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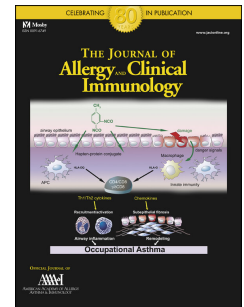
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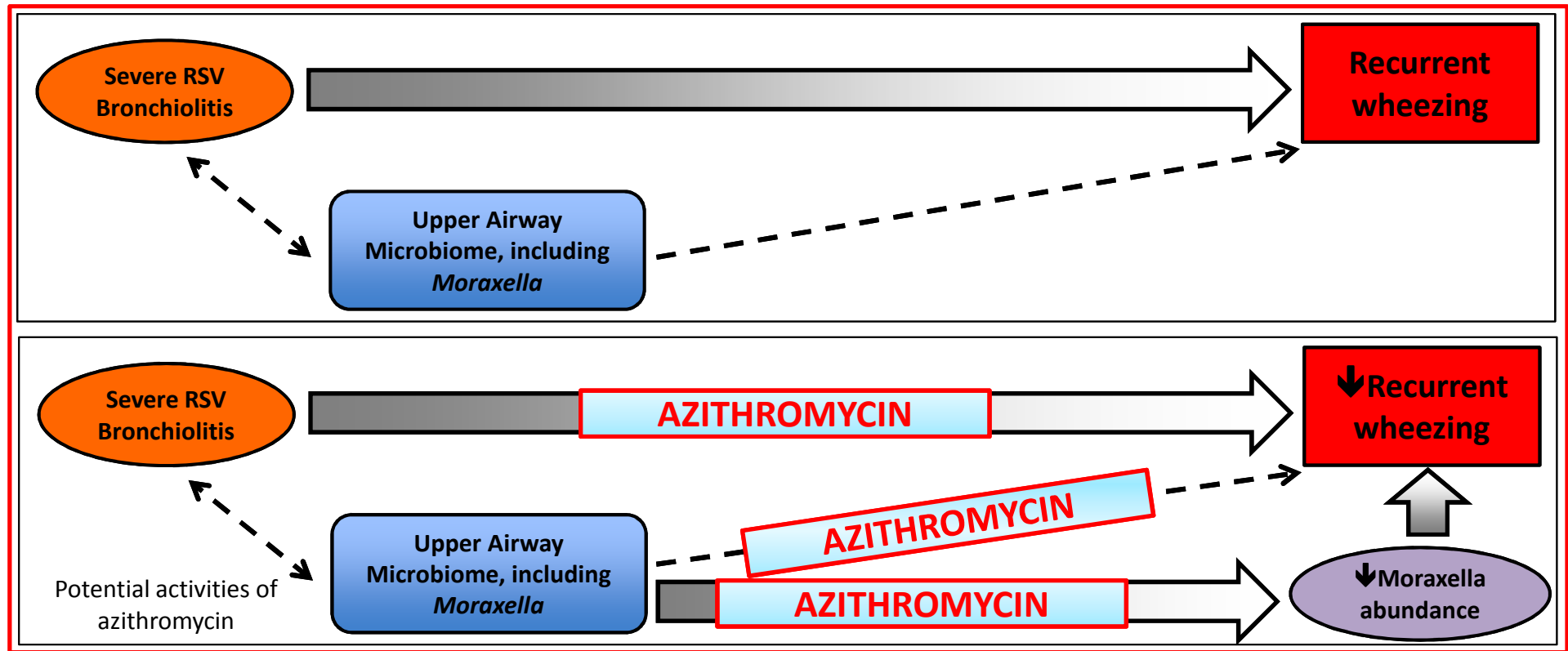
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Azithromycin therapy during RSV bronchiolitis: upper airway microbiome alterations and subsequent recurrent wheeze

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Capsule Summary:

In a proof-of-concept study, azithromycin therapy during severe RSV bronchiolitis was associated with lower *Moraxella* abundance in the upper airway; lower *Moraxella* abundance was associated with lower odds of subsequent recurrent wheeze. Additional studies should evaluate the role of the airway microbiome in the development of post-RSV recurrent wheeze, and whether microbiome modifications may serve as a prevention strategy.

Key Words:

Azithromycin, respiratory syncytial virus (RSV), wheezing, asthma, microbiome, *Moraxella*

Abbreviations:

APW-RSV: Azithromycin to Prevent Wheezing following RSV Bronchiolitis

PERMANOVA: Permutational Multivariate Analysis of Variance

RSV: Respiratory syncytial virus

RW: recurrent wheeze

To the Editor,

Early life RSV bronchiolitis is a risk factor for recurrent wheeze (RW) and asthma^{1,2}. We recently reported that, in a proof-of-concept randomized trial (the APW-RSV trial), azithromycin therapy during RSV bronchiolitis reduced the probability of developing RW during the subsequent year by approximately 50%³. Azithromycin was chosen because it is capable of targeting neutrophilic airway inflammation⁴ and based on our results in a mouse model of viral bronchiolitis⁵. In infants hospitalized with RSV bronchiolitis, we demonstrated anti-inflammatory effects of azithromycin³ reflected by reduced nasal lavage levels of IL-8, a marker of neutrophilic airway inflammation. However, the mechanism of azithromycin's anti-inflammatory effects may have reflected direct effects on the immune response, or an indirect effect through anti-bacterial actions of azithromycin leading to alteration in bacteria abundance and a reduction in neutrophilic inflammation. Therefore, it remains imperative to investigate if the potential beneficial effects of azithromycin for the prevention of post-RSV wheezing are related, at least in part, to its anti-bacterial effects.

Emerging evidence links the airway microbiome, either during periods of well-being or during viral respiratory infections, and the development of RW⁶⁻⁸. Based on these reports, we hypothesized that the beneficial effects of azithromycin on the reduction of post-RSV wheeze observed in our proof-of concept trial were associated with airway microbiome changes. The goal of this study was to characterize upper airway microbiome changes following azithromycin or placebo therapies and if these changes were related to the reduction in post-RSV RW.

A detailed description of the APW-RSV clinical trial, clinical outcome assessment, genetic methods, and statistical analyses is available in the on-line supplement. Briefly, 39 otherwise healthy infants, aged 3.8±2.9 months, hospitalized with RSV bronchiolitis were

randomized to receive oral azithromycin (10 mg/kg once daily for 7 days, followed by 5 mg/kg once daily for 7 additional days), or placebo. The incidence of RW was assessed monthly over the next year while the participants and investigators remained blinded to study treatment assignment. 16S rRNA gene sequencing was performed on nasal lavage samples obtained before and at the end of study treatment to characterize bacterial community composition and abundances. We examined the relationships between bacterial abundance at the end of treatment and the occurrence of RW (≥ 3 episodes) over the subsequent year.

Baseline characteristics of study participants are presented in Table S1, and were not significantly different between the treatment groups.

A detailed description of the airway microbiome characterization methodology is available in the on-line supplement. At randomization, the airway microbiome community structure (bacterial composition and abundance), measured by Permutational Multivariate Analysis of Variance (PERMANOVA) test⁹, did not differ between the groups ($p=0.7$; Figures 1a and S1). The relative abundances of the most abundant bacterial genera are presented in Figure S1. *Streptococcus* and *Moraxella* were the two most abundant genera in both treatment groups at randomization (Figure S1).

At the end of study treatment, the overall bacterial composition and abundance significantly differed between the 2 groups ($p=0.001$; Figure 1b, S1). The directions of microbial change differed between treatment groups (Figure 1c-d). In the placebo group (Figure 2), the relative abundances of *Dolosigranulum* and *Corynebacterium* increased significantly ($qvalue=0.03$, 0.03 ; respectively), while the relative abundance of *Streptococcus* decreased significantly ($qvalue=0.01$). In the azithromycin group (Figure 2), the relative abundance of *Moraxella* decreased significantly ($qvalue=0.03$).

At the end of study treatment, the *Moraxella* genus was the only bacterial taxon whose abundance differed significantly between treatment groups (azithromycin group median 0.05%, IQR 0-7.6% vs placebo group median 9.0%, IQR 0.1 -33.1%; q value=0.009; Figure S2).

Lower *Moraxella* abundance at the end of study treatments, irrespective of treatment assignment, was significantly associated with lower odds of experiencing subsequent RW in the whole study population (OR=0.86; 95% CI 0.75-0.99, p =0.03). This analysis, when confined to the placebo group to minimize potential confounding from other potential azithromycin effects (e.g., anti-inflammatory effects), showed comparable effect size on the odds of RW, but did not reach statistical significance levels (OR 0.86; 0.75 - 1.01, p =0.07). We could not reliably estimate an OR for the azithromycin group alone, since only 3 participants had detectable levels of *Moraxella* at the end of azithromycin treatment, and RW was an infrequent event among participants treated with azithromycin.

In order to investigate potential confounding of the association between *Moraxella* abundance at the end of treatment and RW by baseline characteristics of study participants (Table S1), we included these covariates, one at the time, in a regression analysis and found that none contributed significantly to the model (data not shown).

An additional regression model shows that the magnitude of reduction in *Moraxella* abundance over the 14 days of treatment, adjusted for baseline abundance, was significantly associated with the odds of RW (OR=0.87; 0.74-0.98, p =0.04).

The results of this study have shown that azithromycin therapy during severe RSV bronchiolitis was associated with a reduction in *Moraxella* abundance in the upper airway, and that lower *Moraxella* abundance was associated with lower odds of subsequent RW. A previous report suggested that early life asymptomatic colonization of the upper airway with bacteria

including *Moraxella catarrhalis* is associated with higher risk of developing persistent wheezing and asthma⁶. A more recent study⁷, demonstrated that dominant presence of *Moraxella* in the nasopharynx during RSV infection was associated with a more severe RSV disease evidenced by concomitant fever. Our findings add to these previous reports as we found that greater abundance of *Moraxella* may also be a risk factor for the development of post-RSV RW and hence it is possible that reducing *Moraxella* abundance may reduce that risk.

Lower *Moraxella* abundance was associated with lower odds of RW in the whole study population. A subgroup analysis confined to the placebo group revealed a comparable effect (i.e. the same odds ratio) for the reduction in RW, but with borderline significance ($p=0.07$), most likely a consequence of inadequate power for this subgroup analysis due to small sample size. Thus, we cannot be certain that the association between the reduction in *Moraxella* abundance and the reduction in RW, noted in the whole cohort, is not at least partly confounded by other beneficial effects of azithromycin, including anti-inflammatory effects. Moreover, we cannot exclude that some of this reduction was independent of the effect of azithromycin and reflects the evolution of the airway microbiome after viral infection.

The relatively small sample size is a limitation of this study. However, the goal of this pilot study was to prove the concept that airway microbiome during RSV bronchiolitis is a relevant determinant of post-RSV RW. In addition, our findings were obtained in a single institution, and additional research is required to assess if these findings are valid in a larger and a more diverse population (e.g., a population that includes more diverse minority groups, such as Hispanics children). While *Moraxella* abundance was related to the odds of developing RW, this association does not necessarily reflects causation and future research is needed by modifying *Moraxella* abundance post-RSV bronchiolitis.

In summary, this proof-of-concept study showed that 2 weeks of treatment with azithromycin during RSV bronchiolitis modified the upper airway microbiome, most notably with a reduction in *Moraxella* abundance. Lower *Moraxella* abundance was associated with lower odds of developing RW over the next 12 months. This observation identifies a potential role of *Moraxella* in the pathogenesis of post-RSV RW, and suggests that azithromycin may reduce the risk of development of post-RSV RW through anti-microbial effects on airway microbiome composition. These findings, which are summarized in Figure S3, provide rationale for additional studies that will evaluate the role of the airway microbiome in the development of post-RSV RW, and whether microbiome modifications may serve as a strategy for post-RSV RW prevention.

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Figure legends:

Figure 1. Bacterial community structure in the AZM and Placebo groups before and after the study treatment (2a and 2b), and the dynamics of bacterial community structure changes in the 2 groups over the treatment period (2c and 2d). In this non-metric multidimensional scaling (NMDS) plot, each dot represents the microbiome composition of one sample. Dots that are closer to each other have more similar microbiome composition compared to dots that are farther to each other.

Panel 2a demonstrates no difference microbiome composition between AZM and Placebo groups at randomization. Panel 2b demonstrates clear separation between the 2 groups at the end of study treatments. Panels 2c and 2d indicate that the microbiota shifted considerably, but in different trajectories, between randomization and day 14 in both the azithromycin (3a) and the placebo groups (3b).

Figure 2. Changes in the relative abundance of bacterial genera at the end of study treatments compared to randomization. After 14 days of treatment, *Moraxella* (qvalue=0.03) significantly decreased in azithromycin group. *Dolosigranulum* (qvalue=0.03) and *Corynebacterium* (qvalue=0.03) increased significantly in placebo group, and *Streptococcus* (qvalue=0.01) decreased significantly in placebo group. * qvalue<0.05.

Figure S1. Relative abundance of the dominant bacteria (>1%) before and after the study treatment, as illustrated by heat-map. The colors describe the different abundance of each bacterial genus. Each column corresponds to one sample, while each row represents the bacterial genus.

Figure S2. Relative abundance of *Moraxella* in the azithromycin and Placebo groups at the end of study treatments (day 14). *Moraxella* abundance is significantly lower in azithromycin group compared to the placebo group (qvalue=0.009).

Figure S3. A schematic model of the possible mechanism through which azithromycin decreases the occurrence of post-RSV recurrent wheeze.

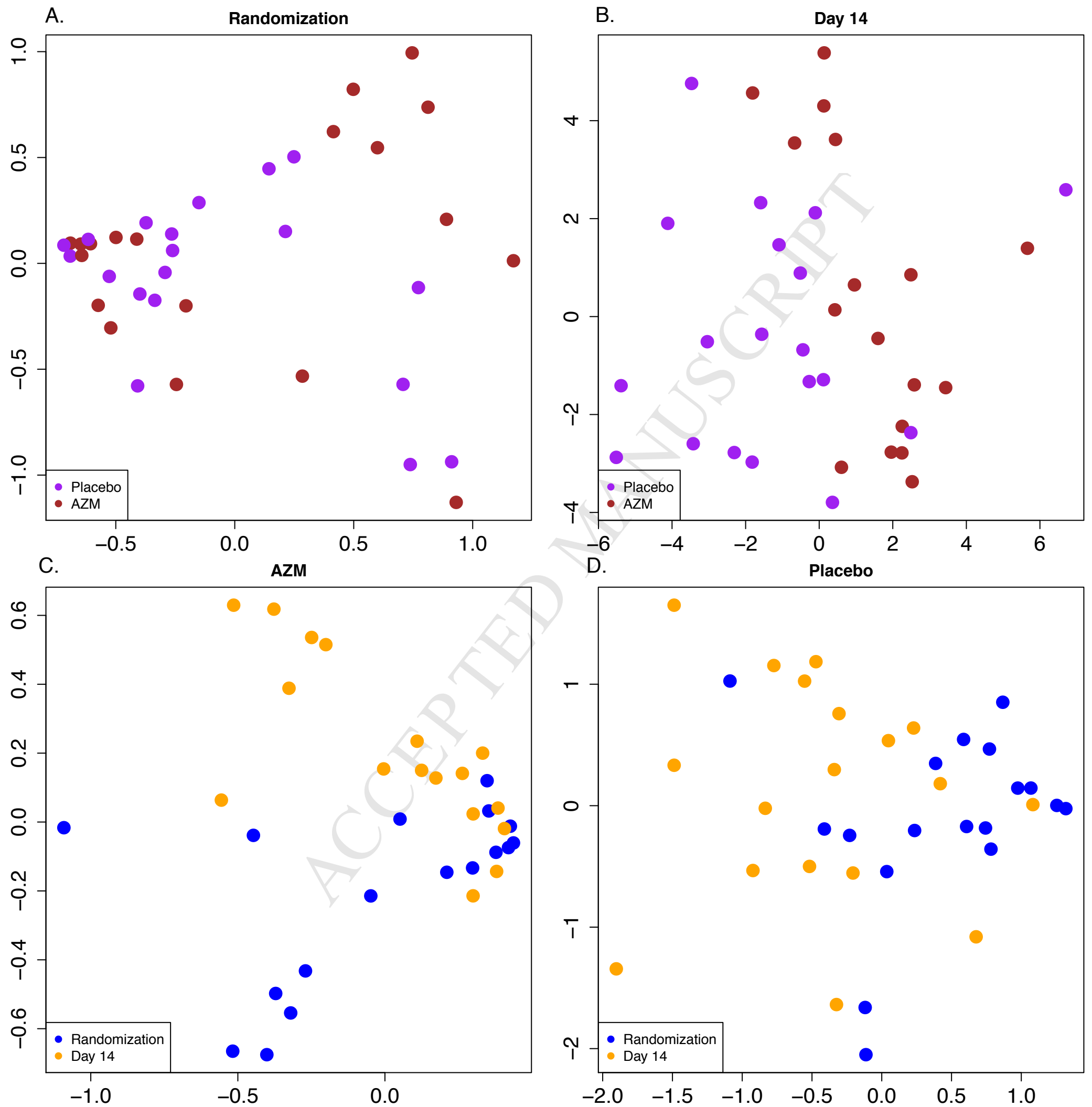
Table 1. Baseline characteristics of the APW-RSV study participants

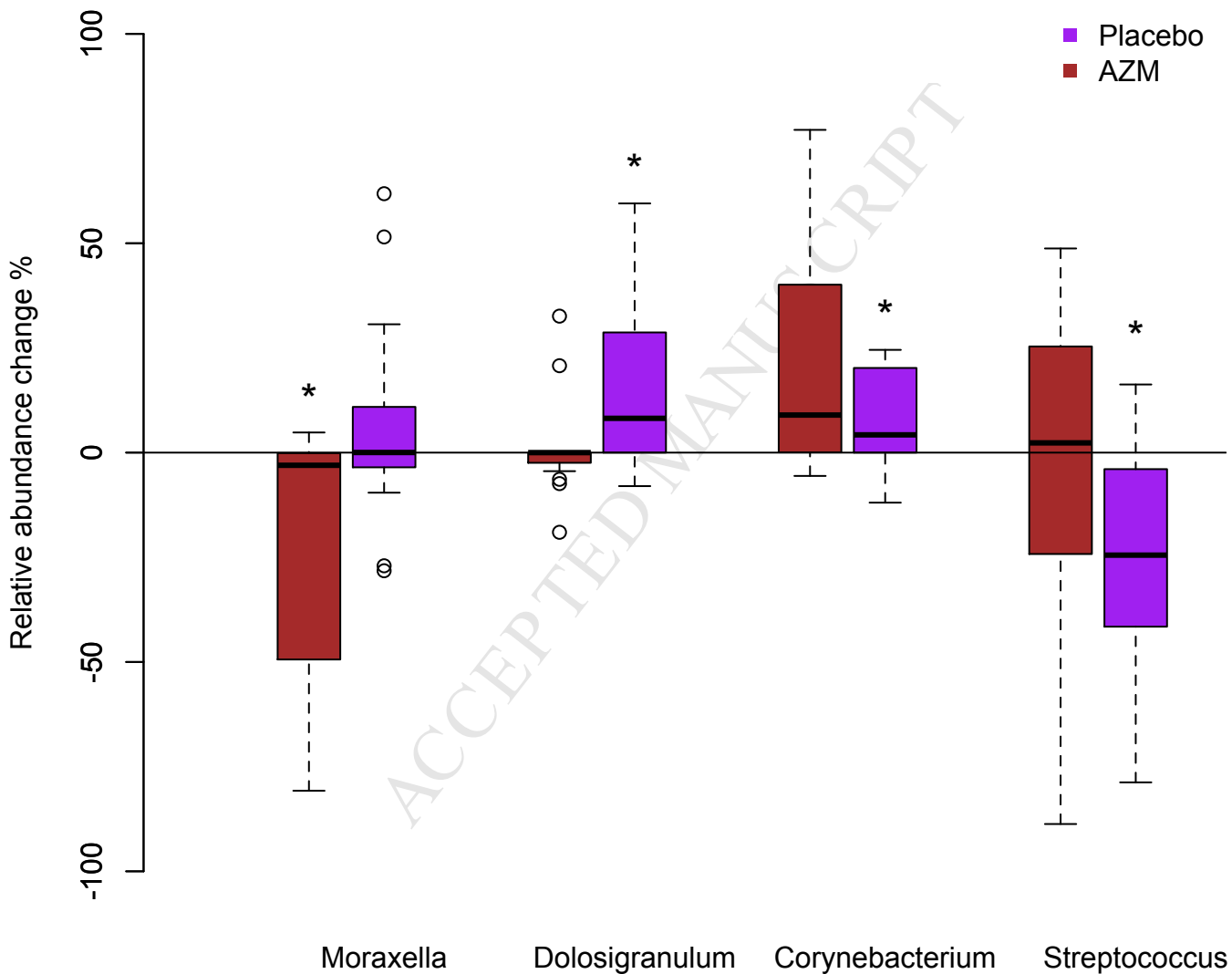
	All participants	Azithromycin (n=19)	Placebo (n=20)
Age at enrollment (months) ^a	3.8 (2.9)	3.7 (3.7)	3.9 (2.0)
Gender (male)	59.0%	47.4%	70.0%
<i>Race:</i>			
Caucasian	64.1%	63.2%	65.0%
African American	35.9%	36.8%	35%
Birth weight (kg) ^a	3.4 (0.5)	3.3 (0.6)	3.4 (0.4)
Length of pregnancy (wks) ^a	38.8 (1.4)	38.9 (1.3)	38.7 (1.4)
Maternal smoking during pregnancy	7.7%	10.5%	5.0%
History of breast feeding	28.2%	31.6%	25.0%
Tobacco smoke exposure	35.9%	42.1%	30%
Pet exposure	61.5%	63.1%	60.0%
History of eczema	17.9%	21.1%	15.0%
Parental history of asthma	41.0%	42.1%	40.0%
Duration of respiratory symptoms prior to randomization (days) ^a	5.4 (1.4)	5.2 (1.3)	5.5 (1.4)
Duration of hospitalization (hours) ^a	63.4 (53.2)	58.0 (41.4)	68.5 (63.2)
Lowest O2 saturation on room air ^a	90.8 (4.3)	91.4 (4.9)	90.3 (3.6)

Data are expressed as proportion of children in each group except as noted.

P>0.05 for the baseline characteristics comparisons between the treatment groups.

^a Data represents the mean (SD).





Online supplement

Methods

Patients and study intervention

A detailed description of the APW-RSV clinical trial has been reported elsewhere¹. Briefly, this was a randomized, double-masked, placebo-controlled, proof-of-concept trial involving 40 infants hospitalized with RSV bronchiolitis. Eligible infants were 1-18 months of age, otherwise healthy, and hospitalized for the first episode of bronchiolitis with confirmed RSV infection. Infants with history of prematurity were excluded. Participants were randomized to receive either AZM oral suspension 10 mg/kg once daily for 7 days, followed by 5 mg/kg once daily for 7 additional days, or an oral placebo suspension containing methylcellulose and cherry syrup. The study protocol was approved by the Washington University Institutional Review Board. Participants' parents provided written informed consent.

Clinical outcomes assessment

The outcome of RW was assessed monthly over a year following the initial RSV bronchiolitis while the participants and investigators remained blinded to study treatment assignment¹. As we have done previously⁵, we defined the occurrence of a wheezing episode as any time that a parent/guardian answered yes to either "Has your child had wheezing with colds?" or "Has your child had wheezing without colds?".

DNA Extraction and quality control

Total nucleic acid was extracted from 200 ul nasal lavage samples using the bioMerieux NucliSENS easyMAG automated extractor kit and following standard protocol. The V1-V3 regions of 16S rRNA gene was amplified, barcoded and sequenced on the Roche 454 Titanium platform using the protocol developed by Human Microbiome Project². Negative controls including reagent control and water control were included in each pool during library preparation and sequencing. If a large number of reads (>500 reads) was presented in any of the negative controls, the pool was discarded and re-sequenced.

DNA Sequencing and sequence data processing

Sequencing data was performed according to standardized protocols developed by the Human Microbiome Project². In brief, samples were binned by allowing one mismatch in the barcodes. Reads were filtered out if the average quality scores were less than 35 and/or read length less than 200 base pairs. Chimeric sequences were removed using Chimera-Slayer software. Samples with less than 1000 reads were excluded from the analysis. Reads passing quality control were then classified from phylum to genus level using the Ribosomal Database Project (RDP) Naive Bayesian Classifier (version 2.5, training set 9). Taxa assigned with less than 0.5 confidence were reassigned to the next higher taxonomic level in which the classification threshold was greater than 0.5.

Statistical analysis of microbiome data

Each sample was rarefied to the lowest read counts among all samples. The abundance of a taxon in a sample was indicated as the relative abundance, which was calculated by dividing the number of reads for a taxon by the total read counts of the sample. We apply Nonmetric Multi-Dimensional Scaling (NMDS) to explore the microbiome data structure based on dissimilarity measurement (Bray-Curtis dissimilarity) between two samples. NMDS is ordination technique, which aims to discover the data pattern in N dimensional spaces. For microbiome data, it allows the investigator to identify the subject relationships based on the bacterial compositions and abundances.

Permutational Multivariate Analysis of Variance (PERMANOVA) was used for formal statistical testing to investigate whether the bacterial community structure differed between different variables³. PERMANOVA is a non-parametric method that tests whether the community structure is significantly different between groups. It uses a permutation test with pseudo-*F* ratios to generate the p-values³. Baseline variables such as age, gender, race and breastfed feeding were included in the model. Metastats analysis was performed to identify the genera that contribute to the difference between two bacterial communities⁴. Metastats is a statistical method based on Fisher's exact test, which was developed for the Human Microbiome Project². The genera were considered to be significantly different if: (1) q value <0.05 and (2) the mean relative abundance for a given genus was at least 1% in one group of the two compared groups. Additional confirmatory analysis that compared *Moraxella* abundance between AZM and Placebo groups, at the end of study treatments utilized Analysis of covariance (ANCOVA), which accounted for the baseline *Moraxella* abundance.

Richness and Shannon diversity were used to describe the complexity of bacterial community as previously described⁵. Richness is defined as the number of different bacteria in a sample. Richness does not acknowledge the taxa abundances. Shannon diversity quantifies both the number of different bacteria and their abundance, and it is a popular univariate diversity index used in ecology and human microbiome studies. Both richness and diversity measurements are taxonomic label-free. The statistical significances of diversity indices were tested by Wilcoxon-rank test. A P value of <0.05 was considered statistically significant. All the analyses, described above, were performed in R (version 2.15.2).

Logistic modeling was used to identify predictors of subsequent 3 or more wheezing episodes. Preliminary logistic modeling was performed on each individual clinical variable including gender, race, age, breastfeeding, and each bacterial genera with mean relative abundance $>1\%$. Each predictor with $P<0.2$ was allowed to be entered into the final model. $P<0.05$ was considered as significant relationship between any potential predictor and the presence of at least 3 subsequent wheezing episodes. Logistic analysis was performed in SAS.

Microbiome sequencing and quality control outcomes

Seventy-eight nasal lavage samples were available for microbiome analyses. These nasal lavage samples and additional negative controls, which included DNA extraction and PCR amplification reagent controls, were subjected to 16S rRNA gene sequencing. In total, 456,457 reads were generated. Four of 78 samples yielded <1000 reads/sample, and were excluded from the analyses. Therefore, the specific analysis that

investigated the change in taxa abundance over the treatment period included 35 study participants. The negative controls contained between 3-400 reads/sample, and the median read depth of our patients' samples is 6268 reads/sample, suggesting that the background noise from the overall sequencing process was minimal. In addition, these low counts of reads in the negative controls should not affect the results of the analysis, as we focused on the relatively high abundance organisms as described in the Methods section. The 16s rRNA gene data are available from the SRA database: accession number PRJNA292612.

Results

Microbial communities in nasal lavage samples obtained at randomization are similar between treatment groups

At randomization, the airway microbiome community structures did not differ between the groups. 27 (IQR 23-34) and 24 (IQR 16-30) genera were detected in the AZM and placebo groups, respectively, suggesting that the nasal lavage samples harbor complex distributed bacterial populations. The relative abundances of the most abundant bacterial genera are presented in Figure 1S. *Streptococcus* and *Moraxella* were the two most abundant genera in both treatment groups (Figure 1S). *Streptococcus* constituted 36.5% (IQR: 13.9%-76.6%) vs. 55.7% (IQR: 31.9%-72.4%) of the whole bacterial population in AZM vs. placebo groups, respectively ($p=0.5$). *Moraxella* constituted 3.1% (IQR: 0.2-29.6%) vs. 5.0% (IQR: 0.7-20.4%) of the whole bacterial population in AZM vs. placebo groups, respectively ($p=0.7$). Richness ($p=0.3$) and Shannon diversity ($p=0.95$) did not differ significantly between the two treatment groups.

To further investigate bacterial community composition and abundance, we performed NMDS to illustrate the data structure. As shown in Figure 1a, no distinct clusters were formed, at randomization, based on the treatment group. PERMANOVA testing³ confirmed no statistically significant differences in composition and abundance of bacteria between AZM and placebo groups samples at randomization ($p=0.7$).

Changes in nasal lavage microbial communities following the study treatments.

Richness ($p=0.4$) and Shannon diversity ($p=0.8$) did not differ between the groups at the end of study treatments. In contrast, the composition and abundance of the bacterial communities changed substantially in both groups, as indicated by Figure 1S and the NMDS plots in Figure 1b. PERMANOVA testing³ confirmed statistically significant differences in community structures in both treatment groups at the end of study treatments compared to baseline ($p=0.004$, $p=0.004$ for the AZM and placebo groups).

The microbial changes over the treatment period showed different trajectories in each treatment group (Figures 1c-d). More specifically, among all the abundant genera ($>1\%$) in the placebo group (Figure 2), the relative abundances of *Dolosigranulum* and *Corynebacterium* significantly increased ($qvalue=0.03$, 0.03 ; respectively), while the relative abundance of *Streptococcus* significantly decreased ($qvalue=0.01$). In the AZM group (Figure 2), the relative abundance of *Moraxella* significantly decreased ($qvalue=0.03$), and only 3 participants had detectable levels of *Moraxella* at the end of the AZM treatment. Despite the similarity of bacteria composition and abundance at randomization, at the end of study treatments, the overall bacterial composition and

abundance measured by PERMANOVA test³ differed significantly between the 2 groups ($p=0.001$).

The *Moraxella* genus was the only bacterial taxon that its abundance was statistically different between the groups at the end of the study treatments. The relative abundance of *Moraxella* was significantly lower in the AZM group (median 0.05%, IQR 0-7.6%) compared to the placebo group (median 9.0%, IQR 0.1 -33.1%; q value=0.009, Figure 2S). Additional confirmatory analysis, which adjusted *Moraxella* abundance at day 14 to its abundance measured at randomization, confirmed the significant difference in *Moraxella* abundance between the groups ($p=0.002$).

Lower *Moraxella* abundance at the end of study treatments was associated with a reduced risk of post-RSV RW

Lower *Moraxella* abundance, measured at the end of study treatments in the whole study population, irrespective of treatment assignment, was significantly associated with lower odds of experiencing subsequent RW (OR=0.86; 95% CI 0.75-0.99, $p=0.03$). This analysis, when confined to the placebo group to minimize potential confounding other potential AZM beneficial effects (e.g., anti-inflammatory effects), showed the same effect size on the reduction in the odds of RW, but did not reach statistical significance levels (OR 0.86; 0.75 - 1.01, $p=0.07$). We could not reliably estimate an OR for the AZM group alone, since only 3 participants had measurable levels of *Moraxella* at the end of the AZM treatment, and recurrent wheezing was an infrequent event among participants treated with AZM.

In order to investigate potential confounding of the association between *Moraxella* abundance and RW by baseline characteristics of study participants (listed in Table 1S), we included these covariates, one at the time, in a regression analysis and found that none of these covariates significantly contributed to the model (data not shown).

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Supplement figure legends:

Figure S1. Relative abundance of the dominant bacteria (>1%) before and after the study treatment, as illustrated by heat-map. The colors describe the different abundance of each bacterial genus. Each column corresponds to one sample, while each row represents the bacterial genus.

Figure S2. Relative abundance of *Moraxella* in the azithromycin and Placebo groups at the end of study treatments (day 14). *Moraxella* abundance is significantly lower in azithromycin group compared to the placebo group (qvalue=0.009).

Figure S3. A schematic model of the possible mechanism through which azithromycin decreases the occurrence of post-RSV recurrent wheeze.

