and phone call interviews as described for the primary outcome. These will be measured as we previously described[17, 40].

The monitoring of potential drug related side effects will start immediately after randomization. The time frame for measurement of all other long-term outcomes starts at the end of the treatment period (2 weeks from randomization) and ends at end of the follow-up period.

3. **OTHER EXPLORATORY OUTCOME VARIABLES MEASURED DURING THE FOLLOW-UP PERIOD (18-48 MONTHS):**

a. Time to 4th wheezing episode.
b. Annualized number of days with wheezing.
c. Annualized number of days with parental absence from work due to child's respiratory symptoms.
d. Annualized number of days with child absence from day care.
e. Annualized number of days with nights with awakening due to respiratory symptoms.
f. Rates of ED and urgent care visits.
g. Rates of hospitalizations for respiratory symptoms.
h. Rates of upper respiratory infections.
i. Proportion of children prescribed asthma controllers medications (ICS, LTRA).
j. Cumulative number of wheezing episodes.
k. Proportion of children with at least one positive specific IgE (SIgE) to inhalant allergen at V3 and at the end of study visit (see Section O for details on the specifics allergy testing).
l. Total IgE level and eosinophil count measured at the last study visit.
m. Determine if demographic (sex, age, race), baseline asthma/allergy phenotypic characteristics (Personal/family atopic history, second hand smoking, pet exposure), RSV serotype (A vs. B), peripheral eosinophil count, and the presence of co-infection with other respiratory viruses during the RSV bronchiolitis (e.g. rhinovirus) and/or bacteria are predictors of response to azithromycin therapy.
n. Changes in upper airway microbiome community structure following study treatments.
o. Upper airway microbiome community structure measured 6 months following randomization.
p. Based on our preliminary data we will specifically evaluate if azithromycin therapy results in reduction of Moraxella abundance and whether lower Moraxella abundance is associated with reduced odds to develop RW.
q. Compositions of the viral and/or the fungal components of the upper airway microbiome, and the composition of the stool microbiome (these will be explored based on availability of additional funding).

Secondary clinical outcomes will be assessed by clinic visits and phone call interviews as described for the primary outcome. These will be measured as we previously described[17, 40].

4. **LABORATORY OUTCOMES MEASURED AT ENROLLMENT AND AT THE END OF THE 2-WEEK STUDY TREATMENT PERIOD:**

a. Nasal lavage levels of IL-8.
b. Nasal lavage levels of neutrophil elastase.
c. Nasal lavage levels of matrix metallopeptidase 9 (MMP-9).
d. Rates of upper airway colonization with macrolide-resistant bacteria (additional sampling at the 6 month clinic visit). See Section O for the specifics of macrolide-resistance studies.

G. RANDOMIZATION

Patients who satisfy all the eligibility criteria at RZ encounter will be randomized to azithromycin or placebo groups based on a blocked randomization allocation sequence. Randomization list will be supplied to the investigational pharmacy at SLCH by the study statistician. Randomization will be stratified by prior use of open-label non-macrolide antibiotic over the past 2 weeks prior to randomization. The investigators will be blinded to treatment assignment and to the size of randomization blocks. Hence, size of randomization blocks will be randomly selected by the randomization software. Once all data collection has been completed study medications will be dispensed.

H. NON STUDY DRUGS

Chronic daily treatment with any medication other than routine vitamins or nutritional supplements, and other than current treatments for the acute RSV bronchiolitis will exclude the child from participation in the study.

Treatment with macrolide antibiotic (erythromycin, clarithromycin or azithromycin) will not be allowed during the active treatment phase of the study due to safety concerns of potential overdosing with macrolides; if this treatment will become mandatory, participation in the study will be terminated.

Other treatments (including antibiotics, inhaled corticosteroids, systemic corticosteroids, montelukast and albuterol) may be prescribed by the SLCH physician and/or the child's primary care physician by their clinical judgment, and will be recorded in the child's research file.

I. RECRUITMENT

We will recruit up to 200 children over 3 consecutive RSV seasons. We have a sufficient pool of hospitalized RSV patients available for this study as approximately 400+ children, aged 1 month (30 days) -18 months, are admitted to SLCH each RSV season. Moreover, over 7 RSV seasons our group has recruited similar number of patients (~ 65 patient/season) in our institution for 2 observational RSV cohorts (RBEL I, II). The SLCH census list will be reviewed twice daily during the RSV season for all potential admissions with RSV bronchiolitis. Furthermore, all children with positive RSV results from the virology lab at SLCH will be identified twice daily to assess for potential eligibility. Based upon these initial screens, all hospitalized 1 month (30 days) -18 month old infants will be further screened by a study coordinator to determine eligibility. Recruitment and retention will be monitored yearly at the end of each RSV season to assure adherence to the accrual milestone as listed below.

	M1 May 1, 2017	M2 May 1, 2018	M3 May 1, 2019
Expected enrollment (for season)	57	66	65
Cumulative enrollment expected	57	123	200
Minimum enrollment	47[1]	118[2]	NA
Minimum retention of subjects enrolled in prior seasons	NA	80%[3]	80%[3]

Table 2. APW-RSV II: accrual milestones.

[1]If fewer than 47 subjects are enrolled in the first RSV season, another recruitment site will be identified.

[2]If fewer than 118 subjects are enrolled, a corrective action plan will be required.

[3]For retention, 80% of cumulative actual enrollment in prior year is required from all prior seasons, based on power calculation assumptions. Subjects who miss more than 3 consecutive visits (6, 18, 30 and 42 months) or calls or both (i.e.; a visit and 2 calls scheduled before or after the visit) will not be considered to be "retained" for the purposes of this evaluation.

In a case that a call(s) is missed, the occurrence of wheezing episodes from the last patient contact will be monitored at the subsequent call. If retention will be lower than 80%, recruitment goals will be adjusted accordingly.

The M2 and M3 reports will also include data on the number (%) of patients who develop RW (a combined report and not by treatment group).

In addition to the yearly monitoring of recruitment and retention, a monthly screening and recruitment report would be provided to the NHLBI's Program Officer during the RSV season(s). This monthly report would include the following data:

Number of RSV patients screened (Positive RSV tests in the SLCH virology lab)	
Number of hospitalized RSV + patients aged 1-18 months	
Number of eligible subjects[1]	
Number of subjects asked to participate	
Number of patients agreed to participate[1]	
Number of subjects randomized	

Table 3. A monthly screening and recruitment report (during the RSV season)

[1] Reason(s) for ineligibility and for declining to participate (Based on parents willingness to provide these data) will be reported.

J. DRUG SUPPLIES

Generic azithromycin will be purchased and will be dispensed by the investigational pharmacy in the SLCH. We have obtained an FDA's Investigational New Drug (IND) approval for using azithromycin as detailed in this protocol (IND: 112359 (Exempt status)).

The investigational pharmacy in the SLCH will also manufacture and dispense the matching placebo. The following formulation was approved by the investigational pharmacy in the St. Louis Children's Hospital after tasting, texture and consistency testing.

The investigational pharmacist will produce the placebo powder based on the following steps (for 30 mL of placebo):

1. Mix 31.5 grams of SyrSpend SF Alka powder with 70 mg FD&C Red #3.
2. Combine 2 grams of SyrSpend SF Alka powder with 30 mg of powder obtained from Step #1.

Suspension for the first week of therapy: the suspension will be produced (reconstituted with water) by the SLCH investigational pharmacy.

Suspension for the second week of therapy: The study medication powder will be provided to the parents in the study medication bottle. Before initiation of the second week therapy, the parents will add sterile water to the study medication bottle based on our instruction (premeasured bottle of water will be provided to the family). The study coordinator will call the parents to assure successful completion of this process.

Since azithromycin suspension, once reconstituted, is only stable for 10 days, we will supply parents with study medication for the first and second week of treatment.

All non-study medications (including albuterol) will be prescribed by the hospital staff and primary care physicians per their clinical discretion and will be recorded as secondary outcomes by the investigators. The patients will be responsible for obtaining all these non-study medications.

K. ADHERENCE

As much as possible, use of study medications will be monitored on designated diary to enhance patient adherence. Volumes of remaining study medications will be measured at clinic visit (V1). Adherence assessment of the azithromycin vs. placebo will be based upon volume remaining and medication diary entries.

L. EDUCATION

Management of respiratory symptoms during or after the acute hospitalization will be directed by the SLCH attending and then by the primary care physician. Therefore, they will provide the education regarding management of wheezing and asthma. The investigators will monitor the clinical management and will be available to answer any questions of the families.

M. RETENTION

We will utilize retention approaches that were used in our RBEL cohorts over the past 14 years and in our APW-RSV pilot trial. Retention efforts will focus on ease of visits and phone calls. Visits will be at times convenient to the parents, many of whom work (thus, hours after day care and preschool will be available). We will make every effort to minimize parking problems and other general inconveniences. Phone calls will be scheduled at times convenient to the parents. A monetary compensation will be given for each visit and upon completion of every phone call. A monetary compensation will be given if a blood draw is completed at a LabCorp facility. Study staff will be available to answer questions about that parents might have throughout the study. In addition, we will keep the families engaged with the study by sending them yearly birthday and holiday cards, and an annual study newsletter. At enrollment, we will provide the parent with a study tote bag to carry study supplies in when they go home from the hospital. We may offer additional small appreciation gifts at each of the clinic visits for the child and possibly snacks when the children are older. We may also provide a parent/legal guardian with a gift card or voucher to the SLCH cafeteria or gift shop if the child remains hospitalized for an extended period of time after enrollment into the study. In addition, we may send out by mail a small appreciation mother's day and/or father's day gift yearly to the parent(s) and/or the study participant. Finally, a yearly retention event may be implemented to maximize retention and to resume contact with patients that were lost to follow-up. This will be a social event that will be conducted in the St. Louis Children's Museum, or similar location that accommodates young toddlers.

N. MONITORING FOR ADVERSE EFFECTS OF THE TREATMENT

Overall, and as discussed in the "RISKS/PROTECTION AGAINST RISKS" Sections, azithromycin is well tolerated in children and has a very good safety profile, although some side effects have been reported. To minimize potential for adverse events we will not enroll children with any chronic disease, or children who take any other daily medications (other than vitamins, nutritional supplements, and medications prescribed during the acute bronchiolitis). The parents will be informed on potential side effects (see RISKS/PROTECTION AGAINST RISKS Sections) and will be requested to report to the investigators in a case of adverse event. A detailed description of monitoring of potential Adverse Events is available in the DATA AND SAFETY MONITORING PLAN section.

O. SPECIAL STUDY PROCEDURES, TECHNIQUES, AND OUTCOME ASSESSMENT

Detection of main clinical outcome (i.e., episode of wheezing): Episode of wheezing will be detected by the question "Has your child's chest sounded wheezy or whistling (with and without a cold)? This question was adopted from the International Study of Asthma and Allergies in Childhood (ISAAC) study [94], and was previously utilized in our pilot APW-RSV study[40] and in the RBEL study[17]. Data collection will be conducted in a standardized way utilizing Data Collection Forms that we have modified from our APW-RSV study[40] and in the RBEL study[17]. The time frame for detection of post-RSV wheezing start at the end of study treatments. Therefore, wheezing episodes that occurs at the first 2 weeks of the study, does not "count" toward the outcome of RW.

Study physical exams:

- Full physical examination: An examination of the status of the following systems/regions: Head, Eyes, Nose and Throat, Cardiovascular, Lungs, Abdominal, Musculoskeletal/Extremities, Neurologic, Skin. Documentation of height and weight. This exam will be conducted at enrolment by a licensed medical practitioner. **This study exam cannot replace the child's attending physician exam performed as a standard of care, since it is study directed, and has different goals than the physical exam performed by child's attending.**
- Brief physical examination: An examination of the status of the following systems/regions: Hair/skin, Eyes, Nose and Throat, Lungs, and as indicated by history. Documentation of height and weight. This exam will be conducted by the clinical coordinator.

Nasal lavage samples: nasal lavage samples will be obtained at each of the study visits using the technique we utilized in our pilot APW-RSV study[40] and in the RBEL study[17]. These samples will be utilized to measure:

- Inflammatory markers (IL-8, matrix metallopeptidase 9 and neutrophil elastase): in samples obtained at RZ and V1.
- Upper airway microbiomes including antibiotic resistance studies: in samples obtained at RZ, V1, and V2.
- Viral multiplex PCR panel: in samples obtained at RZ.
- Remnants of these samples, and sample obtained at the last study visit, will be stored at -70 degrees for future potential studies.

Blood samples: blood samples will be obtained at each of the study visits. If blood is to be obtained at enrollment or a scheduled study visit, but parent opts out of blood draw at that time, this blood may be drawn at a future visit with parent permission. Blood samples will be utilized to measure:

- Eosinophil levels (CBC will be performed at RZ, eosinophil count only will be performed at V4 or last study visit (V3) for year 3 recruits): at RZ, V4 or last study visit (V3) for year 3 recruits.
- Total IgE level: at V1, V4 or last study visit (V3) for year 3 recruits.
- Sensitization to aeroallergens will be measured in samples obtained at V4 , or the last study visit (V3) for year 3 recruits. We will measure the concentrations of SIgE to the following allergens: cat, dog, mite (f,p), mouse, and cockroach.
- Covid-19 antibody testing may be performed, with parent permission, at V5, V4, or last study visit (V3) for year 3 recruits.
- Serum and nucleic acid samples will be obtained and stored at -70 degrees for future genetic, bacterial, viral ancillary and other potential studies.

Blood samples for eosinophil count, Total IgE, and Serum Specific IgE to determine sensitization to aeroallergens that are collected at V4 or the last study visit (V3) for year 3 recruits will be processed thru LabCorp. The blood sample will be labeled with the participants study identification number, initials, and participants year of birth

only (01/01 will be entered as month/day for all study participants). LabCorp will provide the study team results thru their secure, online system Beacon. Beacon will allow our study team to get the results by logging into their secure system utilizing a username and password combination.

If a parent agrees to have their child (study participant) tested for the COVID-19 antibody testing this will also be completed through LabCorp. If the participant's blood test will become positive, we will report this to his/her pediatrician. We may also have to report this positive result to the Centers for Disease Control (CDC), and we may have to report a positive result to any state and local authorities based on state and local guidelines regarding COVID-19. The information provided to LabCorp will be de-identified for testing purposes. However, for any positive results the study team may need to provide the participant's demographics (to possibly include full name, gender, date of birth) and may need to collect contact information for any positive result reporting.

Additionally, if a participant is unable to complete an in person study visit for their V3 (year 3 enrollment), V4 (year 4 enrollment) or V5 (agree to COVID-19 testing and/or blood work that was not obtained at their V4) due to COVID-19 restrictions, or for any other reason such as distance, personal restrictions, etc. However, they would like to have the blood work for the study that they have agreed to completed at their local LabCorp facility that performs pediatric testing instead of at the PCRU located within St. Louis Children's Hospital they may complete this testing there. Their study coordinator will fill out the appropriate lab requisition(s), along with instructions related to obtaining the blood draw, and mail them directly to the participants home address. Any blood testing performed at LabCorp will be completed prior to April 30, 2021.

Stool samples: stool samples will be collected at RZ, V1, V2, V4, or last study visit (V3) for year 3 recruits, and will be saved for future microbiome studies.

Urine samples: urine sample may be collected at RZ and V1. This will be saved for future studies investigating biomarkers related to allergy and asthma development. The urine sample will be collected utilizing a pediatric urine collection bag placed in the child's diaper.

Nasal brush collection: nasal brush samples will be obtained at RZ, V1, V2 using the technique we utilized in our pilot APW-RSV study[40] and in the RBEL study[17]. These will be utilized:

- Epithelial cells collected by the brush will be stored at -70 degrees for future studies.

Macrolide antibiotic resistance studies: The method for macrolide antibiotic resistance studies has been adopted from our NHLBI's AsthmaNet study: **A**zithromycin for preventing the development of upper **R**espiratory tract **I**llness into **L**ower respiratory tract symptoms in children (APRIL)[75]. Briefly, nasal wash samples will be obtained at three time points: randomization, the end of study treatments, and 6 months later. Samples will be inoculated onto sheep's blood agar containing azithromycin, and evaluated at after 18-24 hours. The absence or presence of normal upper respiratory tract flora will be assessed, and pathogenic organisms will be isolated and identified. Additional susceptibility testing will performed on pathogenic bacteria using disk diffusion for azithromycin, erythromycin, clindamycin, clarithromycin. In addition, cefoxitin resistance will be assessed for *Staphylococcus aureus*.

Remnant of all biological samples will be saved for future analyses based on parent's consent to use these samples in future studies. Patients will be also offered to have their biological samples stored in the Asthma and Airway Translational Research Unit (AATRU) Biobank (PI: Dr. Mario Castro (an investigator in the APW-RSV II study), which

is a bio-repository of biological samples collected by our group from participants of Asthma/Allergy studies. Dr. Mario Castro's AATRU biobank is located at the University of Kansas Medical Center Research Institute. Dr. Beigelman is an investigator in the AATRU bio-bank protocol. Enrollment in the AATRU bio-bank is not mandatory for APW-RSV II participants, and would be performed based on separate independent consent of the AATRU protocol.

P. POTENTIAL RISKS

Potential risks include potential adverse effect of azithromycin, study burden from phone calls and clinic visits, pain and discomfort from blood testing, and mild discomfort from nasal wash and/or brush collection. Participants will be reimbursed for time and travel costs limiting financial burden

It is expected that some of the children participating in this study will experience worsening of their RSV bronchiolitis, and will require intensive care management. In addition, it is expected that most study participants will develop wheezing episodes and asthma and some may require hospitalization. These are expected outcomes during/following RSV bronchiolitis and likely to occur even if the child will not participate in the study. See the "Protection Against Risks" Section for description of the management of these events

***P.1. Potential risks associated from study medication (Azithromycin or placebo) treatment*:**

Azithromycin is one of the most commonly prescribed antibiotics in the US. In 2010, 51.5 million outpatient oral azithromycin prescriptions were prescribed; and azithromycin was the most frequently prescribed antibiotic agent during that year[95]. By 2004 oral azithromycin has been prescribed to more than 80 million children[96].

See the section "*Rare potential adverse effects of azithromycin in children* " for description of FDA's safety report that presents SAE data on azithromycin treatment in children, including 4.3 million children that were younger than 1 year.

Safety of azithromycin in children and common potential side effects:

a. *Safety of prolonged azithromycin therapy among extremely premature infants*[51, 52]:

Two double-blinded placebo-controlled trials investigated the utility of azithromycin vs. placebo for the prevention of Bronchopulmonary Dysplasia (BPD) in 2 cohorts of 263 critically ill, mechanically ventilated, extremely premature infants. These infants were given azithromycin (10 mg/kg/day daily for one week, following by a dose of 5 mg/kg/day for additional 5 weeks), or placebo for up to 6 weeks. No adverse events were seen disproportionately in azithromycin group (including no higher rate of infections, feeding problems, or arrhythmias). In our IND application to the FDA for our previous pilot APW-RSV study we indicated that we have chosen to use this treatment regimen (albeit for a shorter duration: i.e., 2 weeks instead of 6 weeks) based on its safety profile in these young and critically ill infants.

b. The rate of azithromycin related (or possibly related) side effects in our previous APW-RSV pilot trial (n=39)[40]

Gastrointestinal adverse events (diarrhea, vomiting, or abdominal pain) during the active treatment phase of the APW-RSV study were recorded in 7 children treated with azithromycin and 8 children treated with placebo, and

none were severe enough to warrant discontinuation of study medication[40]. We did not detect any non-gastrointestinal side effects of the study medication.

c. The rate of azithromycin related side effects vs. the rate of side effects of other common antibiotics in children

Safety data analysis based on 43 open label, randomized, comparative pediatric trials identified a total of 2655 children (6 months-16 years) that were treated with azithromycin (10 mg/kg once daily for 3 days) and a comparison group that included 1844 children treated with various other antibiotics[97]. Approximately 11% of the children included in this analysis were younger than 2 years. In the azithromycin group 8.7% of the children experienced adverse effects, compared with 9.8% in the comparison group[97].

A similar analysis that have utilized safety data from pediatric trials included 1213 children treated with the same azithromycin dosing regimen and 1212 children treated with other antibiotics[98]. The occurrence of adverse effects was 7.9% in the azithromycin group and 11.5% among children treated with other antibiotics (p=0.003). Significantly fewer gastrointestinal events were recorded for azithromycin than for the other antibiotics (6.5 vs. 9.9%, P=0.002), and their duration was significantly shorter (mean 2.3 vs. 5.0 days, P=0.0001)[98]. This analysis concluded that azithromycin pediatric oral suspension is well tolerated and associated with significantly fewer adverse events compared to other common pediatric antibiotics.

Additional analysis that have summarized the results of these 2 safety reports have shown that azithromycin adverse events were mild to moderate and very seldom necessitate withdrawal of the treatment (1-1.3%)[96]. The most common side effects are gastrointestinal side effects, which include abdominal pain (2%), vomiting (1.8%) and diarrhea (1.3). Skin rash can occur in 1-1.3% of children[96].

d. Potential induction of antibiotic resistance

As with any antibiotic, azithromycin therapy has the potential to induce antibiotic resistance. We will monitor this potential adverse effect in our study.

In summary, based on the above data, azithromycin therapy is well tolerated in children, and its overall safety profile is comparable to the safety profile of other antibiotics that are commonly used in children.

Rare potential adverse effects of azithromycin that have been reported in children:

a. Potential higher risk of infantile pyloric stenosis

Infantile hypertrophic pyloric stenosis occurs in approximately 2 – 3.5 per 1000 live births. Therapy with erythromycin, another macrolide antibiotic, in early life is a known risk factor for the development of infantile pyloric stenosis. Until recently, this risk was not reported with azithromycin use. A recent retrospective study that was conducted based on utilization of billing data, has reported that azithromycin use in the newborn period increases the risk of developing pyloric stenosis[99]**. The strongest association was among infants that were treated during the first 2 weeks of life**, and the association was much less profound, albeit still statistically significant, among children between 2 and 6 weeks of age. The association was not significant among infants older than 6 weeks[99]. **Infants less than one month of age are not eligible to enroll in this study.**

b. *Other potential adverse effects reported in children*

Serious allergic reactions (e.g., angioedema, anaphylaxis, Stevens Johnson Syndrome, toxic epidermal necrolysis) are very rare; however, fatalities have been reported. As with other anti-infective agents, use of azithromycin may result in overgrowth of non-susceptible bacteria or fungi, particularly Clostridium difficile in the colon. Clostridium difficile associated diarrhea (CDAD) may range in severity from mild diarrhea to fatal colitis. We will advise patients and parents about the possibility of bloody or moderate to severe watery diarrhea. Should this occur, the study medication will be stopped and the clinical center contacted. If CDAD is suspected or confirmed, azithromycin will be discontinued and the patient will be treated appropriately.

Other rare side effects include chest pain, increased stomach gas, or jaundice, yeast infections of the mouth or vagina, dizziness, headache, tiredness, sleeplessness, ringing in the ears or sleepiness. These have occurred mostly in adults taking azithromycin. In addition, Azithromycin may cause a temporary change in the number of blood cell levels: such as red blood cells (RBC), white blood cells (WBC), and/or platelets.

Severe adverse events (SAE) of azithromycin in children are very rare. An FDA report, which evaluated azithromycin safety, suggests that ~28.3 million pediatric patients (0 to 16 years old) received a prescription for azithromycin from October 2005 to September 2009; **~4.3 million of them were in the age of 0 to 1 year olds** [78]. Severe adverse events (AE) since market approval (June 2005) were very rare and reported as the following:

1. 4 unduplicated reports of serious AE cases, in children (0-16yr), were labeled to Zmax (azithromycin):

- No death was reported
- Labeled SAE cases (n=4)
    - Black stools and black nasal discharge in 1 year old exposed to clarithromycin then Zithromac® fine granules
    - Swelling, rash and welts in an 8 year old
    - Syncopal episode in an 8 year old (Zithromac® fine granules)
    - Elevated glucose (472) in a 15 year old with diabetes

2. 9 unduplicated reports of serious AE cases, in children (0-16yr), were labeled to azithromycin (all formulation):

- One event of death was reported: Choking in 15 month female
- Serious or life-threatening (n=9)
    - Anaphylaxis (n=1)
    - Stevens-Johnson syndrome (n=3)
        - Cardiac arrest (n=2) in patients receiving IV boluses (IV administration is contrary to labeled instructions)
    - Acute severe liver injury (n= 3)
        - 1 liver transplant
        - Unclear causality: congestive heart failure, hepatotoxic drugs, and acute viral hepatitis remain alternative etiologies

Given the fact that ~28.3 million pediatric patients (0 to 16 years old) received a prescription for azithromycin from October 2005 to September 2009, **this rate of serious adverse reactions in children is extremely low (0.46 events per one million).**

Rare potential adverse effects of azithromycin that have been reported in adults:

A study that included 1577 adults with chronic obstructive pulmonary disease (COPD) that were treated daily with azithromycin or placebo for a year showed that hearing decrements were slightly more common in the azithromycin group than in the placebo group (25% vs. 20%, P=0.04)[100]; this potential risk is very unlikely to be relevant to our study participants as the treatment duration in our study is very short compared to the duration used in the adult COPD study (2 weeks vs. one year). In addition, we will treat otherwise healthy children vs. older COPD patients that were treated in the adult study. These older COPD patients were prone to have hearing loss based on their age and other risk factors, as evident by 20% of participants in the placebo group who had hearing loss.

In 2013, the U.S. Food and Drug Administration (FDA) issued a warning that azithromycin can potentially cause fatal arrhythmia. The FDA identified specific populations who have an increased risk of the disease: patients with known cardiac risk factor, patients with electrolyte imbalance, or the concomitant use of some anti-arrhythmic medications[101]. The FDA's safety data have acknowledged that azithromycin is one of the most common prescribed medication in the US, and recommends that health care professional should consider the risk of fatal heart rhythms with azithromycin when considering treatment options **for patients who are already at risk for cardiovascular events.**

The FDA's recommendation was issued after the publication of the results of an observational study[102] that was conducted in **adult population** (mean age 49 yrs) of Medicaid beneficiaries in the US, **a population characterized by a high prevalence of coexisting conditions and high mortality rates**. The study results showed that the use of azithromycin was associated with a risk of death from cardiovascular causes that was 2- 3 times as high compared to the risk associated with no use of antibiotics, or the risk associated with amoxicillin treatment[102]. The risk was most pronounced among patients in the highest decile for cardiac risk based upon the cardiovascular disease risk score (245 additional deaths (95% CI 63-576) per 1 million courses), in contrast to 9 additional deaths per million courses (95% CI 2-21) among patient in the lowest 5 deciles. Additional study among US veterans showed similar increased risk of death during azithromycin treatment compared to amoxicillin treatment[103]. These 2 studies were conducted in adults with mean ages 49[102]- 57[103] yrs, which were enriched with subjects who had risk factors for cardiac morbidity. In contrast, **a similar study conducted in a younger population** (mean age 40 yrs), which was more similar in its characteristics to the typical population seen in a general clinical practice, **did not detect higher risk of death associated with azithromycin use compared to penicillin V use[104]. The authors suggested that azithromycin could have effect on cardiovascular mortality only in selected populations with risk factors for cardiovascular morbidity, but not in the general population[104].**

Since these initial reports two additional recent studies did not detect an increased risk for mortality and/or for arrhythmia among elderly patients treated with azithromycin:

1. Mortensen all[105] reported that 90-day mortality was a significantly lower in patients (> 65 yrs) who received azithromycin for pneumonia than in those who received non-macrolide antibiotics. There was no difference in the risk of arrhythmia between the groups.
2. Trac et all[106] reported that among older adults (>65yrs), macrolide antibiotics (48% received azithromycin), compared with non-macrolide antibiotics, were not associated with a higher risk of ventricular arrhythmia measured over 30 days. In this study, macrolide were actually associated with a significantly lower risk of all-cause mortality.

Based on these recent date we conclude that it is not clear if azithromycin is associated with an increased risk of arrhythmia <u>even in older adults</u>. Moreover, and to the best of our knowledge, this potential rare adverse effect among older adults has never been reported in children. The 2010 FDA's pediatric safety review (see

previous section)[78], which included azithromycin safety data, did not detect any such events that may be related to oral azithromycin use. Moreover, several prospective placebo controlled clinical trials have been performed with prolonged macrolide therapy (dose ranges 5-15mg/kg/day) in children with cystic fibrosis, asthma, and bronchopulmonary dysplasia (BPD) which have not demonstrated increased mortality or cardiovascular related side effects. Out of these studies, the 2 most relevant studies for our study population are 2 double-blinded placebo-controlled trials using azithromycin or placebo for the prevention of PBD in 263 critically-ill, preterm infants that were treated with azithromycin or placebo for up to 6 weeks (10 mg/kg/day daily for one week, following by a dose of 5 mg/kg/day for additional 5 weeks)[51, 52] . In these studies, azithromycin was not associated with an increase in adverse events (including arrhythmia) when compared to placebo. The significance of these safety data, over other studies, is related to the continuous monitoring of these pre-term infants in the Neonatal Intensive Care Unit. Therefore, any potential cardiac arrhythmia would have been detected.

In summary, based on the current data we believe that the potential rare risk of cardiac arrhythmia reported in 2 out of 5 studies performed in adults is very unlikely to be relevant to our study population which is very young, otherwise healthy, and do not have known risk factors for heart disease. Children with other significant medical history including heart disease cannot enroll in this study based on its exclusion criteria. In addition, chronic use of daily medications is not allowed per our study protocol. Nevertheless, the parents will be notified on this potential risk, which has been detected in some studies performed among adults, during the consent process.

Risk associated with the placebo:

There are no known risks associated with the placebo product as it is used simply to maintain the blind for the other treatment arms.

***P.2. Potential risks associated from study procedures*:**

Phlebotomy: Venipuncture may cause a slight pain for the child. An infection may arise at the site of the needle stick, but this is highly unlikely. Bruising may occur around the venipuncture site.

Nasal lavage/ brush: Nasal lavage has no adverse effect except for some mild discomfort with the procedure. Nasal brushing/swab causes irritation of the nasal mucosa when it is being performed. In a very rare cases, and if the nasal mucosa is inflamed, it might cause mild nasal bleeding.

Microbiome analyses: Microbiome genomic sequences will be analyzed using the computing facilities at Washington University's The Genome Institute. Any human sequences will be removed to protect patient privacy, and only microbial sequences will be analyzed and deposited in the public Short Read Archive Database.

Stool collection: A stool sample will be collected from a diaper at the randomization visit by the study coordinator. In addition, parents will collect stool at home (from diapers) prior to the 2-week, 6-month visit, and last clinic visit. Parents will be provided with kits (including disposable gloves) and instruction for collecting these samples. There is a small potential risk of transmission of pathogens from the child's stool to the parents; however, this risk is not different and not higher than the risk associated with the child's routine diaper changes, which the parents perform multiple time during the day. Therefore, participation in the study will not increase this potential existing risk. Otherwise, there is no risk associated with stool collection.

Urine collection: A urine sample may be collected from a diaper using a pediatric urine collection bag at the randomization visit and at visit 1 by the study coordinator. There is a rare risk of experiencing a mild skin rash due to the adhesive on the collection bag.

Other than avoiding the phlebotomy, there is no alternative treatment or procedure for the above, other than nonparticipation. However, families may opt to participate in the trial, but not to have the blood testing.

P.3. Potential risk of breach of confidentiality:

For all participants, there is a small risk of breach of confidentiality if participant identity is revealed. There are no other psychological risks or financial or legal risks.

Q. ADEQUACY OF PROTECTION AGAINST RISKS

Q.1. Specific measures to assure appropriate consent process:

An informed consent document will be approved by the Washington University IRB. Parents of children identified for participation in this study will be approached, after discussion potential participation with their attending physician. Informed consent documents will be provided and discussed with the parents/legal guardians. Informed consent will be obtained by one of the study investigators, or their appropriately trained designees when the participants are hospitalized for RSV bronchiolitis in St. Louis Children's Hospital. The consent process will be conducted in a private area and with adequate time for the parents/guardians to evaluate the study. Assent forms will not be used at enrollment due to the age of the participant population

The rights and welfare of human subjects included in this investigation and the methods for obtaining informed consent have been and will be carried out in conformity with the Washington University Medical Center Human Studies Committee guidelines and Assurance of Compliance with HHS/FDA adopted by Washington University Medical Center. The information outlined in the informed consent will be discussed with the subjects to assure that they understand the nature of the research and have voluntarily decided to participate. If there were language barriers or other impediments to communication, appropriate measures will be taken to ensure subject's understanding. Discussions regarding the research and subject's desire to continue to participate will continue throughout the current proposal.

Q.2. Specific measures to protect confidential research data and patient safety:

All clinical data obtained from the patients will be collected by paper clinical research forms. Then, the data will be entered into the electronic database (RedCap) and the research charts will be kept in locked file cabinets. Lab results will be entered directly to the database.

Access to the database will be restricted and password protected. All investigative staff involved in this proposal have undergone training in Good Clinical Practice guidelines for research studies. There are no unusual risks to confidentiality for participants in this study.

All procedures will be carefully explained to the child's parents/legal guardian, in order to maximize cooperation from the child. Parent/legal guardian will be cautioned about the potential side effects of phlebotomy, nasal brushing/washing, and the azithromycin treatment.

Each infant's parents/legal guardian will be given the name of the investigator and a phone number where one of the investigators may be reached should problems develop following the test or during the study treatment. All testing will be performed on site at the Pediatric Research Unit (PRU) at St. Louis Children's Hospital, or at the inpatient unit during the acute hospitalization. During the above testing, an emergency crash cart is readily available.

Q.3. Specific measures to secure biologic samples:

Participants' bio-samples collected for this study will be labeled only with a study ID, the Child's initials, and blood/sample draw date. Samples will be transferred to Dr. Mario Castro's laboratory, which is part of the Asthma and Airway Translational research Unit (AATRU) Biobank. Dr. Mario Castro's AATRU biobank is located at the University of Kansas Medical Center Research Institute. Biological samples collected for our study will be sent to this biobank for storage and processing. However, any stool specimens that have been collected or will be collected as part of this study will remain at Washington University. All specimens that will be transferred for processing and storage will only have the child's study ID, initials (initials may not be on frozen aliquots) and sample collection date, no PHI will be stored on collected/stored samples. Stool specimens remaining at Washington University will be be stored with the child's study ID, initials, and sample collection date, no PHI will be stored on collected/stored samples. Dr. Castro is an investigator in this study, and his lab will serve as the biobank for this study. The samples will be kept in a secure space in Dr. Castro's lab and only authorized personals of our research team will be allowed to process the sample. During the consent process, the parents will be asked if they are willing that the child's samples will be saved and potentially shared for future studies. This potential future research will include only deidentified samples.

Q.4. Specific measures to minimize potential risk of having adverse effects related to azithromycin treatment:

1. Taking azithromycin with food will be recommended in order to minimize possible gastrointestinal side effects (the most common side effect).
2. Only previously healthy children (without any other chronic disease) who are not regularly taking daily medications will be eligible to participate this study.
3. Participants may not enroll in this study if there is a known allergy to any of the antibiotics similar to azithromycin, such as erythromycin (Ilosone®) or clarithromycin (Biaxin®). Open-label treatment with macrolide during the treatment phase of the study is one of our exclusion criteria.
4. Parents will be instructed to stop the study medication if any rash, significant episodes of vomiting or diarrhea, or other adverse reaction occur and to call the study investigator.
5. Parents will be contacted at least once a week during the treatment phase to monitor adherence to the study medication, and to inquire on potential adverse effects.

Q.5. Specific measures to minimize potential risk of research in children:

The objective of the study is to prevent the development of wheezing episodes and subsequent asthma in young children hospitalized with RSV bronchiolitis. As such, it is imperative that young children (infants and toddlers) hospitalized with RSV bronchiolitis will be enrolled to this study. The available safety record of azithromycin in clinical trials in children with the extensive post-marketing safety record suggests that this intervention does not represent a meaningful increase in risk for the study participants.

The investigative team has extensive experience in dealing with children in this age category. Drs. Beigelman and Bacharier are pediatricians who take care of hospitalized RSV patients as part of their General Pediatrics Attending duties in the St. Louis Children's Hospital. In addition, they have an extensive experience performing clinical trials in this age group as investigators in the NHLBI's Childhood Asthma Research and Education Network (CARE), and AsthmaNet: and the NIAID's Inner City Asthma Consortium (ICAC).

Q.6. Plans for ensuring necessary medical or professional intervention in the event of adverse effects to the subjects:

As noted above, individual participants will have frequent contact with study staff and will have the ability to contact staff with acute events in a real-time manner with subsequent clinical follow-up determined by the nature of the event. Parents/Legal guardians will be asked to call in case their child experiences any potential adverse effect that might be related to the study medication.

Respiratory adverse events during the hospitalization will be treated by the child's attending physician. The infants will be treated according to a predefined care-path, the SLCH Bronchiolitis Pathway, a set of orders based on the American Academy of Pediatrics guidelines for treatment of bronchiolitis[79]. The SLCH Bronchiolitis Pathway is an evidence-based pathway that focuses on supportive treatments such as: nasal suctioning, supplemental oxygen, and IV fluids for infants who are not able to feed adequately. The Pathway strongly discourages use of systemic corticosteroids and antibiotics. All decisions regarding medical treatment during the hospitalization, other than those related to study participation, will made by the child's primary attending physician.

Respiratory adverse events after the hospitalization (including wheezing and asthma exacerbations) will be treated by the child's primary care physician. All these events including respiratory-related ED visits and hospitalization will be monitored by the study investigators.

All study population has an increased risk for respiratory adverse events, particularly worsening of the RSV bronchiolitis and the development of subsequent wheezing episodes and/or asthma. These are expected events after severe RSV infection, and are likely to occur even if the child will not participate in the study.

Finally, the study will be monitored by a DSMB, IRB and the NHLBI.

R. POTENTIAL BENEFITS OF PROPOSED RESEARCH TO SUBJECTS AND OTHERS

If our preliminary finding from our proof-of concept APW-RSV trial will be confirmed, then participants treated with azithromycin will be less likely to develop RW and potentially less asthma.

Knowledge regarding allergy blood testing may be helpful to the child's primary care physician in their management. In addition, all children and their parents will receive close follow-up by the nurses and physicians; we believe this close follow-up is a valuable educational resource for the parents, since they may be able to identify the presence of wheezing episodes if these will occur. If a child will develop wheezing episodes, we will refer the child to his/her primary pediatrician for short-term and long-term management of these wheezing episodes.

The result of the study might benefit other patients in the future, as if our hypothesis will be confirmed, other children will benefit from azithromycin treatment for the prevention of post-RSV wheezing and asthma.

The risks to subjects are reasonable in relation to the anticipated benefits to research participants and others

Overall, azithromycin is one of the most commonly prescribed antibiotics in childhood, which had a longstanding safety record. As such, there are only slightly greater than minimal risks to the children involved in the proposed project predominantly related to minor study burden and blood draws. Therefore, the risk/benefit ratio is favorable to individual participants as the risks are minimal, and based upon the results of our pilot APW-RSV trial the likelihood of benefit is meaningful, as this intervention may result in significant decrease in the risk of post-RSV wheezing and asthma.

S. IMPORTANCE OF THE KNOWLEDGE TO BE GAINED

This proposed project is designed to establish that a simple, inexpensive, and safe treatment with azithromycin, in infants hospitalized with RSV bronchiolitis, substantially reduces the occurrence of post-RSV RW, and potentially representing the first successful post-RSV asthma prevention strategy.

Early life RSV bronchiolitis is a major risk factor for RW and asthma- the most chronic disease of childhood. We will conduct a comprehensive clinical trial for the prevention of these outcomes, based on our preliminary data that have shown that azithromycin treatment reduced the likelihood of developing post-RSV RW by approximately 50%, measured over the year following the RSV hospitalization.

If our preliminary data will be confirmed, the results of this trial has the potential to change the way we treat severe RSV bronchiolitis patients with the goal of preventing post-RSV RW, and asthma**.** A reduction in post-RSV RW would have significant impact on society and the health system through reductions in morbidity and health care utilization. The clinical trial will be accompanied by microbiome studies that are design to provide insight on pathways through which the azithromycin modifies the microbiome, and how these changes relate to the development of RW. This knowledge may inform the development of additional microbiome-directed therapies for asthma prevention.

T. ANTICIPATED RESULTS

Based upon the results of our RBEL I study[17], we anticipate that over the follow up period, 72% of the participants in the placebo group will develop RW. Based on the anticipated effect size of the intervention, we anticipate that no more than 45% of the participants in the azithromycin group will develop RW. However, the actual proportion of participants in the azithromycin group that will develop RW might be even smaller as our power calculations were very conservative compared to the effect size noted in our APW-RSV pilot study[40].

U. STUDY TIME-LINE AND FUTURE PLANS

As detailed in **Figure 7** the study database will be locked after 54 months. If our hypothesis is confirmed, and azithromycin is an effective treatment modality for the prevention of post-RSV RW, we will apply for a renewal of this grant in order to continue following this cohort beyond the preschool age. This will allow us to evaluate long-term efficacy of the intervention for our long-term goal: the prevention of asthma development, which could not be definitely accessed within the time frame of this proposed project.

	2016				2017				2018				2019				2020			
Quarter	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Study Initiation																				
Year 1 recruitment																				
Year 1 pts f/u																				
Year 2 recruitment																				
Year 2 pts f/u																				
Year 3 recruitment																				
Year 3 pts f/u																				
Recruitment /retention monitoring						**M1**				**M2**				**M3**						
Analysis/ publication																				

Figure 7: study time line

VI. DATA AND SAFETY MONITORING PLAN (including adverse events)

Please refer to section V.P. above for a detailed description of potential risks and protections again risks

The APW-RSV II clinical trial will be overseen by the Washington University Human Research Protection Office (HRPO). We applied for a FDA's Investigational New Drug (IND) to allow use of azithromycin in children one-6 months of age, given the current indication for azithromycin is limited to children aged 6 months or older. The FDA reviewed our applications and concluded that an IND is not required to conduct this study (PIND 112359).

The PIs, Co-Investigators, the Washington University HRPO and a National Heart, Lung, and Blood Institute (NHLBI) Data and Safety Monitoring Board (DSMB) will monitor the study for adverse events, adherence to protocol, and patient accrual and withdrawal. All adverse events will be reported to the Washington University School Medical Center IRB and the DSMB. In addition, we will follow the NHLBI criteria for expedited reporting of severe adverse events (SAE) and unanticipated problems: these will be reported to the IRB, NHLBI and if required will also be reported to the FDA (see below the AE/SAE section or reporting timelines and reporting procedures).

A. DATA AND SAFETY MONITORING BOARD (DSMB)

The DSMB that will monitor this project will be convened by the NHLBI. In brief, the DSMB will review safety data, study conduct, data management, and analysis/interpretation. To do so, the DSMB will be asked to review the protocol, informed consent form/process, protocol amendments, data on recruitment and retention, protocol adherence, safety events, any emerging information impacting the safety or ethics of study participation, and manuscripts. A DSMB charter will be approved by the DSMB and will include a detailed description of the DSMB membership, responsibilities, and policies. DSMB reviews will occur at least yearly, and more frequently if warranted as described in the DSMB Charter. Recommendations (minutes/reports) from the DSMB will be provided to the Principal Investigator (PI), Dr. Avraham Biegelman, and NHLBI Program Official from the DSMB. The PI will distribute the Minutes to the IRB.

B. SAFETY REPORTING: COLLECTION AND REPORTING OF ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

B.1. Definitions: Adverse events, serious adverse events and unanticipated problems:

Adverse Event (AE)- Any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related. An adverse event can also be any unfavorable and unintended sign (e.g., an abnormal laboratory finding), symptom, or disease temporally associated with the use of a drug, without any judgment about causality. Determination of the relationship between the event and study drug, or a study mandated procedure must be made by the PI. This definition will also include changes in the medical status that may be related to study procedures. All adverse events will be categorized as being possibly, probably, definitely, or not related to study drug.

Serious Adverse Event (SAE) - An adverse event is considered "serious" if, it results in any of the following outcomes:

1. Death
2. A life-threatening adverse reaction
3. Hospitalization or prolongation of existing hospitalization
4. A persistent or significant disability interfering with the ability to conduct normal life functions.

5. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples may include: new diagnosis of cancer or renal insufficiency.

Unanticipated Problem (UP)- any incident, experience, or outcome that meets all of the following criteria: 1) unexpected 2) related or possibly related to participation in the research; and 3) suggests that the research places subjects or others at a greater risk of harm than was previously known or recognized. UPs are not necessarily associated with AEs.

Unexpected (Adverse Event) - An adverse event is considered "unexpected" if it is not listed in the consent document or it is more severe than previously described,; or is not consistent with the risk information described in the general investigational plan.

B.2. Collection, reporting, and management of AE and SAE:

Reporting period:

The safety-reporting period will begin once the child enrolls into the study and ends with completion of study participation (last visit or phone call). It should be noted, as described in the section on determination of relatedness, that the intervention (study drug administration) ends 14 days after enrollment and based on the half-life of azithromycin, all drug is eliminated within 15 days after the last dose. Therefore, while reporting will continue to 48 months from enrollment, the *study-related* risks to the safety of children in the final 47 months of participation will be limited to monitoring procedures at the 4 study visits (V3-V6). Therefore, all SAE's and AEs should be collected and reported from enrollment to time of Phone Call-1 (2 months following randomization). After Phone call 1, AE reporting will be focused on the AEs of Special Interest (related to study procedures) for the remainder of the study period. SAEs will be continued to be reported until the end of study participation.

Collection and reporting of AE and SAE:

Eliciting adverse events other than those related to respiratory health will be spontaneous or in response to an open-ended question, such as "How are you?" or "Have you noticed any changes in your health since your last visit?" Directed questions will be used to follow up spontaneous reporting of events for clinically relevant details.

Determination of the relationship between the event and study drug, or a study mandated procedure must be made by the investigator. All adverse events will be categorized as being possibly, probably, definitely, or not related to study drug or study participation.

The following conventions will be used for reporting AEs:

- If a specific diagnosis is unclear at the time of reporting, descriptive terms will be used. For example, a patient with cough and a headache may later be diagnosed with influenza. Initially "cough" and "headache" will be reported and later changed to "influenza" after the diagnosis is evident.

- Multiple symptoms or signs related to the same event will be reported as a single event, unless complications of the same event are considered medically relevant and not adequately captured in an existing

AE. For example, the single AE " influenza" will be reported rather than headache, cough, sore throat and myalgia if these symptoms are considered related to the infection . Therefore, AEs will be recorded by the study team based on their interpretation of the patient's report. However, if the same patient develops renal failure due to severe dehydration with influenza, the renal failure will be reported as another AE.

- The onset of the AE is the date of the first symptom or sign as noted by the patient or a care provider. For example, a patient may have a cough for several days followed by fever and shortness of breath before the diagnosis of pneumonia is made. The AE will be reported as "pneumonia", with the onset being the date of the first symptom or sign (cough, in this case).

- Worsening of pre-existing conditions will be reported with a description of the change in status. For example, "worsening asthma" may be reported in a patient with a medical history that includes asthma while reporting in "asthma" in such an individual is not informative.

- Lab or other test results outside the normal range will be reported as AEs if there are associated signs or symptoms or further evaluation or more frequent monitoring is required.

Grading severity of adverse events:

The severity of AE's will be determined according to the following criteria:

Mild: Event imposes no limitations on individual's ability to perform usual activities

Moderate: Event interferes with ability to perform usual activities

Severe: Event prevents the individual from performing usual activities

Determining AE relatedness:

The PI will determine if an AE is "possibly related", "probably related", "definitely related", or "not related" to the study drug or a study mandated procedure. The determination will be based on the biologic plausibility, the temporal relationship, and the presence or absence of an alternative explanation for an AE. This protocol requires up to 47 months of follow up after a 14 day treatment period (with either azithromycin or placebo). Given even in the case of active drug (azithromycin), the drug will be cleared within 15 days ($T_{1/2}$ = 68 hours x 5 = 340 hours or 14.2 days), *study related* risks during the final 47 months of follow up will be from study procedures/monitoring or long term effects of prior drug exposure (e.g., a future allergic reaction). Therefore the timing of an adverse event will have a significant role in the determination of relatedness.

AEs will be categorized as having one of the following causalities:

- Not Related: The AE is clearly not related to the study drug.
- Possibly Related: The AE follows a reasonable, temporal sequence with administration of the study drug and/or follows a known response pattern to treatment with the study drug but could readily have been produced by the subject's clinical state, environmental factors, or other modes of therapy or concomitant drugs administered to the subject.
- Probably Related: The AE is uncommon in the subject population and follows a reasonable, temporal sequence from administration of study drug; follows a known response pattern to treatment with the study drug; and is not likely to have been produced by the subject's clinical state, environmental factors, or other modes of therapy or concomitant drugs administered to the subject.
- Definitely Related: The AE is uncommon in the subject population and follows a reasonable, temporal sequence from administration of study drug; follows a known response pattern to treatment with the study

drug; and cannot have been produced by the subject's clinical state, environmental factors, or other modes of therapy or concomitant drugs administered to the subject.

Reporting requirements:

As with all study data, AEs will be reported in a timely fashion. In all cases reporting to IRB's, NHLBI, and the FDA will be commensurate with regulatory requirements. Time sensitive reporting will be completed based on the shortest regulatory requirement of either the FDA or the IRB

Specific expedited safety reporting requirements are applicable as follows:

- SAE, which is suspected of being related to study drug or a study mandated procedure and is unexpected (**Suspected Unexpected Serious Adverse Reaction; SUSAR**):
    - SAE that is life threatening: it must be reported to the Washington University' s HRPO, the NHLBI, the FDA and DSMB within 7 working days of initial receipt of information. **If the event results in the death of a study participant, it will be reported within one working day.**
    - SAE that is non-life threatening: it must be reported to the Washington University' s HRPO, the NHLBI, and the FDA and DSMB within 10 working days of initial receipt of information.
- Unanticipated problem that is not an SAE will be reported to the Washington University' s HRPO, and the NHLBI within 14 working days of initial receipt of information.
- Adverse events that do not meet the definition of an unanticipated problem involving risks to participants or others will be reported to the Washington University' s HRPO at the time of continuing review.
- It is expected that most study participants will have acute exacerbation of wheezing and/or asthma and some of them would require hospitalization. This is an expected outcome following hospitalization for RSV bronchiolitis, and hence does not require an expedited report.
- **Any actions issued by the WU HRPO will be reported to the NHLBI by the PI within 14 business days.**

Management of SAEs :

The PI or one of the other investigators, which will be designated by the PI, will decide if based on his/her best clinical judgment a SAE may be related to the study medication. If it will be determined that a SAE is possibly related, probably related, or definitely related to the study medication, then the study therapy will be discontinued. As with all AE/SAE, the participants will be followed until resolution of this event. We will not withdraw the participants from the study, as our analysis approach is an intention to treat approach.

The procedures for collection and reporting of SAEs are described above.

Required follow up of adverse events:

All adverse events must be followed (and documented on case report forms) until one of the following criteria is met:

- Resolution
- A documented plan for further evaluation and management, including the overseeing care provider, is provided.
- The event is considered and documented to be stable and adequately managed, though ongoing.

C. PROTOCOL DEVIATION

- Major Protocol Deviation - A change made without prior IRB approval that has the potential to negatively impact the rights, safety, or welfare of a participant or to negatively impact the ability of a study to yield scientifically valid results.
    - Major protocol Deviation will be reported to the Washington University' s HRPO within 10 working days (if the event results in death of a participant, then 1 working day).
- Minor Protocol Deviation - A change made without prior IRB approval that does not have the potential to negatively impact the rights, safety, or welfare of a participant or to negatively impact the ability of a study to yield scientifically valid results.
    - Minor protocol Deviation will be reported to the Washington University' s HRPO at the time of continuing review.

D. UNBLINDING FOR SAFETY

Treatment assignment information for each subject ID will be kept in a secure location in the St. Louis Children Hospital Pharmacy.

In the event of medical necessity, a treating physician may contact the PI (or on of the co-investigators who may cover for him) with a request for unblinding.

The PI or his designee will evaluate the request and if justified, per his/her clinical judgment, will approve the investigational pharmacist to disclose the treatment assignment to the treating physician. As much as possible, this communication regarding the actual treatment assignment should be done by the pharmacist and should not involve the PI or other research team members.

Disclosing treatment assignment would only be done in circumstances of a medical emergency in which knowing treatment assignment would impact the child's medical care. Therefore, we would not unblind if a child had an event a few months after last dose of study medication, unless there is a specific medical concern and if knowing the treatment could change patient care.

All unbinding events will be reported to the DSMB during the following DSMB meeting.

E. CONFLICTS OF INTERESTS

All investigators identified potential Conflict of Interest (COI) by utilizing a COI Form that has been designed by the Washington University Office of Human Research. All investigators will repeat this procedure before the establishment of the research fund and then yearly.

The DSMB members will identify any potential COI at the beginning of each DSMB meeting.

F. INTERIM ANALYSIS

An interim analysis was not specified in this protocol, as we do not believe it will serve the purpose of detecting early signal of efficacy, and it is unlikely to protect patients' safety.

This trial is design to investigate if the beneficial effects of azithromycin for the prevention of post-RSV RW, which we previously detected over one year of follow-up, are durable over the preschool years. In order to achieve this goal, this trial will include a longer follow up period (up to 48 months). Hence, interim analysis is unlikely to detect an efficacy signal and may result in false negative results.

We believe that interim analysis is unlikely to serve the purpose of protecting patients' safety, as azithromycin, which is the study intervention, is an antibiotics with a well proven safety profile, which is very commonly used in children in the clinical practice (for details see the Protection of Human Subject Section). Furthermore, the rate of adverse reactions in our pilot trial was not different between children treated with azithromycin or placebo. Nevertheless, the rate of adverse reactions will be monitored throughout the study by the investigators, the IRB, and the DSMB.

G. STOPPING RULES

We have no stopping rules for efficacy given the potential for a false negative if the study will be stopped too early (i.e., we are not over-powered to detect the effect of the intervention).

VII. STATISTICAL DESIGN AND ANALYSIS

A. ANALYSIS PLAN

General approach:

The goal of this clinical trial is to test the null hypothesis that RW is present at the same rate in the azithromycin group as compared to the placebo group. The primary analysis will be an intent-to-treat, comparing treatment groups in a Kaplan-Meier survival curves. Then, a Cox proportional hazards regression model will be used to estimate the magnitude of the treatment effect in terms of the hazard ratio for the occurrence of RW.

Rationale for using survival analysis methodology for the primary outcome assessment:

In order to maximize the duration of follow-up in this trial which will recruit subjects over 3 consecutive RSV seasons, participants will have variable follow up durations based upon the year in which each participant will be recruited: the first season's recruits will be followed for up to 48 months, the second season will be followed up to 36 months, and the third season will be followed for at least 18 months**.** Maximizing the duration during which we will monitor the effect of the intervention will result in a higher likelihood of detecting the difference in outcomes between treatment groups. **Based on this study design with variable duration of follow-up, survival analysis methodology is most appropriate.**

Primary outcome analysis:

The initial analytic strategy will be a log-rank test, which compares the Kaplan Meier survival curves describing the time to the third episode of wheezing.

Then, the primary analysis of the primary endpoint will be conducted using a stepwise Cox regression model that would include 2 covariates: race and parental history of asthma. These 2 covariates were significant predictors of childhood asthma development following severe RSV bronchiolitis in our RBEL cohort[1]. If the proportional hazards assumption is violated, we will follow the recommendations of Allison[15] and will address the problem by including in the model an interaction term between time and the variable that violates the proportional hazards assumption.

At the next step, we will conduct multivariate analysis that will include other covariates that may be associated with the primary outcome. Chi square tests, t-tests, and potentially Wilcoxon's test will be used to compare all non-time dependent covariates listed below* across groups in order to identify variables that should be included in subsequent multivariate models. Those fixed-time covariates that yield at least some suggestion of a between-group difference ($p < 0.1$) will then be included in a stepwise Cox regression analysis along with all of the time dependent covariates (e.g., pet exposure). Additional analyses will include the use of an Anderson-Gill model[16] in which wheezing episodes are treated as recurrent events when compared between groups. In Cox models that do not contain time dependent covariates, we will evaluate the proportional hazards assumption by assessing the parallelism of the log-log plots and using martingale residuals and the method of Lin, and Ying[17]. The Cox models which contain time-dependent covariates will be tested using Schoenfeld residuals[18].

The key statistic to be generated by the Cox regression analyses, for primary outcome reporting, will be covariate adjusted hazard rate ratios that compare the RW hazard rate in the azithromycin group with the corresponding hazard rate in the control group. Ninety-five percent confidence bounds will be generated for all calculated hazard rate ratios.

Finally, we will explore for potential interactions between study treatment assignment and pre-specified covariates to evaluate whether these covariates are predictors of response to azithromycin therapy:

a. Demographic: Sex, age, race.
b. Baseline asthma/allergy phenotypic characteristics: Personal history of eczema, parental history of asthma, peripheral eosinophil count on enrolment.
c. Environmental phenotypic characteristics: second hand smoking, pet exposure.
d. Acute bronchiolitis characteristics: Duration of respiratory symptoms before enrolment, and the presence of co-infection with other respiratory viruses during the RSV bronchiolitis (e.g. rhinovirus).

*The following covariates will be compared between the groups and would be considered to be included in the Cox models:

a. Age at enrollment (months)
b. Gender
c. Race
d. Birth weight
e. Length of pregnancy (wks)
f. Maternal smoking status during pregnancy
g. Breast feeding status at the time of enrollment

h. Tobacco smoke exposure (at the time of enrollment and during f/u visits)
i. Pet exposure (at the time of enrollment and during f/u visits)
j. History of eczema
k. Parental history of asthma
l. Duration of respiratory symptoms prior to randomization
m. Duration of hospitalization (hours: measured from the time of the Emergency Room visit that triggered the admission and until time of discharge)
n. Lowest O2 saturation recorded on room air
o. Antibiotics use during the acute hospitalization. Antibiotic use during the follow up period will be measured as one of the secondary outcomes.
p. Co-infection with other respiratory virus on enrollment (mainly Rhinovirus)
q. Complete compliance with study medication (defined as >80% compliance)
r. Antipyretic (acetaminophen and Ibuprofen) use during the initial RSV bronchiolitis
s. Day care attendance
t. Number of siblings in the house
u. Eosinophil count at enrollment

Censoring considerations: All subjects will be censored at the time of the last contact with the subject confirms that they had not experienced the outcome measure. This applies whether the subject is a dropout or has remained on study until the end.

Sensitivity analyses:

In the primary outcome analysis, we will implement LASSO using the R package glmpath in models that contain only the fixed covariates. After using LASSO to select a best set of fixed covariates, we will potentially add one or more of the three time dependent covariates to that list using stepwise procedures.

The primary analysis, in subjects who are lost to follow-up, will include only the available data until the time they lost to follow-up. However, we would like to be cautions in interpreting these results because a potential non-random dropout could bias the results. Hence, we will perform sensitivity analyses using multiple imputation.

Additional pre-specified statistical analysis will examine subset differences between children who were intubated during the acute RSV bronchiolitis and children who were not intubated in order to better interpret the results of the primary analysis based on the entire data. This will be performed by the following steps:

1. We will examine potential differences in baseline covariates between children who were and were not intubated. If this analysis will yield at least some suggestion of a between-group difference ($p < 0.1$), the presence/absence of intubation will be included as a covariate in the model that will be used for the primary outcome analysis.
2. We will explore potential interaction between intubation and study treatment assignment although power to detect such differences may be low (no preliminary data is available to project the expected power).

3. We will repeat steps #1 and #2 for examining the effect of any mechanical ventilation (intubation, CPAP, BiPAP).

Secondary and exploratory outcomes analyses:

Secondary outcome measures will be analyzed using a variety of statistical approaches. Biological outcome measures such as IL-8 levels that will be measured at baseline and at two weeks when the treatment is terminated will be evaluated using analysis of covariance with the post treatment measure as the dependent variable and with predictor variables that include the baseline value, the treatment group, and other pre-defined covariates.

Secondary outcome measures that are time to event variables such as asthma diagnosis will be compared across groups using the same survival and Cox regression approaches as were discussed for the primary outcome variable. Other secondary outcome measures are continuous measures that will be based on patient reports at phone calls that are made every two months. These include the number of days during the prior two months in which the subject experienced respiratory symptoms, used the rescue inhaler, or awakened at night due to such symptoms. These frequently occurring events will be evaluated using mixed model repeated measures analysis of variance in which the repeated outcome will be quantified every two months. However, because the number of days for these outcome measures is likely to vary substantially from one two-month interval to another, we will seek to stabilize these data and plan to use six month intervals in the analysis. For each variable, the number of days over six months will be the sum of the three preceding two-month measures, with an appropriate multiplicative factor being used in the calculation if one of two of the data points in a given six month interval is missing. We anticipate using an autoregressive or Toeplitz covariance structure in the repeated measures analyses because we expect within subject correlation coefficients to be smaller when data points are close together. However, the determination of the appropriate covariance structure will be based on more formal assessments of these correlation coefficients as well as on the Schwarz-Bayesian criterion and Akaike's information criterion.

Data regarding antibiotic use, systemic corticosteroids use, and the occurrence of upper respiratory infections will be also collected every two months. These data will be used to determine the rate of these events during each six months study interval, and these repeated measures data will be compared across groups using Generalized Estimating Equations (GEE).

Missing data in the mixed model repeated measures analyses will be handled in the following ways. First, subjects randomized during the second year may provide data only up to 30 months while subjects randomized during the third year may provide only up to 18 months of data. Since missing data caused by randomization in year two or three (as oppose to randomization at year one) will be completely at random, we will analyze the data we have without imputation and with no associated bias. Second, we will also assume random missingness in the situation where a subject may have missed a particular phone call but completed subsequent phone calls. Therefore, we will calculate values for a given six-month interval in which one of the two-month phone calls was missing by imputing for a particular missing two-month value the average of the values for the outcome measure in the relevant six-month interval that were not missing. For example, if the 4 month phone call was missing and if the two-month and 6 month call yielded 12 days and 24 days for a given variable, we will impute 18 days (the average of 12 and 24) for the missing four month value. In all of these mixed model analyses, the focus will be on the statistical contrasts that compare between-group differences in the change from baseline to 18 months, from baseline to 30 months, and from baseline to 42 months.

A.2. Microbiome outcomes:

We will use the streamlined 16S analytical pipeline developed in The Genome Institute for the Human Microbiome Project to perform the microbiome analysis[110, 111]. Bacteria counts will be rarified to the same number across all the samples to minimize the bias potentially caused by read depth. To evaluate the effect of azithromycin treatment on the upper airway and gut microbiome, we will compare the bacterial community structure between azithromycin and placebo groups at each of the following 3 time points: randomization, week 2, and month 6, utilizing multivariate statistical testing that are widely used for 16S microbiome analysis [112]. First, NMDS based on Bray-Curtis dissimilarity will be used to identify the microbial clustering patterns and assess the similarity between the two groups in a reduced dimensional space at two individual time points. Then, we will use PERMANOVA to test the significant differences of the overall bacterial community between the two groups[113]. To correct for potential confounding factors, we will test individual variables in a PERMANOVA model. All the variables that show a trend towards an association with the treatment assignment ($P< 0.15$) will be included in the model, as described previously[114] . To identify the signature bacteria taxa that contribute to the difference between two compared groups, we will apply Metastats, a statistical approach developed in Human Microbiome Study for differential bacteria feature identification[114]. P-values from multiple comparisons will be corrected by the false discovery rate (FDR) approach. Lastly, we will measure both alpha and beta diversity of the bacterial community in the two groups. Alpha diversity index such as Shannon diversity and richness to evaluate the complexity of the whole microbial community; Wilcox-sum-Rank testing will be used to test the difference of diversity between compared groups. Beta diversity represented by Bray-Curtis dissimilarity will be used to indicate the inter-subjects variation in the bacterial composition. All analysis will be performed by R (http://www.r-project.org/) and Bioconductor. The above analyses will allow us to characterize the microbial community at each individual sampling point, and to evaluate how it is affected by azithromycin treatment.

To examine the relationship between microbiome changes and the diagnosis of RW, we will apply a linear mixed model to bacteria of interest such as the bacteria that are significantly different between the two groups identified at individual time points, as we have previously performed[115]. Microbiome data will be normalized to satisfy the assumption of normality by a logarithmic transformation. In the linear mixed model, transformed bacterial abundance will be modeled as the response, and the sample collection time points, treatment groups and RW outcome as fixed effects and patients as random effect. P-values from multiple comparisons will be corrected by the false discovery rate (FDR) approach.

Finally, we will perform a whole genome sequencing (WGS) on a subset of the bio-samples. Among all samples obtained at the end of study treatments, we will choose 50 samples with the strongest positive associations and 50 with the strongest negative associations with RW: these 100 samples will be selected for WGS sequencing. The library preparation, whole genome shotgun sequencing, and data processing will be performed as previously described in the Human Microbiome Project[116]. We will characterize the microbial community structure at species the level, identify other microbial components such as viruses, and reconstruct the metabolic pathways of the bacterial community for each sample[117]. Similar statistical strategies will be used for the WGS data as described in the 16S rRNA gene data analysis. We will be alert to any changes in data quality; if needed, we will modify methods or repeat samples to obtain rigorously validated results.

B. SAMPLE SIZE JUSTIFICATION

B.1. Clinical outcome:

Power calculations were performed using the program Power and Precision, Version 4[19]. They account for recruitment of patients over 3 consecutive RSV seasons (~5 months per year) with a follow up period of 18-48 months (**Figure 1: Study Design**). Our power computations are based on an evaluation of the four year survival and hazard curves generated by 190 participants of the RBEL I cohort[1] who share the same characteristics as the controls in this proposed research, and hence used to estimate the RW rate in the control group. The survival pattern observed in our randomized pilot study (APW-RSV)[14] was utilized to estimate the effect of the intervention. The RBEL survival curves show a low placebo group hazard rate (HR) of about 2% during the first three months after hospitalization. This HR increases steadily to about 5% per month at the end of the first year. After that, HRs decrease steadily to 3% per month by the end of year 2 to a steady monthly rate of 2% during year 3, and 1% during year 4. These assumptions yield a four-year hazard rate of 71.6%, nearly identical to the actual rate of 70.4% observed in RBEL I. We used the HR as described as the basis for the placebo group in our power computation. These projected hazard rates yield a four-year hazard rate of 55.1%. Thus, the power computations summarized below represents hazard rates of 71.6% in the placebo group and 55.1% in the azithromycin group over the follow-up period. Based on the parameters described above, two-sided tests at the 0.05 level of significance, and assuming a dropout rate of 20%, **power computations yield a requirement of 94 randomized subjects per group for a power of 0.9, and 70 per group for a power of 0.8.** Based on these considerations, we will randomize a total of up to 200 subjects or 94 per group, numbers that yield the target power of 0.9. In evaluating these computations, it is important to note that we have assumed a hazard ratio during the first 18 months of at least 2. While this is a substantial hazard ratio, we emphasize in the next paragraph that our pilot randomized trial with one year of follow-up yielded a hazard ratio of 0.34 (comparing azithromycin with placebo) meaning that the observed risk associated with placebo compared to azithromycin was nearly 3.

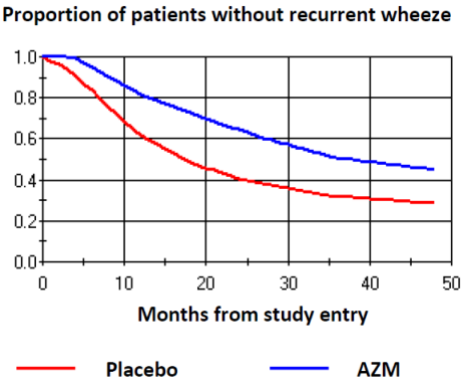


Figure 3. Anticipated effect size of the intervention: proportion of patients who will not develop recurrent wheeze in the 2 treatment groups

We strongly believe that these power computations yield an adequate and indeed conservative sample size for the following reasons: First the excellent target power of 0.9 and the need for only 70 subjects per group to achieve a power of 0.8 suggest that we will still have adequate power if recruitment is slower than it has been in the past. Second, the assumed HR ratios (55.1/71.6 = 0.77) are substantially greater than the 0.34 observed in our APW-RSV pilot study. Although this large hazard rate ratio difference between those projected in this study (0.77) and the pilot study (0.34) are mitigated somewhat by the fact that the pilot study follow-up period was only one year, we have based our computations on hazard rate ratios that are closer to 1 than in the pilot study, even during the first year. Therefore, we are powered to detect between group differences that are smaller than were observed in our pilot study. Third, we assumed a HR of 1 during years 3-4. This is a very conservative approach as we believe that prevention of inflammatory damage to the airway during the acute RSV bronchiolitis will result in ongoing preventative effect for wheezing prevention during childhood

B.2.Microbiome outcomes:

We will utilize the whole study population (n=200) for this aim. This sample size provides 90% power to detect the difference for a given taxa between two groups, with effect size (5%) and standard deviation (SD1=2 and SD2=10) and alpha=0.05. However, 141 patients will be sufficient to provide 80% power to detect the same effect size. All sample size calculations assume 10% sample failed rate, which may be from different stages of the experiments,

such as patient drop out, sample preparation and sequencing failure (the combined failure rate related to the last 2 reasons in our APW-RSV trial was less than 4%). These power calculations were performed based on the assumption that we will observe half of the effect size and standard deviation detected in our preliminary data, in which *Moraxella* decreased significantly after azithromycin treatment. We used a decreased effect size to infer the sample size as *Moraxella* was a relatively high abundance taxon in the data and was affected by the intervention in our preliminary studies. In this proposal, we are also interested in comprehensive detection of the difference of relatively rare taxa in those two groups. We will be more confident to classify relatively rarely taxa in the Miseq deep sequencing platform (we anticipate 4x more reads/sample) than with the Roche 454 sequencing platform, in which our preliminary data was generated.

VIII. SIGNIFICANCE

Severe RSV bronchiolitis is a major risk factor for subsequent wheezing episodes and eventually asthma. Previous interventions in the setting of severe RSV bronchiolitis targeted allergic (i.e. eosinophilic) inflammation and were found to be ineffective in preventing subsequent wheezing episodes. We will use an innovative intervention (azithromycin) that is safe for use in children, is capable of attenuating neutrophilic inflammation, and has the mechanistic rationale to be an effective intervention.

This trial may identify the first effective intervention to apply during severe RSV bronchiolitis with the goal of preventing post-RSV RW, and ultimately asthma. A meaningful reduction in post-RSV RW would have significant impact on society and the health system through reductions in morbidity and health care utilization. This trial will be conducted by a multidisciplinary team, including experts in asthma/RSV clinical trials, infectious diseases, and microbiome research. Understanding the pathways through which the azithromycin modifies the microbiome, and how these changes relate to the development of RW, may inform the development of additional microbiome-directed therapies for asthma prevention.

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X. APPENDIX 1.

COMBINED LIST OF DRUGS THAT PROLONG QT AND/OR CAUSE TORSADES DE POINTES (TDP)

CredibleMeds® has reviewed available evidence for the drugs on this list and concluded that they either 1) have a Known Risk of TdP ⚠ 2) prolong the QT interval and therefore have a Possible Risk of TdP ? or 3) have a Conditional Risk of TdP , that is, under certain conditions such as overdose, drug - drug interactions or when administered to certain high-risk individuals (e.g. congenital long QT syndrome).

Generic Name	Brand Name
Alfuzosin	Uroxatral®
Amantadine	Symmetrel® and others
Amiodarone	Cordarone® and others
Amisulpride	Solian® and others
Amitriptyline	Elavil® (Discontinued 6/13) and others
Anagrelide	Agrylin® and others
Apomorphine	Apokyn® and others
Aripiprazole	Abilify® and others
Arsenic trioxide	Trisenox®
Artenimol+piperaquine	Eurartesim®
Asenapine	Saphris® and others
Astemizole	Hismanal®
Atazanavir	Reyataz®
Atomoxetine	Strattera®
Azithromycin	Zithromax® and others
Bedaquiline	Sirturo®
Bepridil	Vascor®
Bortezomib	Velcade® and others
Bosutinib	Bosulif®
Buprenorphine	Butrans® and others
Capecitabine	Xeloda®
Ceritinib	Zykadia®
Chloral hydrate	Aquachloral® and others
Chloroquine	Aralen®
Chlorpromazine	Thorazine® and others
Cilostazol	Pletal®
Ciprofloxacin	Cipro® and others
Cisapride	Propulsid®
Citalopram	Celexa® and others
Clarithromycin	Biaxin® and others

Generic Name	Brand Name
Clomipramine	Anafranil®
Clozapine	Clozaril® and others
Cocaine	Cocaine
Crizotinib	Xalkori®
Cyamemazine (cyamepromazine)	Tercian®
Dabrafenib	Tafinlar®
Dasatinib	Sprycel®
Degarelix	Firmagon®
Delamanid	Deltyba®
Desipramine	Pertofrane® and others
Dexmedetomidine	Precedex® and others
Diphenhydramine	Benadryl® and others
Disopyramide	Norpace®
Dofetilide	Tikosyn®
Dolasetron	Anzemet®
Domperidone	Motilium® and others
Donepezil	Aricept®
Doxepin	Sinequan® and others
Dronedarone	Multaq®
Droperidol	Inapsine® and others
Eribulin mesylate	Halaven®
Erythromycin	E.E.S.® and others
Escitalopram	Cipralex® and others
Ezogabine (Retigabine)	Potiga® and others
Famotidine	Pepcid® and others
Felbamate	Felbatol®
Fingolimod	Gilenya®
Flecainide	Tambocor® and others
Fluconazole	Diflucan® and others
Fluoxetine	Prozac® and others

Generic Name	Brand Name
Foscarnet	Foscavir®
Furosemide (frusemide)	Lasix® and others
Galantamine	Reminyl® and others
Gatifloxacin	Tequin®
Gemifloxacin	Factive®
Granisetron	Kytril® and others
Grepafloxacin	Raxar®
Halofantrine	Halfan®
Haloperidol	Haldol® (US & UK) and others
Hydrochlorothiazide	Apo-Hydro® and others
Hydrocodone - ER	Hysinglaâ„¢ ER and others
Hydroxychloroquine	Plaquenil® and others
Hydroxyzine	Atarax® and others
Ibutilide	Corvert®
Iloperidone	Fanapt® and others
Imipramine (melipramine)	Tofranil®
Indapamide	Lozol® and others
Isradipine	Dynacirc®
Itraconazole	Sporanox® and others
Ivabradine	Procoralan® and others
Ketoconazole	Nizoral® and others
Lapatinib	Tykerb® and others
Lenvatinib	Lenvima®
Leuprolide	Lupron® and others
Levofloxacin	Levaquin® and others
Levomepromazine	Nosinan® and others
Levomethadyl	Orlaam®
Lithium	Eskalith® and others
Loperamide	Imodium® and many other OTC and Rx brands
Mesoridazine	Serentil®

Generic Name	Brand Name
Methadone	Dolophine® and others
Metoclopramide	Reglan® and others
Metronidazole	Flagyl® and many others
Mifepristone	Korlym® and others
Mirabegron	Myrbetriq®
Mirtazapine	Remeron
Moexipril/HCTZ	Uniretic® and others
Moxifloxacin	Avelox® and others
Nelfinavir	Viracept®
Nicardipine	Cardene®
Nilotinib	Tasigna®
Norfloxacin	Noroxin® and others
Nortriptyline	Pamelor® and others
Ofloxacin	Floxin®
Olanzapine	Zyprexa® and others
Ondansetron	Zofran® and others
Osimertinib	Tagrisso®
Oxaliplatin	Eloxatin®
Oxytocin	Pitocin® and others
Paliperidone	Invega® and others
Panobinostat	Farydak®
Pantoprazole	Protonix® and others
Papaverine HCl	none
Paroxetine	Paxil® and others
Pasireotide	Signifor®

Generic Name	Brand Name
Pazopanib	Votrient®
Pentamidine	Pentam®
Perflutren lipid microspheres	Definity®
Pimozide	Orap®
Pipamperone	Dipiperon (E.U) and others
Posaconazole	Noxafil® and others
Probucol	Lorelco®
Procainamide	Pronestyl® and others
Promethazine	Phenergan®
Propofol	Diprivan® and others
Quetiapine	Seroquel®
Quinidine	Quinaglute® and others
Quinine sulfate	Qualaquin®
Ranolazine	Ranexa® and others
Rilpivirine	Edurant® and others
Risperidone	Risperdal®
Ritonavir	Norvir®
Roxithromycin	Rulide® and others
Saquinavir	Invirase®(combo)
Sertindole	Serdolect® and others
Sertraline	Zoloft® and others
Sevoflurane	Ulane® and others
Solifenacin	VESIcare®
Sorafenib	Nexavar®
Sotalol	Betapace® and others

Generic Name	Brand Name
Sparfloxacin	Zagam®
Sulpiride	Dogmatil® and others
Sunitinib	Sutent®
Tacrolimus	Prograf® and others
Tamoxifen	Nolvadex®(discontinued 6/13) and others
Telaprevir	Incivo®
Telavancin	Vibativ®
Telithromycin	Ketek®
Terfenadine	Seldane®
Tetrabenazine	Nitoman® and others
Thioridazine	Mellaril® and others
Tizanidine	Zanaflex® and others
Tolterodine	Detrol® and others
Toremifene	Fareston®
Torsemide	Demadex® and others
Trazodone	Desyrel® (discontinued 6/13) and others
Trimipramine	Surmontil® and others
Tropisetron	Navoban® and others
Vandetanib	Caprelsa®
Vardenafil	Levitra®
Vemurafenib	Zelboraf®
Venlafaxine	Effexor® and others
Voriconazole	VFend®
Vorinostat	Zolinza®
Ziprasidone	Geodon® and others

Note: Medicines on this list are reviewed on an ongoing basis to assure that the available evidence supports their continued placement on this list. Because, the list changes regularly, we recommend always checking the website at crediblemeds.org for the most up-to-date information. Most drugs have multiple brand names and it is not practical to list them on this form. The CredibleMeds.org website provides a partial list of the more common brands.

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XI. APPENDIX 2.

SARS-COV-2 MEASUREMENT TESTING:

Background: The APW-RSV II clinical trial

The APW-RSV II clinical trial is investigating if the addition of azithromycin to routine bronchiolitis care, among infants hospitalized with RSV bronchiolitis, reduces the occurrence of recurrent wheeze during the preschool years.

We recruited 200 participants during 3 consecutive RSV seasons. Study participants were randomized to receive azithromycin or placebo for 2 weeks. The treatment phase for the last participant ended in spring 2019 and study follow-up will continue until fall 2020, and thus the final year of observation with include the COVID-19 pandemic.

COVID-19 and its potential implications on the APW-RSV II clinical trial

COVID-19, caused by the novel coronavirus, SARS-CoV-2, started in China in late 2019 and became a global pandemic affected millions worldwide. On April 6, the CDC COVID-19 Response Team reported that occurrence of clinical COVID-19 was lower in children than in adults (https://www.cdc.gov/mmwr/volumes/69/wr/mm6914e4.htm). However, a very recent report from China (https://doi.org/10.1101/2020.03.03.20028423) revealed that among contacts of subjects infected with SARS-CoV-2, the infection rate was similar between children and adults. The difference between these 2 reports may be related to the high rate of asymptomatic SARS-CoV-2 infections among children: estimated as up to 28% of young infected childen[1]. However, children have not been targeted for testing in the United States, and thus the rate of both symptomatic and asymptomatic infection remains unknown.

Given these uncertainties in terms of the prevalence and severity of this infection in young children, it is plausible to suggest that COVID-19 infection has the potential to impact the outcomes of the APW-RSV II clinical trial. The primary outcome of the APW-RSV II clinical trial is: "*The occurrence of a third episode of post-RSV wheezing measured over a follow up duration of 18-48 months, quantified by a Cox proportional hazards regression model, and compared between the groups*."

In addition, almost all secondary outcomes of the APW-RSV II clinical trial may be affected by COVID-19. These include:

g. Time to physician asthma diagnosis.

h. Time to asthma diagnosis OR to the third episode of wheezing.

i. Annualized number of days with: any respiratory symptoms (wheezing, cough, or shortness of breath), or albuterol use.

j. Rate of oral corticosteroid courses use.

k. Rate of antibiotic courses use.

In the APW-RSV clinical trial we are looking for the development of recurrent wheezing/asthma in children who have already demonstrated susceptibility to respiratory viral infections, as all were hospitalized due to RSV bronchiolitis. It is unknown if these children are more likely to be infected with SARS-Cov-2 and what will be the impact of COVID -19 on short and long-term respiratory symptoms in these children. As COVID-19 infection has the potential to affect the clinical trial outcomes, understanding the rate of its occurrence is essential in evaluating this exposure on our outcomes of interest.

Methods: SARS-CoV-2 measurements

In order to determine the prevalence of SARS-CoV2 infection in the study cohort, we will assess SARS-CoV-2 infection status as demonstrated by presence of SARS-COv2 antibody (IgG and IgM) in peripheral blood. This approach was selected instead of direct viral detection using nasopharyngeal swabs for the following reasons:

1. Most importantly, antibody testing will give us valuable information on past infection while a PCR based test can only provide information on the current infection status (one point in time).
2. Antibody testing conducted by a blood test is safer for the research team and family as it is not associated with virus transmission risk, as opposed to the small risk associated with obtaining a nasal swab.

We plan to utilize the COVID 19 IgG serology test of the LabCorp lab and not a research lab kit, in order to increase the reliability and the validity of the test and to allow us to share the results with participant families.

After obtaining IRB approval, we will approach the caregivers of study participants (currently 190 participants are retained) and will request their consent for SARS-CoV-2 serology testing. SARS-CoV-2 testing will be voluntary; unwillingness to provide consent will not affect continued participation in the APW-RSV II clinical trial. It is anticipated that parents will likely agree to participate as knowing the SARS-Cov-2 status of their children will be valuable for them.

The test will be obtained at the last study clinic visit that will be conducted late summer-fall 2020. If clinic visit will not be able to be conducted due to Washington University COVID 19 regulations, we will send the participants to a LabCorp lab to obtain the test.

Methods: Primary outcome

The primary outcome of the APW-RSV clinical trial will remain the same (the occurrence of a third episode of post-RSV wheezing); however, the primary outcome assessment will include the SARS-Cov-2 infection status as an additional exposure covariate.

Methods: Exploratory outcome

Wheezing rates among our study populations 6-months before and 6-months after SARS-Cov-2 activity started in the St. Louis area (March 2020).

Methods: Analysis plan

In order to evaluate the effect of the exposure (SARS-Cov-2 infection) we will conduct a sensitivity analysis to assess whether SARS-Cov-2 infection status of the study participants is associated with primary outcome of the APW-RSV II clinical trial (recurrent wheeze). We will include infection status as a time-dependent covariate in our Cox regression model. As it is not possible to determine the exact date of infection for study participants, for those participants testing positive for SARS-Cov-2 we will assume an infection date of March 1, 2020. We will compare the results of this more complex model with our original pre-specified model, and if the results are not appreciably different then we will retain the original model as the primary analysis and the model including infection status will be included as a supplement.

We will also employ sensitivity analyses that include infection status for the secondary outcomes.

In addition, we will conduct exploratory analyses that will entail: (1) estimates of the proportion of participants infected with SARS-Cov-2 overall and stratified by treatment arm, and (2) for SARS-CoV-2 positive participants, a comparison of the wheezing rates in the 6-months prior to the SARS-Cov-2 activity in the St. Louis area (September 2019 to February 2020) to the wheezing rates 6-months after activity in the region (March 2020 to September 2020). For the analysis of wheezing rates, we will test the statistical null hypothesis of no difference in wheezing rates for SARS-Cov-2 positive participants in pre vs. post SARS-Cov-2 activity in St. Louis using a generalized linear model assuming a Poisson or negative binomial regression distribution. If we reject the null hypothesis ($P < 0.05$) and the wheezing rates are elevated in the post-outbreak period then we will conclude that wheezing rates were higher in the post-outbreak period for participants that tested positive for SARS-Cov-2.

Biohazard consideration

Blood drawing and PPE measures will be conducted according to all regulations that will be in place at that time at Washington University School of Medicine, in order to assure the safety of the child, its family, and the research team.

If conducted at the LabCorp facility, it will be conducted according to all LabCorp regualtions.

Reference

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