

Long-Term Azithromycin Reduces *Haemophilus influenzae* and Increases Antibiotic Resistance in Severe Asthma

Steven L. Taylor^{1,2}, Lex E. X. Leong^{1,2}, Fredrick M. Mobegi^{1,2}, Jocelyn M. Choo^{1,2}, Steve Wesselingh^{1,2}, Ian A. Yang^{3,4}, John W. Upham^{3,5}, Paul N. Reynolds^{6,7}, Sandra Hodge^{6,7}, Alan L. James^{8,9}, Christine Jenkins^{10,11}, Matthew J. Peters^{11,12}, Melissa Baraket^{13,14}, Guy B. Marks^{13,14,15}, Peter G. Gibson^{15,16}, Geraint B. Rogers^{1,2*}, and Jodie L. Simpson^{16*}

¹South Australian Health and Medical Research Institute, Adelaide, South Australia, Australia; ²South Australian Health and Medical Research Institute Microbiome Research Laboratory, College of Medicine and Public Health, Flinders University, Bedford Park, South Australia, Australia; ³Faculty of Medicine, The University of Queensland, St. Lucia, Queensland, Australia; ⁴Department of Thoracic Medicine, The Prince Charles Hospital, Chermide, Queensland, Australia; ⁵Translational Research Institute, Princess Alexandra Hospital, Woolloongabba, Queensland, Australia; ⁶Department of Thoracic Medicine, Lung Research Unit, Royal Adelaide Hospital, Adelaide, South Australia, Australia; ⁷School of Medicine, The University of Adelaide, Adelaide, South Australia, Australia; ⁸Department of Pulmonary Physiology and Sleep Medicine, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia; ⁹School of Medicine and Pharmacology, University of Western Australia, Crawley, Western Australia, Australia; ¹⁰Respiratory Trials, The George Institute for Global Health, New South Wales, Australia; ¹¹Department of Thoracic Medicine, Concord General Hospital, New South Wales, Australia; ¹²Australian School of Advanced Medicine, Macquarie University, New South Wales, Australia; ¹³Respiratory Medicine Department and Ingham Institute, Liverpool Hospital, New South Wales, Australia; ¹⁴South Western Sydney Clinical School, University of New South Wales, Sydney, New South Wales, Australia; ¹⁵Woolcock Institute of Medical Research, Glebe, New South Wales, Australia; and ¹⁶Respiratory and Sleep Medicine, Priority Research Centre for Healthy Lungs, The University of Newcastle, Callaghan, New South Wales, Australia

ORCID IDs: 0000-0003-4357-8243 (S.L.T.); 0000-0002-5073-2272 (L.E.X.L.); 0000-0003-0554-9919 (F.M.M.); 0000-0001-9500-8360 (J.M.C.); 0000-0002-0017-3433 (J.W.U.); 0000-0002-6018-0547 (A.L.J.); 0000-0003-2717-5647 (C.J.); 0000-0002-9649-8741 (M.J.P.); 0000-0001-5865-489X (P.G.G.); 0000-0003-4054-1947 (G.B.R.); 0000-0001-5299-4236 (J.L.S.).

Abstract

Rationale: The macrolide antibiotic azithromycin reduces exacerbations in adults with persistent symptomatic asthma. However, owing to the pleiotropic properties of macrolides, unintended bacteriological consequences such as augmented pathogen colonization or dissemination of antibiotic-resistant organisms can occur, calling into question the long-term safety of azithromycin maintenance therapy.

Objectives: To assess the effects of azithromycin on the airway microbiota, pathogen abundance, and carriage of antibiotic resistance genes.

Methods: 16S rRNA sequencing and quantitative PCR were performed to assess the effect of azithromycin on sputum microbiology from participants of the AMAZES (Asthma and Macrolides: The Azithromycin Efficacy and Safety) trial: a 48-week, double-blind, placebo-controlled trial of thrice-weekly 500 mg oral azithromycin in adults with persistent uncontrolled asthma. Pooled-template shotgun metagenomic sequencing, quantitative PCR, and isolate whole-genome sequencing were performed to assess antibiotic resistance.

Measurements and Main Results: Paired sputum samples were available from 61 patients ($n = 34$ placebo, $n = 27$ azithromycin). Azithromycin did not affect bacterial load ($P = 0.37$) but did significantly decrease Faith's phylogenetic diversity ($P = 0.026$) and *Haemophilus influenzae* load ($P < 0.0001$). Azithromycin did not significantly affect levels of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Moraxella catarrhalis*. Of the 89 antibiotic resistance genes detected, five macrolide resistance genes and two tetracycline resistance genes were increased significantly.

Conclusions: In patients with persistent uncontrolled asthma, azithromycin reduced airway *H. influenzae* load compared with placebo but did not change total bacterial load. Macrolide resistance increased, reflecting previous studies. These results highlight the need for studies assessing the efficacy of nonantibiotic macrolides as a long-term therapy for patients with persistent uncontrolled asthma.

Keywords: asthma; macrolides; *Haemophilus influenzae*; antibiotic resistance

(Received in original form September 22, 2018; accepted in final form March 13, 2019)

*Joint senior authors.

Supported by Australian National Health and Medical Research Council grant no. 569246 and a grant from the John Hunter Hospital Charitable Trust.

Author Contributions: Conception and design: S.L.T., P.G.G., G.B.R., and J.L.S. Acquisition of data: S.L.T., L.E.X.L., F.M.M., and J.M.C. Analysis and interpretation of data: S.L.T., L.E.X.L., F.M.M., J.M.C., and G.B.R. Drafting or revising of manuscript: S.L.T., G.B.R., and J.L.S. Final approval of manuscript: S.L.T., L.E.X.L., F.M.M., J.M.C., S.W., I.A.Y., J.W.U., P.N.R., S.H., A.L.J., C.J., M.J.P., M.B., G.B.M., P.G.G., G.B.R., and J.L.S.

Sequences are available at the European Nucleotide Archive, accession numbers PRJEB26356 and PRJEB27079.

Correspondence and requests for reprints should be addressed to Geraint B. Rogers, Ph.D., 5D332 Flinders Medical Centre, Flinders Drive, Bedford Park, South Australia 5042, Australia. E-mail: geraint.rogers@sahmri.com.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 200, Iss 3, pp 309–317, Aug 1, 2019

Copyright © 2019 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201809-1739OC on March 15, 2019

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Oral azithromycin has been shown to significantly reduce exacerbation rates and improve asthma-related quality of life in those with persistent uncontrolled asthma. Although the mechanism of benefit is poorly understood, long-term macrolide treatment in other respiratory contexts has been shown to significantly alter the composition of lower airway microbiology.

What This Study Adds to the

Field: This is the first study based on a randomized, double-blind, placebo-controlled trial to investigate the impact of azithromycin treatment on the asthma airway microbiome. The absence of selective increases in pathogenic bacteria with azithromycin treatment suggests that long-term therapy is not associated with deleterious impacts on airway microbiology. Indeed, significant changes in both airway microbiota diversity and levels of the important respiratory pathogen, *Haemophilus influenzae*, in those receiving azithromycin identifies potential contributors to treatment-associated clinical benefit. Through the combined application of metagenomics and culture-based methods, we report the impact of azithromycin treatment on the microbiota-wide carriage of antibiotic resistance genes and relate this to specific bacterial taxa. Although increases in resistance gene carriage were largely limited to viridans streptococci, identifying these changes greatly informs our understanding of the wider implications of long-term azithromycin therapy.

Asthma is a global health issue affecting an estimated 235 million people worldwide (1). For the majority of patients, standard corticosteroids and bronchodilator therapies manage symptoms effectively. However, for an estimated 50 million patients, asthma is characterized as persistent and uncontrolled (2). These patients experience more severe airway inflammation (3), exhibit an altered airway

microbiology (4, 5), and, importantly, are at a higher risk of severe exacerbations, which disproportionately contribute to asthma-associated hospitalizations and healthcare costs (6–8). Recently, long-term azithromycin therapy was shown to reduce exacerbations and improve quality of life in this patient population (9), signifying azithromycin therapy as a valuable addition to existing regimens for treating moderate to severe asthma (10). However, although clinical side effects from azithromycin were minimal, the potential for detrimental consequences of long-term therapy on airway microbiology remains poorly understood.

Azithromycin is a member of the macrolide drug class that can modulate multiple pathways of host immunity (11–13) and also inhibit bacterial protein synthesis (14) and reduce pathogen virulence (15). The use of macrolides has therefore been found to alter the composition of bacteria in the airways (16–19), including the growth of pathogenic organisms. In bronchiectasis, treatment with another macrolide antibiotic, erythromycin, has been shown to increase the relative abundance of the airway pathogen *Pseudomonas aeruginosa* in the lungs (16). In persistent uncontrolled asthma, the lower airway microbiota is associated with multiple markers of asthma severity, including lower lung function (4), neutrophilic inflammation (5), and corticosteroid resistance (20); however, the effect of azithromycin on the airway microbiota in these patients is largely unknown.

Along with selection of airway pathogens, the administration of macrolides has been previously reported to increase the carriage of macrolide-resistant bacteria (18, 21–27). Although macrolide resistance has not been shown to directly alter the capacity of macrolides to reduce exacerbations (21), prolonged therapy might yet have deleterious microbiological consequences. Macrolide resistance can be acquired in bacteria intrinsically, through genetic mutations, or extrinsically, through acquisition of transmissible macrolide resistance genes from an existing macrolide-resistant organism. Acquired macrolide resistance is a global health concern, particularly relating to the treatment of infections where macrolides are routinely prescribed, such as in nontuberculous *Mycobacterium* infections

(28) and atypical infections in community-acquired pneumonia (29) and sexually transmitted infections (30). Identifying reservoirs of transmissible resistance genes is a global health strategy to limit the dissemination of antibiotic resistance to the wider community; however, this has not been investigated in patients receiving azithromycin in asthma.

In this analysis from the AMAZES (Asthma and Macrolides: The Azithromycin Efficacy and Safety) study, we used undirected approaches to test the hypothesis that azithromycin affects the abundance of opportunistic pathogens and carriage of antibiotic resistance genes in the airways of patients with persistent uncontrolled asthma.

Methods

Study Population

AMAZES was a 12-month, double-blind, randomized, placebo-controlled trial (ACTRN12609000197235) to determine whether oral azithromycin decreases the frequency of asthma exacerbations in adults (≥ 18 yr) with symptomatic asthma despite current use of inhaled corticosteroids and long-acting bronchodilators who had no hearing impairment or abnormal prolongation of the corrected QT interval (9). Patients were randomly assigned (1:1) to receive azithromycin 500 mg or placebo three times per week for 48 weeks. Of the 420 patients recruited for the trial, sputum samples from 61 patients were analyzed in this study, on the basis of paired sample availability.

Sputum Collection and DNA Extraction

Induced sputum was collected at baseline and after 48 weeks of the trial, as described previously (9). Nucleic acid extraction was performed on frozen sputum samples as described previously, using a combined physical, enzymatic, and heat-based cell lysis, followed by phenol-chloroform extraction and DNA recovery using EZ-10 Spin columns (Bio Basic, Inc.) (5).

16S rRNA Gene Amplicon Sequencing and Shotgun Metagenomic Sequencing

The V1–3 hypervariable region of the bacterial 16S rRNA gene was sequenced

Table 1. Characteristics of Patients at Baseline

	Placebo	Azithromycin	P Value
<i>n</i>	34	27	
Age, yr*	57.7 (17.4)	59.4 (12.5)	0.682
Age at asthma diagnosis, yr [†]	9.50 (0.00–61.0)	23.0 (1.0–70.0)	0.132
Asthma duration [†]	41.8 (2.14–70.0)	30.2 (3.4–62.4)	0.076
Female [‡]	12 (35)	15 (56)	0.129
Height, cm*	168 (9.69)	167 (9.8)	0.699
Weight, kg [†]	86.7 (50.9–115)	83.5 (49.1–167)	0.691
Body mass index, kg/m ^{2*}	30.5 (5.46)	30.9 (6.85)	0.769
Atopy [‡]	31 (94)	21 (78)	0.124
Ever-smoker [†]	9 (26)	8 (30)	>0.999
Pack-years [†]	6.00 (0.20–19.0)	25.8 (0.50–60.0)	0.086
ACQ score*	1.71 (0.83)	1.68 (0.91)	0.783
Medications			
ICS dose, BDP, µg/d [†]	1,000 (200–4,000)	1,000 (400–2,640)	0.520
ICS/long-acting β-agonist combination [†]	34 (100)	25 (93)	0.192
Leukotriene modifier [†]	1 (2.9)	0	>0.999
Long-acting antimuscarinic [†]	6 (18)	8 (30)	0.361
Theophylline [†]	0	2 (7.4)	0.192
Oral corticosteroid [†]	0	1 (3.7)	0.443
Pre-B2 spirometry*			
Pre-B2 FEV ₁ %	70.9 (16.7)	69.3 (16.9)	0.698
Pre-B2 FVC%	81.2 (15.2)	82.2 (12.0)	0.780
Pre-B2 FEV ₁ /FVC%	66.9 (9.18)	66.0 (11.9)	0.755
Blood eosinophils, ×10 ⁹ /L [†]	0.30 (0.11–12.4)	0.30 (0.10–0.90)	0.592
Sputum cell counts [†]			
Total cell count, ×10 ⁶ /ml	5.76 (1.08–69.9)	5.13 (0.72–36.0)	0.462
Neutrophils, %	37.5 (0.25–95.8)	35.5 (0.68–83.5)	0.620
Eosinophils, %	1.75 (0.00–23.8)	2.0 (0.0–83.0)	0.818
Sputum phenotype [†]			
Eosinophilic	14 (41)	11 (44) [§]	0.862
Neutrophilic	7 (21)	3 (12) [§]	0.321
Paucigranulocytic	11 (32)	10 (40) [§]	0.888
Mixed	2 (5.9)	1 (4.0) [§]	0.696
Sputum bacterial count, ×10 ⁵ /µl of DNA [†]	5.66 (0.124–77.7)	3.71 (0.059–95.8)	0.423
Sputum microbiota diversity [†]			
Faith's phylogenetic diversity	11.48 (5.52–25.0)	11.2 (3.56–15.3) [§]	0.547
Simpson's evenness	0.117 (0.023–0.205)	0.117 (0.032–0.210) [§]	0.423
Shannon-Wiener diversity	5.66 (2.41–7.19)	5.60 (1.22–6.42) [§]	0.885
Taxon richness	56 (27–83)	52 (11–85) [§]	0.645

Definition of abbreviations: ACQ = asthma cough questionnaire; B2 = β₂-adrenergic agonist; BDP = beclomethasone dipropionate; ICS = inhaled corticosteroids.

*Mean (SD), Student's *t* test.

[†]Median (minimum–maximum), Mann-Whitney *U* test.

[‡]*n* (%), Fisher exact test.

[§]Data available for 25 participants.

from sputa DNA (5). Briefly, reads were processed using QIIME v1.9.1 and assigned taxonomy on the basis of 97% sequence similarity, using the SILVA database v1.23 as a reference. Shotgun metagenomic sequencing was performed on DNA extracts pooled according to treatment group and time point as described previously (31). Pooled template was sequenced on an Illumina HiSeq 4000 platform with paired-end 150-bp reads. After quality filtering and human DNA removal (see Table E1 in the online supplement), reads were *de novo* assembled using IDBA (32) and open

reading frames identified using MetaGeneMark (33). Screening of antibiotic resistance genes was performed by mapping genes to the Comprehensive Antibiotic Resistance Database (CARD) v1.1.3 (34). Levels of transmissible resistance genes that were increased in the azithromycin group from baseline but not in the placebo group were verified by targeted quantitative PCR (qPCR) assays.

qPCR

Table E2 describes the qPCR conditions used to quantify levels of total bacteria

(16S rRNA gene), *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus*. Table E3 describes the qPCR conditions used to quantify resistance genes levels for 23S ribosomal RNA methyltransferases (*erm*[B] and *erm*[F]), ATP-binding cassette ribosomal protection proteins (*mel* and *msr*[E]), major facilitator superfamily antibiotic efflux pump (*mef*), and tetracycline-resistant ribosomal protection proteins (*tet*[M] and *tet*[W]).

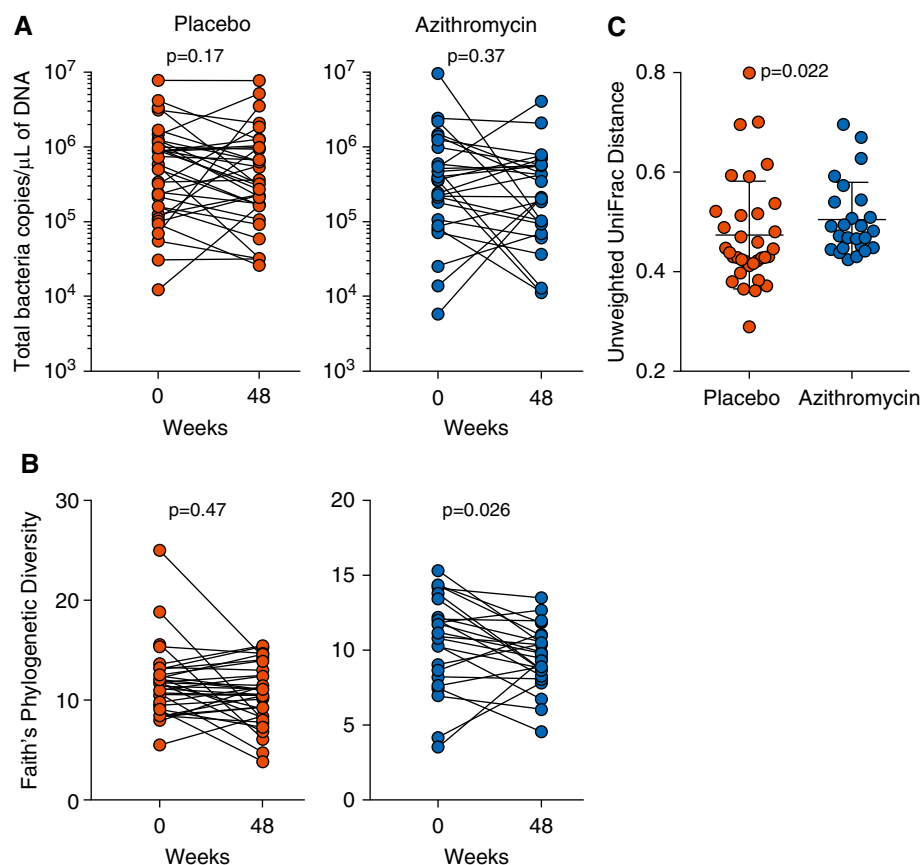


Figure 1. Effect of azithromycin on sputum bacterial load and microbiota composition. (A) Total bacterial load before and after placebo (left) or azithromycin (right). (B) Faith's phylogenetic diversity before and after placebo or azithromycin. (C) Unweighted UniFrac distance in placebo and azithromycin groups, where a higher dissimilarity distance indicates greater change in microbiota composition between samples before and after placebo or azithromycin.

Bacterial Culture and Whole-Genome Sequencing

H. influenzae, *Haemophilus parainfluenzae*, streptococci, and *S. aureus* were cultured from selected paired sputum samples from 15 of 27 patients collected at baseline and after 48 weeks of azithromycin therapy. Azithromycin sensitivities were determined according to European Committee on Antimicrobial Susceptibility Testing guidelines, by disk diffusion assay (35). Resistant isolates were subjected to whole-genome sequencing (WGS) and resistance determinants were identified using the Nullarbor pipeline v20170519 and by mapping genes to CARD.

Statistical Analysis

All continuous data were tested for normality using GraphPad Prism v7.03. Unpaired parametric data were analyzed by Student's *t* test, unpaired nonparametric data were

analyzed by the Mann-Whitney *U* test, paired nonparametric data were analyzed by the Wilcoxon matched-pairs signed rank test, and proportional data were analyzed by Fisher exact test. Permutational multivariate ANOVA of 16S rRNA taxonomic profiles was performed using the PRIMER 6 software (PRIMER-E Ltd.). 16S rRNA taxonomic changes were analyzed by linear discriminant analysis (LDA) effect size, with $\alpha = 0.05$, LDA score threshold = 2, and a "one versus all" test (36). All methods are described in detail in the online supplement.

Results

Baseline Demographics

The AMAZES study took place between June 12, 2009, and Jan 31, 2015; adults with persistent uncontrolled asthma were randomly assigned to either 500 mg of

azithromycin or placebo, three times per week for 48 weeks (9). Of these participants, raw sputum was available at both baseline and after 48 weeks from 27 patients who received azithromycin and 34 who received placebo. Patients had similar clinical characteristics and sputum microbiological measures at baseline between groups (Table 1) and were broadly representative of the total trial population, except sex (64% female in total trial vs. 44% in subgroup; $P = 0.007$) and blood eosinophil count (0.20×10^9 cells/L in total trial vs. 0.30×10^9 cells/L in subgroup; $P = 0.005$). For full details of baseline characteristics, see Tables E4–E6.

Azithromycin Therapy Reduces Sputum Phylogenetic Diversity

Paired comparisons of sputum between baseline and after 48 weeks of treatment indicated that azithromycin was not associated with a significant change in total bacterial load (Figure 1A). However, bacterial diversity was significantly reduced in the azithromycin group ($P = 0.026$) but not in placebo ($P = 0.47$; Figure 1B). Pairwise permutational multivariate ANOVA testing identified a significant difference in microbiome composition in the azithromycin group ($P = 0.014$; $t = 1.30$), which was not seen in the placebo group ($P = 0.988$; $t = 0.827$). This change was also reflected in higher unweighted UniFrac distance in the azithromycin group compared with the placebo group ($P = 0.022$; Figure 1C). Together, these results demonstrate that, in the lower airways, azithromycin therapy reduces bacterial diversity while total bacterial levels remain unchanged.

Azithromycin Therapy Reduces *H. influenzae* Load

Of all the taxa identified in the lower airways, Gammaproteobacteria showed the greatest reduction after azithromycin ($P = 0.039$; log LDA score = 4.68; Figure 2A) but not placebo (Figure E1). A range of species belong to Gammaproteobacteria, of which the absolute levels of the notable respiratory pathogen, *H. influenzae*, fell significantly in patients who received azithromycin ($P < 0.0001$) but not placebo ($P = 0.62$; Figure 2B). Other lower airway pathogens, including *S. pneumoniae*, *S. aureus*, *P. aeruginosa*, and *M. catarrhalis*, were not significantly affected by azithromycin (Figure E2). The changes in pathogen loads were also compared between placebo and azithromycin groups, with supportive findings (Table E7).

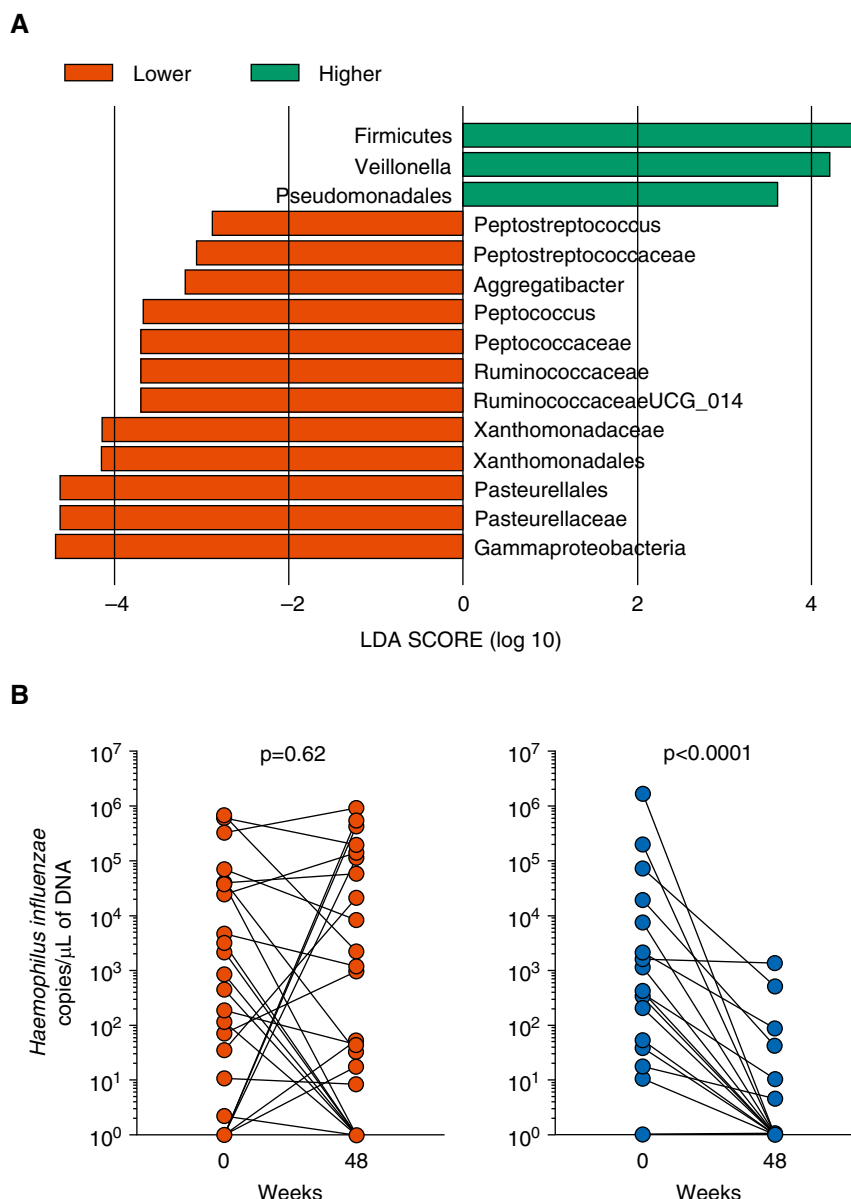


Figure 2. Effect of azithromycin on individual taxa. (A) Linear discriminant analysis (LDA) effect size of taxa that significantly changed in relative abundance after azithromycin. Red indicates taxa that were lower and green indicates taxa that were higher after azithromycin (B) *Haemophilus influenzae* copy number before and after either placebo (left) or azithromycin (right).

Azithromycin Therapy Increases Carriage of Antibiotic Resistance Genes

Carriage of antibiotic resistance genes was screened for by pooled template shotgun metagenomic analysis. A total of 89 antibiotic resistance genes were detected across all samples, of which seven transmissible genes (*tet*[W], *mel*, *msr*[E], *tet*[M], *erm*[F], *mef*[A], and *erm*[B]) were more highly represented after azithromycin but not placebo (Figure E3). Five of these

genes (*erm*[B], *erm*[F], *msr*[E], *mef*[A], and *mel*) confer resistance to macrolides, whereas two (*tet*[W] and *tet*[M]) confer resistance to tetracyclines (34). This effect was confirmed by qPCR, with all seven resistance genes significantly increased after azithromycin (Figure 3) but not placebo (Figure E4). The fold changes in antibiotic resistance gene carriage between placebo and azithromycin groups showed similar increased resistance gene carriage in the azithromycin group (Table E8).

Macrolide Resistance Genes Identified from Sputum Isolates

To investigate which species harbored transmissible resistance genes, bacteria were isolated from a subgroup of patients before and after azithromycin ($n=15$) and subjected to azithromycin susceptibility testing. The total proportion of azithromycin-resistant isolates increased significantly after azithromycin therapy ($P<0.0001$; Table 2), of which viridans streptococci were the only significant subgroup ($P=0.001$; Table 2). WGS identified macrolide resistance genes *erm*(B), *mel*, and *mef*(A), as well as *tet*(W) and *tet*(M) in resistant viridans streptococci (Table E9). Six of the seven streptococci with both *erm*(B) and *tet*(M) were found to contain both genes on a Tn916 mobile genetic element. Of the four azithromycin-resistant *H. parainfluenzae* isolated, two contained the putative mutation in the 50S ribosomal protein L4 (G65A) associated with macrolide resistance (37), and two had no recognized genetic mechanism for macrolide resistance (Table E9). Of the two azithromycin-resistant *S. aureus* isolates, one contained *erm*(A) and one contained *msr*(C) (Table E9).

Discussion

Long-term azithromycin treatment effectively reduces exacerbation rates in adults with persistent uncontrolled asthma (9). However, concerns about the potential negative effects on the lower airway microbiology and antimicrobial resistance impede its widespread adoption to the clinical setting. The results presented here show that although azithromycin has a selective effect on the lower airway microbiota, it does not alter total bacterial levels, nor does it increase the abundance of pathogenic bacteria. In fact, the amount of *H. influenzae* significantly decreased with azithromycin compared with placebo. However, regarding antimicrobial resistance carriage, azithromycin increased carriage of macrolide and nonmacrolide resistance genes. As these results were determined using an undirected metagenomic approach, they provide a comprehensive assessment of the effect of azithromycin on the airway microbiome and resistome in severe asthma.

In addition to asthma, azithromycin has been shown to be effective in a range of chronic respiratory diseases, including chronic obstructive pulmonary disease

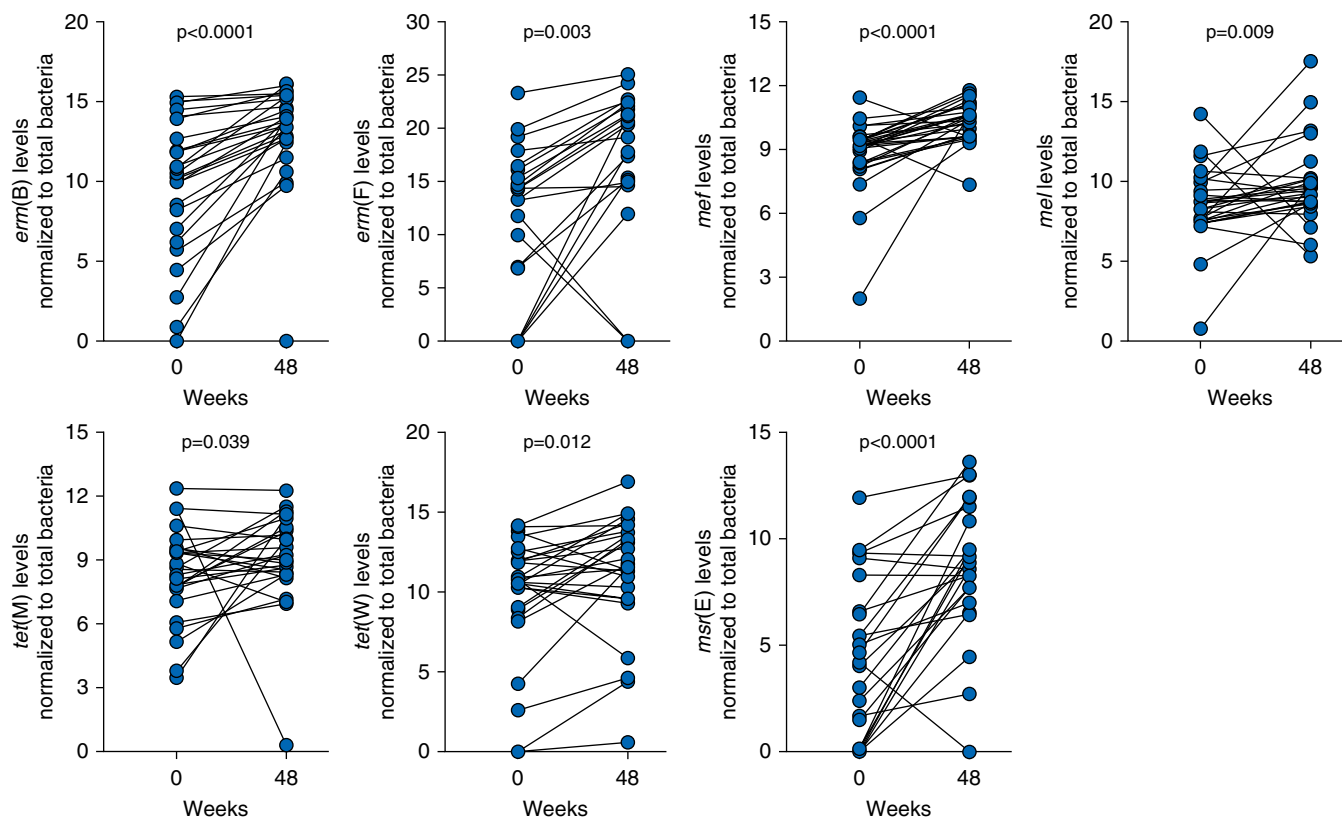


Figure 3. Effect of azithromycin on levels of screened antibiotic resistance genes. Levels of the seven antibiotic resistance genes before and after azithromycin. Genes identified by pooled-template shotgun metagenomic sequencing were quantified by quantitative PCR and normalized to total bacteria.

(COPD) (38), cystic fibrosis (CF) (39), bronchiectasis (21, 40), and diffuse panbronchiolitis (41). However, the effect of macrolides on carriage of respiratory pathogens in these diseases is mixed. In childhood bronchiectasis, for example, 12 to 24 months of azithromycin was associated with a reduction in cultured *H. influenzae* (40), reflecting the results reported here. In adult bronchiectasis, 48 weeks of erythromycin was also associated with a reduction in *H. influenzae*; however, an increase in *P. aeruginosa* predominance was reported (16). In childhood cystic fibrosis, 18 months of azithromycin did not affect positive culture of *P. aeruginosa* (42).

Furthermore, in diffuse panbronchiolitis, erythromycin therapy significantly improved symptoms in patients infected with *H. influenzae* or *P. aeruginosa*, with no change found in the bacterial flora (41). We observed no change to *P. aeruginosa* abundance; however, *Pseudomonas* is uncommon in persistent adult asthma (5). Investigation of the potential for *Pseudomonas* to colonize the airways of patients with asthma receiving azithromycin is now warranted.

Azithromycin can act via multiple pathways in chronic airway diseases, which makes identifying the mechanism of action difficult. We report no significant decline in

total bacterial load in patients receiving azithromycin, which supports previous findings (19, 43) and suggests that any broad antibiotic effects are minimal. However, we found a decline in phylogenetic diversity, extending the findings of another study of 8 weeks of azithromycin therapy in COPD (19). The decline in phylogenetic diversity indicates azithromycin has a potentially selective effect on particular bacteria. This is further evident in the weighted and unweighted UniFrac results, where unweighted UniFrac distances were significantly higher in azithromycin than in placebo, but weighted UniFrac distances were not ($P = 0.74$; data not shown). Unweighted UniFrac distance is calculated by the presence or absence of detected taxa, whereas weighted UniFrac accounts for relative abundance and is useful for examining differences in community structure (44). The discrepancy between weighted and unweighted UniFrac indicates that azithromycin has specific selective effects that result in the loss of detection of certain taxa, rather than a change in their relative abundance. The

Table 2. Proportion of Azithromycin-Resistant Isolates before and after Therapy

	Before	After	P Value
<i>Haemophilus influenzae</i>	0/4 (0%)	0/0 (0%)	>0.99
<i>Haemophilus parainfluenzae</i>	2/13 (15%)	2/2 (100%)	0.057
Viridans streptococci	24/33 (73%)	33/33 (100%)	0.001
<i>Staphylococcus aureus</i>	1/2 (50%)	2/2 (100%)	>0.99
Total species	27/52 (52%)	37/37 (100%)	<0.0001

Fisher exact test was used; data were available for 15 patients.

stark decline in *H. influenzae* load after azithromycin, but the relative stability of *S. pneumoniae* levels, exemplifies this. Results from LDA effect size analysis indicate that other members of the Gammaproteobacteria class may also be affected by azithromycin; however, qPCR analysis demonstrated that this was not the case for *P. aeruginosa* and *M. catarrhalis*.

The selective impact of azithromycin specific to *H. influenzae* (and potentially other Gammaproteobacteria) could reflect a number of mechanisms. First, azithromycin has been shown to affect *H. influenzae* biofilm formation (45), which may impair its ability to persist in the lower airways. Second, azithromycin has been shown to improve immune-mediated bacterial clearance. For example, azithromycin improves both macrophage efferocytic function and immune-driven clearance of bacteria, both *in vitro* (46, 47) and when given at low dose to patients with COPD (48–50). In these studies, microbial colonization of the airway, especially by *H. influenzae*, was speculated to contribute to the defective alveolar macrophage phagocytic function in COPD and subsequent airway inflammation. Third, azithromycin may reduce airway inflammation through its effects on bacterial antiinflammatory metabolite production. Such a mechanism was previously shown in a study of azithromycin in COPD (19).

The other primary aim of this study was to assess the carriage of antibiotic resistance genes after azithromycin, as resistance is a known effect (18, 21–27). After screening for all characterized antibiotic resistance genes, we found that seven transmissible resistance genes were higher in the airways after azithromycin (compared with

baseline) but not higher in placebo, of which five (*mel*, *msr*[E], *erm*[F], *mef*[A], and *erm*[B]) confer resistance to azithromycin. These genes had a median fold increase between 2 and 22 compared with baseline. The increase in *tet*(W) and *tet*(M) are of interest, as both are tetracycline resistance genes. From the streptococcal WGS data, *tet*(M) was found on the same mobile genetic element as *erm*(B), which is a well-characterized mobile genetic element (51) and has been shown previously to increase streptococci after macrolide therapy (23). The observed increase in *tet*(W) appears to be novel in relation to macrolide therapy. *tet*(W) was detected in an azithromycin-resistant *Streptococcus oralis* isolate; however, further investigation is required to determine whether carriage of this gene occurs within other prominent species. These findings indicate the wider consequences of azithromycin therapy beyond macrolide resistance alone and potentially support further research into the efficacy of nonantibiotic macrolides (47).

This study has several limitations. Although the pooled-template shotgun metagenomics analysis identified the top resistance genes increased across all included patients, other resistance genes that were not found at high abundance across multiple patients may have been missed. Furthermore, the limited culture-based analysis failed to isolate any bacteria carrying *erm*(F) and *msr*(E), despite their increased carriage rate. It is likely that organisms other than streptococci, *Haemophilus*, and *S. aureus* harbor these genes but were not cultured in this study. For example, sequenced *Prevotella* and *Porphyromonas* isolates, which are common in the airways,

have regions that map to *erm*(F), whereas sequenced *Rothia* and *Pseudomonas* isolates have regions that map to *msr*(E). Furthermore, changes to microbiology and resistance gene carriage between 0 and 48 weeks were analyzed between treatment arms. Analysis at shorter collection intervals would provide additional insight into rate of change for microbiological parameters, and analysis beyond week 48 weeks will allow the persistence of these changes to be assessed. This study did not assess the contribution of potentially confounding variables to changes in airway microbiology, including inhaled corticosteroids dose, gastroesophageal reflux, or acute antibiotics prescribed for exacerbations.

In conclusion, we found that azithromycin did not affect the total bacterial load in the lower airways of patients with persistent uncontrolled asthma. However, azithromycin was associated with a significant decrease in the total diversity of taxa, including a significant decline in the pathogenic species *H. influenzae*, an effect that potentially contributes to treatment-associated benefit. A significant increase in the carriage of antibiotic resistance genes was also found, which included macrolide resistance genes and tetracycline resistance genes. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Flinders Medical Centre Microbiology Facility and Lito Papanicolas for their generous assistance with bacterial culture and species identification, and Heather Powell for assistance in collection of clinical data.

References

- World Health Organization. Asthma: key facts. 2017 [accessed 2019 Jun 27]. Available from: <http://www.who.int/news-room/fact-sheets/detail/asthma>.
- Peters SP, Ferguson G, Deniz Y, Reisner C. Uncontrolled asthma: a review of the prevalence, disease burden and options for treatment. *Respir Med* 2006;100:1139–1151.
- Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, *et al*. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343–373. [Published erratum appears in *Eur Respir J* 52:1352020.]
- Green BJ, Wiriyaichaiorn S, Grainge C, Rogers GB, Kehagia V, Lau L, *et al*. Potentially pathogenic airway bacteria and neutrophilic inflammation in treatment resistant severe asthma. *PLoS One* 2014;9:e100645.
- Taylor SL, Leong LEX, Choo JM, Wesselingh S, Yang IA, Upham JW, *et al*. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J Allergy Clin Immunol* 2018;141:94–103, e15.
- Wang W, Li JJ, Foster PS, Hansbro PM, Yang M. Potential therapeutic targets for steroid-resistant asthma. *Curr Drug Targets* 2010;11:957–970.
- Calhoun WJ, Haselkorn T, Mink DR, Miller DP, Dorenbaum A, Zeiger RS. Clinical burden and predictors of asthma exacerbations in patients on guideline-based steps 4–6 asthma therapy in the TENOR cohort. *J Allergy Clin Immunol Pract* 2014;2:193–200.
- Ivanova JI, Bergman R, Birnbaum HG, Colice GL, Silverman RA, McLaurin K. Effect of asthma exacerbations on health care costs among asthmatic patients with moderate and severe persistent asthma. *J Allergy Clin Immunol* 2012;129:1229–1235.

9. Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, *et al.* Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. *Lancet* 2017;390:659–668.
10. Brusselle G, Pavord I. Azithromycin in uncontrolled asthma. *Lancet* 2017;390:629–630.
11. Altenburg J, de Graaff CS, van der Werf TS, Boersma WG. Immunomodulatory effects of macrolide antibiotics: part 2. Advantages and disadvantages of long-term, low-dose macrolide therapy. *Respiration* 2011;81:75–87.
12. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am J Respir Crit Care Med* 2008;177:148–155.
13. Gielen V, Johnston SL, Edwards MR. Azithromycin induces anti-viral responses in bronchial epithelial cells. *Eur Respir J* 2010;36:646–654.
14. Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA. The structures of four macrolide antibiotics bound to the large ribosomal subunit. *Mol Cell* 2002;10:117–128.
15. Imperi F, Leoni L, Visca P. Antivirulence activity of azithromycin in *Pseudomonas aeruginosa*. *Front Microbiol* 2014;5:178.
16. Rogers GB, Bruce KD, Martin ML, Burr LD, Serisier DJ. The effect of long-term macrolide treatment on respiratory microbiota composition in non-cystic fibrosis bronchiectasis: an analysis from the randomised, double-blind, placebo-controlled BLESS trial. *Lancet Respir Med* 2014;2:988–996.
17. Slater M, Rivett DW, Williams L, Martin M, Harrison T, Sayers I, *et al.* The impact of azithromycin therapy on the airway microbiota in asthma. *Thorax* 2014;69:673–674.
18. Choo JM, Abell GCJ, Thomson R, Morgan L, Waterer G, Gordon DL, *et al.* Impact of long-term erythromycin therapy on the oropharyngeal microbiome and resistance gene reservoir in non-cystic fibrosis bronchiectasis. *mSphere* 2018;3:e00103-18.
19. Segal LN, Clemente JC, Wu BG, Wikoff WR, Gao Z, Li Y, *et al.* Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax* 2017;72:13–22.
20. Goleva E, Jackson LP, Harris JK, Robertson CE, Sutherland ER, Hall CF, *et al.* The effects of airway microbiome on corticosteroid responsiveness in asthma. *Am J Respir Crit Care Med* 2013;188:1193–1201.
21. Altenburg J, de Graaff CS, Stienstra Y, Sloos JH, van Haren EH, Koppers RJ, *et al.* Effect of azithromycin maintenance treatment on infectious exacerbations among patients with non-cystic fibrosis bronchiectasis: the BAT randomized controlled trial. *JAMA* 2013;309:1251–1259.
22. Serisier DJ, Martin ML, McGuckin MA, Lourie R, Chen AC, Brain B, *et al.* Effect of long-term, low-dose erythromycin on pulmonary exacerbations among patients with non-cystic fibrosis bronchiectasis: the BLESS randomized controlled trial. *JAMA* 2013;309:1260–1267.
23. Malhotra-Kumar S, Lammens C, Coenen S, Van Herck K, Goossens H. Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. *Lancet* 2007;369:482–490.
24. Brusselle GG, Vanderstichele C, Jordens P, Deman R, Slabbynck H, Ringoet V, *et al.* Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial. *Thorax* 2013;68:322–329.
25. Mustafa MH, Khandekar S, Tunney MM, Elborn JS, Kahl BC, Denis O, *et al.* Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients. *Eur Respir J* 2017;49:1601847.
26. Saiman L, Anstead M, Mayer-Hamblett N, Lands LC, Kloster M, Hocevar-Trnka J, *et al.*; AZ0004 Azithromycin Study Group. Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA* 2010;303:1707–1715.
27. Kastner U, Guggenbichler JP. Influence of macrolide antibiotics on promotion of resistance in the oral flora of children. *Infection* 2001;29:251–256.
28. Egelund EF, Fennelly KP, Peloquin CA. Medications and monitoring in nontuberculous mycobacteria infections. *Clin Chest Med* 2015;36:55–66.
29. Waterer GW, Rello J, Wunderink RG. Management of community-acquired pneumonia in adults. *Am J Respir Crit Care Med* 2011;183:157–164.
30. Workowski KA, Bolan GA; Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep* 2015;64:1–137. [Published erratum appears in *MMWR Recomm Rep* 64:924.]
31. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Burr LD, Wesselingh S, *et al.* Understanding the impact of antibiotic therapies on the respiratory tract resistome: a novel pooled-template metagenomic sequencing strategy. *Multidiscip Respir Med* 2018;13:30.
32. Peng Y, Leung HC, Yiu SM, Chin FY. IDBA-UD: a *de novo* assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 2012;28:1420–1428.
33. Zhu W, Lomsadze A, Borodovsky M. Ab initio gene identification in metagenomic sequences. *Nucleic Acids Res* 2010;38:e132.
34. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, *et al.* CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2017;45:D566–D573.
35. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect* 2014;20:O255–O266.
36. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
37. Peric M, Bozdogan B, Jacobs MR, Appelbaum PC. Effects of an efflux mechanism and ribosomal mutations on macrolide susceptibility of *Haemophilus influenzae* clinical isolates. *Antimicrob Agents Chemother* 2003;47:1017–1022.
38. Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JAD Jr, Criner GJ, *et al.*; COPD Clinical Research Network. Azithromycin for prevention of exacerbations of COPD. *N Engl J Med* 2011;365:689–698. [Published erratum appears in *N Engl J Med* 366:1356.]
39. Southern KW, Barker PM, Solis-Moya A, Patel L. Macrolide antibiotics for cystic fibrosis. *Cochrane Database Syst Rev* 2012;11:CD002203.
40. Valery PC, Morris PS, Byrnes CA, Grimwood K, Torzillo PJ, Bauert PA, *et al.* Long-term azithromycin for Indigenous children with non-cystic-fibrosis bronchiectasis or chronic suppurative lung disease (Bronchiectasis Intervention Study): a multicentre, double-blind, randomised controlled trial. *Lancet Respir Med* 2013;1:610–620.
41. Kudoh S, Uetake T, Hagiwara K, Hirayama M, Hus L-H, Kimura H, *et al.* Clinical effects of low-dose long-term erythromycin chemotherapy on diffuse panbronchiolitis [in Japanese]. *Nihon Kyobu Shikkan Gakkai Zasshi* 1987;25:632–642.
42. Mayer-Hamblett N, Retsch-Bogart G, Kloster M, Accurso F, Rosenfeld M, Albers G, *et al.*; OPTIMIZE Study Group. Azithromycin for early *Pseudomonas* infection in cystic fibrosis: the OPTIMIZE randomized trial. *Am J Respir Crit Care Med* 2018;198:1177–1187.
43. Brill SE, Law M, El-Emir E, Allinson JP, James P, Maddox V, *et al.* Effects of different antibiotic classes on airway bacteria in stable COPD using culture and molecular techniques: a randomised controlled trial. *Thorax* 2015;70:930–938.
44. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl Environ Microbiol* 2007;73:1576–1585.
45. Starnes TD, Shrout JD, Parsek MR, Appelbaum PC, Kim G. Subinhibitory concentrations of azithromycin decrease nontypeable *Haemophilus influenzae* biofilm formation and diminish established biofilms. *Antimicrob Agents Chemother* 2008;52:137–145.

46. Ween M, Ahern J, Carroll A, Hodge G, Pizzutto S, Jersmann H, *et al.* A small volume technique to examine and compare alveolar macrophage phagocytosis of apoptotic cells and non typeable *Haemophilus influenzae* (NTHi). *J Immunol Methods* 2016;429:7–14.
47. Hodge S, Tran HB, Hamon R, Roscioli E, Hodge G, Jersmann H, *et al.* Nonantibiotic macrolides restore airway macrophage phagocytic function with potential anti-inflammatory effects in chronic lung diseases. *Am J Physiol Lung Cell Mol Physiol* 2017;312:L678–L687.
48. Hodge S, Hodge G, Jersmann H, Matthews G, Ahern J, Holmes M, *et al.* Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;178:139–148.
49. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. *Respirology* 2012;17: 802–807.
50. Hodge S, Jersmann H, Reynolds PN. The effect of colonization with potentially pathogenic microorganisms on efferocytosis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2016; 194:912–915.
51. Clewell DB, Flannagan SE, Jaworski DD. Unconstrained bacterial promiscuity: the Tn916-Tn1545 family of conjugative transposons. *Trends Microbiol* 1995;3:229–236.

Long-term azithromycin reduces *Haemophilus influenzae* and increases antibiotic resistance in severe asthma

Steven L. Taylor, Lex E.X. Leong, Fredrick M. Mobegi, Jocelyn M. Choo,
Steve Wesselingh, Ian A. Yang, John W. Upham, Paul N. Reynolds, Sandra Hodge,
Alan L. James, Christine Jenkins, Matthew J. Peters, Melissa Baraket, Guy B. Marks,
Peter G. Gibson, Geraint B. Rogers, and Jodie L. Simpson

ONLINE DATA SUPPLEMENT

Online Supplement

Supplementary Methods

16S rRNA gene amplicon sequencing and bioinformatic processing

The V1-3 hypervariable region of the bacterial 16S rRNA gene was amplified from sputum

DNA using modified 27F (5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGRGTGTTGATCMTGGCTCAG-

3') and 519R (5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTNTTACNGCGGCKGCTG-3')

primers, where the underlined region indicates the Illumina adapter overhang sequences.

Amplicons were cleaned, indexed and sequenced according to the Illumina MiSeq 16S

Metagenomic Sequencing Library Preparation protocol. 16S rRNA sequence data was

processed as previously described (E1) using QIIME v1.9.1. Briefly, Operational Taxonomic

Unit (OTU) assignment was performed using an open reference approach with the UCLUST

algorithm based on a 97% sequence similarity to the SILVA reference database v1.23. Prior to

OTU assignment, sequences with less than 80% similarity to the reference databases were

discarded. Spurious OTUs were removed systematically using previous reports of common

laboratory sequencing contaminants (E2). Sequence data were subsampled to 1,833 reads. Two

samples from the azithromycin group did not reach this threshold and were removed.

Sequencing data are deposited in the European Nucleotide Archive database (PRJEB26356).

Microbiome diversity metrics (Faith's phylogenetic diversity and UniFrac distances) were

computed using QIIME v1.9.1.

Pooled-template shotgun metagenomic sequencing and bioinformatic processing

Resistance genes that increased following azithromycin therapy were screened using pooled-

template shotgun metagenomic analysis, as described previously (E3). Sputum DNA extracts

were pooled by treatment type (azithromycin or placebo) and time point (at baseline and after

48 weeks). These four pooled samples were then processed for shotgun metagenomic sequencing using the Illumina TruSeq Nano DNA kit and sequenced on an Illumina HiSeq 4000 with paired-end 150bp reads. Sequencing data are deposited in the European Nucleotide Archive database (PRJEB27079). Reads were trimmed using trimmomatic v0.36 (E4), human reads removed using BBMap v37.25 (E5). Summary of read counts are displayed in Table E1. The remaining trimmed, paired, non-human reads were *de novo* assembled into contigs using IDBA v1.1.3 (E6). Unpaired reads were separately assembled into contigs using IDBA v1.1.3. Genes were identified from both paired and unpaired contigs using MetaGeneMark v3.26 (E7) and collapsed using CDHit v4.6.6 (E8).

50,361 genes with greater than 100 bp were identified and concatenated into a non-redundant gene catalogue. Blast+ v2.6.0 was used to identify antimicrobial resistance genes from the CARD database v1.1.3 (E9) with an evalue score $< 1 \times 10^{-20}$. Validation of resistance gene assignment was performed by mapping the gene-catalogue to the MEGARes database v1.01 (E10). Reads from each pooled sample were then aligned to the gene-catalogue using Soap v2.21 (E11) and antimicrobial resistance genes quantified. Sample counts were normalised to counts per million (CPM) total reads and change in CPM (ΔCPM) was calculated by $\text{CPM}_{\text{post treatment}} - \text{CPM}_{\text{pre treatment}}$.

Determination of differential resistance gene carriage

Differential carriage of antimicrobial resistance genes was identified from the pooled-template shotgun metagenomic analysis using the following criteria: 1) The gene is increased in the azithromycin group from baseline, 2) The gene is not increased in the placebo group, and 3) The gene has the potential to be transmitted between species. Of the 89 antimicrobial resistance genes identified, genes *tet(W)*, *mel*, *msr(E)*, *tetM*, *erm(F)*, *mef(A)*, and *erm(B)* matched all 3 criteria.

Resistance gene and bacterial species quantification

Levels of resistance genes *erm*(B), *erm*(F), *mel*, *tet*(M) and *tet*(W) were assessed using SYBR Green assays, and *mef* gene (detecting both *mef*(A) and *mef*(E)) was assessed using Taqman assay based on previously described primer pairs (Table E2). Primers were designed to assess levels of *msr*(E) using the assembled *msr*(E) gene from the metagenomic sequencing data. Primer optimization and specificity was determined by PCR and gel electrophoresis using a *Rothia mucilaginosa* isolate that was confirmed to harbour this *msr*(E) gene through WGS as a positive control. The 163 bp amplicon was confirmed to match *msr*(E) by Sanger sequencing. Levels of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* were quantified using SYBR Green assays and *Haemophilus influenzae* and *Staphylococcus aureus* were quantified using Taqman assays based on previously described primer pairs (Table E3). Determination of *Moraxella catarrhalis* was performed using a SYBR Green assay based on a modified primer pair from a previously described assay as described in Table E3. For SYBR Green qPCR assays, 1 µL of DNA extract, 0.2 µM of each primer, 17.5 µL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. For Taqman qPCR assays, 1 µL of DNA extract, 0.2 µM of each primer, 0.1 µM of target probe, 17.5 µL of 2X KAPA Probe Fast qPCR Master Mix (KAPA Biosystems Inc., Wilmington, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. Quantitative real-time PCR were performed on three technical replicates, at 10 µL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Mean cycle threshold (Ct) of resistance gene replicates were normalized to 16S mean Ct to give ΔCt . $\Delta Ct_{\max} - \Delta Ct$ was calculated so that a higher value represents higher gene carriage. Fold change in resistance gene was determined using the $2^{-\Delta \Delta Ct}$ method,(E12) using the 16S gene as the reference. Total bacteria (as determined by 16S quantification) and specific bacterial species were quantified to

a copy number per μL of extracted DNA (copies/ μL of DNA) by comparing sample Ct to a standard curve with known copy numbers.

Bacterial culture and whole genome sequencing and bioinformatic processing

Sputum was diluted and plated on HBA, Choc-B and SAIDE agar plates to isolate streptococci, haemophilus sp. and *Staphylococcus aureus*, respectively. Plates were cultured for 18-48 h at 35 °C and with 5% CO₂ (for HBA, Choc-B plates). Individual colonies were subcultured and identified by Matrix Assisted Laser Desorption/Ionization-time of flight mass spectrometry (MALDI-TOF), using a Bruker Microflex MS score ≥ 2.0 . Identification of *S. pneumoniae* was determined by optochin sensitivity. Azithromycin sensitivity was determined by disk-diffusion according to EUCAST protocols (E13) and using EUCAST Clinical Breakpoints from v. 8.1. This is the most current version and states the clinical breakpoint for *Haemophilus* is conflicting and should be guided by the epidemiological cut-offs. *Haemophilus* breakpoint sensitivity to azithromycin was therefore determined by comparing inhibition zones to that of macrolide-sensitive *H. influenzae* NTCC8468.

Following disk-diffusion testing, single isolate bacterial lawns were then suspended at approximately 5 MacFarland, in 10% glycerol/saline solution and stored at -80 °C. DNA extraction was performed on 50 μL of bacterial solution using a modified AxyPrep Mag Tissue-Blood gDNA Purification kit protocol (Corning, Big Flats, NY, USA), with an additional 2.9 mg/ml lysozyme and 0.14 mg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO, USA) 1 h at 37 °C incubation for gram positive bacteria. Bacterial DNA from selected resistant isolates were processed for whole genome sequencing (WGS) using Illumina Nextera XT DNA Library Preparation Kit according to preparation guidelines. The libraries were normalised to 2 nM and sequenced on an Illumina NextSeq 550. Sequences were *de novo* assembled using the Nullarbor pipeline v20170519 (E14) and the inferred protein sequences were used to identify

antibiotic resistance gene carriage using Blast+ v2.6.0 against the CARD database v1.1.3 with an evaluate threshold of $\leq 1 \times 10^{-20}$.

Table E1: Summary of pooled sample metagenomic reads

	Total reads	Quality human reads	Quality non-human reads
Azithromycin pre	112,860,634	92,617,788	6,135,414
Azithromycin post	130,838,966	107,280,206	5,647,466
Placebo pre	153,440,890	122,872,560	7,170,758
Placebo post	148,938,168	126,622,016	7,291,460

Table E2: Primers used to quantify antibiotic resistance gene carriage

Target gene	Sequence (5' - 3')	Amplicon size (bp)	Reference
	Resistance gene quantitative PCR		
<i>erm</i> (B)	GAAAGCCRTGCGTCTGACATC CGAGACTTGAGTGTGCAAGAGC	105	(E15)
<i>erm</i> (F)	CGGGTCAGCACTTTACTATTG GGACCTACCTCATAGACAAG	466	(E16, E17)
<i>mef</i>	TATGGAGCTACCTGTCTGGA GGTACTAAAAGTGGCGTAACC [HEX]- CCGTAGCATTGGAACAGCTTTTC- [BHQ1]	85	(E18)
<i>mel</i>	GAACGTAAGAGCCAAGCTGCA GGCACGTTCCGCAATAAATT	51	(E19)
<i>msr</i> (E)	TCGATACGAAGAGGCGATGC CTTCTGTTTGGTGCCGGTTG	163	This study
<i>tet</i> (M)	CAGAATTAGGAAGCGTGGACAA CCTCTCTGACGTTCTAAAAGCGTAT	67	(E20)
<i>tet</i> (W)	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	(E21)
16S	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	466	(E22)

Table E3: Primers used to quantify bacterial species levels

Organism	Sequence (5' - 3')	Amplicon (bp)	Reference
	Bacterial quantitative PCR		
<i>Haemophilus influenzae</i>	ATTAAATGTTGCATCAACGC GACTTTTGCCACGCAC [FAM]- ACGRTTTTACCATAGTTGCACTTTCT C-[BHQ1]	140	(E23)
<i>Streptococcus pneumoniae</i> / <i>Streptococcus pseudopneumoniae</i>	GTGCTITGAAATTCTATGCTTC GTGGAGCTACCTTATTTTTTAC	135	(E24)
<i>Staphylococcus aureus</i>	AAATTACATAAAGAACCTGCGACA GAATGTCATTGGTTGACCTTTGTA [FAM]- AATTAAACCGTATCACCATCAATCG CTTT-[BHQ1]	87	(E25)
<i>Pseudomonas aeruginosa</i>	CGAGTACAACATGGCTCTGG ACCGGACGCTCTTTACCATA	117	(E26)
<i>Moraxella catarrhalis</i>	GTGAGTGCCGCTTTTACAACC TGTATCGCCTGCCAAGACAA	72	(E27)

Table E4: Characteristics of patients at baseline for the subgroup analysed, and remaining patients from the AMAZES cohort

	AMAZES cohort	Subgroup	p-value
N	359	61	
Age, yrs*	57.7 (14.4)	58.5 (15.3)	0.693
Age asthma diagnosis, yrs†	22.0 (0.0, 80.0)	16.0 (0.0, 70.0)	0.551
Asthma duration†	31.9 (0.39, 75.6)	33.8 (2.14, 70.0)	0.207
Female‡	228 (64%)	27 (44%)	0.007
Height, cm*	166 (11.0)	168 (9.68)	0.238
Weight, kg†	82.2 (42.2, 176)	84.8 (49.1, 166)	0.329
Atopy‡	267 (76%)	52 (87%)	0.067
Ever-smoker‡	144 (40%)	17 (28%)	0.087
Pack years, yrs †	7.50 (0.0, 120)	7.20 (0.20, 60.0)	0.807
ACQ score*	1.53 (0.77)	1.69 (0.86)	0.131
Medications			
ICS dose, BDP µg/day †	1400 (160, 4500)	1000 (200, 4000)	0.225
ICS/ Long-acting beta agonist combination‡	353 (98%)	59 (97%)	0.328
Leukotriene modifier‡	13 (3.6%)	1 (1.6%)	0.703
Long-acting anti-muscarinic‡	59 (16%)	14 (23%)	0.206
Theophylline‡	11 (3.1%)	2 (3.3%)	>0.999
Oral corticosteroid‡	13 (3.6%)	1 (1.6%)	0.703
Pre B2 spirometry*			
Pre B2 FEV ₁ %	73.4 (20.25)	70.2 (16.7)	0.239
Pre B2 FVC%	83.0 (15.88)	81.7 (13.8)	0.528
Pre B2 FEV ₁ /FVC%	68.1 (12.72)	66.5 (10.4)	0.357
Blood eosinophils (× 10 ⁹) per L†	0.20 (0.00, 2.00)	0.30 (0.10, 12.4)	0.005
Sputum cell counts†			
Total cell count (× 10 ⁶) per mL	3.87 (0.31, 102.0)	5.13 (0.72, 69.9)	0.066
Neutrophils (%)	34.1 (0.00, 96.00)	37.3 (0.25, 95.8)	0.687
Eosinophils (%)	2.00 (0.00, 77.00)	1.75 (0.00, 83.0)	0.834
Sputum phenotype‡			
Eosinophilic	119 (44%)	25 (42%)	0.886
Neutrophilic	36 (13%)	10 (17%)	0.532
Paucigranulocytic	104 (38%)	21 (36%)	0.768
Mixed	13 (4.8%)	3 (5.1%)	>0.999

* Mean (SD), Student's t-test; † Median (min, max), Mann Whitney U test; ‡ n(%), Fisher's exact test; ACQ: asthma cough questionnaire, ICS: inhaled corticosteroids, BDP: beclometasone dipropionate, FEV₁ %: forced expiratory volume in one second % predicted, FVC: forced vital capacity % predicted.

Table E5: Characteristics of patients at baseline for placebo subgroup analysed and placebo group of remaining AMAZES cohort

	AMAZES placebo	Subgroup placebo	p-value
N	173	34	
Age, yrs*	57.0 (14.7)	57.7 (17.4)	0.787
Age asthma diagnosis, yrs [†]	23.0 (0.00, 80.0)	9.50 (0.00, 61.0)	0.116
Asthma duration [†]	30.0 (0.96, 72.2)	41.8 (2.14, 70.0)	0.019
Female [‡]	109 (63%)	12 (35%)	0.004
Height, cm*	167 (9.67)	168 (9.69)	0.375
Weight, kg [†]	80.6 (42.2, 176)	86.7 (50.9, 115)	0.176
Atopy [‡]	132 (78%)	31 (94%)	0.032
Ever-smoker [‡]	72 (42%)	9 (26%)	0.124
Pack years, yrs [†]	7.50 (0.10, 100)	6.00 (0.20, 19.0)	0.358
ACQ score*	1.51 (0.78)	1.71 (0.83)	0.196
Medications			
ICS dose, BDP µg/day [†]	1000 (250, 4500)	1000 (200, 4000)	0.709
ICS/ Long-acting beta agonist combination [‡]	170 (98%)	34 (100%)	>0.999
Leukotriene modifier [‡]	5 (2.9%)	1 (2.9%)	>0.999
Long-acting anti-muscarinic [‡]	27 (16%)	6 (18%)	0.798
Theophylline [‡]	6 (3.5%)	0	0.592
Oral corticosteroid [‡]	6 (3.5%)	0	0.592
Pre B2 spirometry*			
Pre B2 FEV ₁ %	74.1 (19.2)	70.9 (16.7)	0.372
Pre B2 FVC%	83.3 (15.14)	81.2 (15.2)	0.468
Pre B2 FEV ₁ /FVC%	68.5 (12.37)	66.9 (9.18)	0.453
Blood eosinophils (× 10 ⁹) per L [†]	0.25 (0.00, 1.74)	0.30 (0.11, 12.4)	0.057
Sputum cell counts [†]			
Total cell count (× 10 ⁶) per mL	3.96 (0.31, 27.9)	5.76 (1.08, 69.9)	0.075
Neutrophils (%)	32.4 (0.00, 96.0)	37.5 (0.25, 95.8)	0.401
Eosinophils (%)	2.63 (0.00, 77.0)	1.75 (0.00, 23.8)	0.379
Sputum phenotype [‡]			
Eosinophilic	63 (48%)	14 (41%)	0.565
Neutrophilic	18 (14%)	7 (21%)	0.296
Paucigranulocytic	44 (33%)	11 (32%)	>0.999
Mixed	7 (5.3%)	2 (5.9%)	>0.999

* Mean (SD), Student's t-test; [†] Median (min, max), Mann Whitney U test; [‡] n(%), Fisher's exact test; ACQ: asthma cough questionnaire, ICS: inhaled corticosteroids, BDP: beclometasone dipropionate, FEV₁ %: forced expiratory volume in one second % predicted, FVC: forced vital capacity % predicted.

Table E6: Characteristics of patients at baseline for azithromycin subgroup analysed and azithromycin group of remaining AMAZES cohort

	AMAZES Azithromycin	Subgroup Azithromycin	p-value
N	186	27	
Age, yrs*	58.3 (14.0)	59.4 (12.5)	0.711
Age asthma diagnosis, yrs [†]	21.0 (0.0, 75.0)	23.0 (1.0, 70.0)	0.391
Asthma duration [†]	32.9 (0.39, 75.6)	30.2 (3.4, 62.4)	0.466
Female [‡]	119 (64)	15 (55.6)	0.402
Height, cm*	165 (12.1)	167 (9.8)	0.468
Weight, kg [†]	83.4 (44.1, 164)	83.5 (49.1, 167)	0.959
Atopy [‡]	135 (74)	21 (78)	0.815
Ever-smoker [‡]	72 (39)	8 (30)	0.403
Pack years, yrs [†]	5.70 (0.0, 120.0)	25.8 (0.50, 60.0)	0.209
ACQ score*	1.54 (0.77)	1.68 (0.91)	0.403
Medications			
ICS dose, BDP µg/day [†]	1600 (160, 4000)	1000 (400, 2640)	0.157
ICS/ Long-acting beta agonist combination [‡]	183 (98%)	25 (93%)	0.122
Leukotriene modifier [‡]	8 (4.3%)	0	0.600
Long-acting anti-muscarinic [‡]	32 (17%)	8 (30%)	0.184
Theophylline [‡]	5 (2.7%)	2 (7.4%)	0.218
Oral corticosteroid [‡]	7 (3.8%)	1 (3.7%)	>0.999
Pre B2 spirometry*			
Pre B2 FEV ₁ %	72.8 (21.2)	69.3 (16.9)	0.409
Pre B2 FVC%	82.8 (16.6)	82.2 (12.0)	0.867
Pre B2 FEV ₁ /FVC%	67.7 (13.1)	66.0 (11.9)	0.540
Blood eosinophils (× 10 ⁹) per L [†]	0.20 (0.00, 2.00)	0.30 (0.10, 0.90)	0.062
Sputum cell counts [†]			
Total cell count (× 10 ⁶) per mL	3.87 (0.44, 102.0)	5.13 (0.72, 36.0)	0.440
Neutrophils (%)	37.0 (0.0, 95.25)	35.5 (0.68, 83.5)	0.817
Eosinophils (%)	1.63 (0.0, 73.75)	2.0 (0.0, 83.0)	0.652
Sputum phenotype [‡]			
Eosinophilic	56 (40%)	11 (44%)	0.826
Neutrophilic	18 (13%)	3 (12%)	>0.999
Paucigranulocytic	60 (43%)	10 (40%)	0.830
Mixed	6 (4.3%)	1 (4.0%)	>0.999

* Mean (SD), Student's t-test; [†] Median (min, max), Mann Whitney U test; [‡] n(%), Fisher's exact test; ACQ: asthma cough questionnaire, ICS: inhaled corticosteroids, BDP: beclometasone dipropionate, FEV₁ %: forced expiratory volume in one second % predicted, FVC: forced vital capacity % predicted.

Table E7: Resistance genes and detected macrolide mutations from macrolide resistant isolates

Azithromycin resistant species*	Disk zone (mm)	Week ^a	<i>erm</i> (B)	<i>mef</i> (A)	<i>mel</i>	<i>tet</i> (M)	<i>tet</i> (W)	<i>erm</i> (A)	<i>msr</i> (C)	L4 mutation ^b
<i>Haemophilus parainfluenzae</i>	13	0								
<i>Haemophilus parainfluenzae</i>	14	0								
<i>Haemophilus parainfluenzae</i>	8	48								G65A
<i>Haemophilus parainfluenzae</i>	13	48								G65A
<i>Staphylococcus aureus</i>	11	48						✓		
<i>Staphylococcus aureus</i>	6	48							✓	
<i>Streptococcus australis</i>	8	0		✓	✓					
<i>Streptococcus infantis</i>	11	0		✓	✓					
<i>Streptococcus infantis</i>	11	0		✓	✓					
<i>Streptococcus mitis</i>	6	0	✓			✓				
<i>Streptococcus mitis</i>	9	0		✓	✓					
<i>Streptococcus parasanguinis</i>	11	0		✓	✓					
<i>Streptococcus parasanguinis</i>	9	0		✓	✓	✓				
<i>Streptococcus parasanguinis</i>	9	0		✓	✓					
<i>Streptococcus parasanguinis</i>	11	0		✓	✓					
<i>Streptococcus parasanguinis</i>	9	0		✓	✓					
<i>Streptococcus salivarius</i>	7	0		✓	✓					
<i>Streptococcus salivarius</i>	8	0		✓	✓					
<i>Streptococcus salivarius</i>	6	0	✓			✓				
<i>Streptococcus salivarius</i>	11	0		✓	✓	✓				

<i>Streptococcus anginosus group</i>	8	48	✓			✓				
<i>Streptococcus australis</i>	10	48		✓	✓					
<i>Streptococcus gordonii</i>	9	48		✓	✓	✓				
<i>Streptococcus infantis</i>	9	48		✓	✓					
<i>Streptococcus infantis</i>	6	48	✓	✓	✓					
<i>Streptococcus infantis</i>	12	48		✓	✓	✓				
<i>Streptococcus infantis</i>	10	48		✓	✓					
<i>Streptococcus mitis</i>	8	48		✓	✓					
<i>Streptococcus oralis</i>	6	48	✓	✓	✓	✓	✓			
<i>Streptococcus oralis</i>	6	48	✓	✓	✓	✓				
<i>Streptococcus oralis</i>	6	48	✓			✓				
<i>Streptococcus parasanguinis</i>	6	48	✓	✓	✓	✓				
<i>Streptococcus parasanguinis</i>	10	48		✓	✓					
<i>Streptococcus parasanguinis</i>	6	48	✓	✓	✓					
<i>Streptococcus parasanguinis</i>	8	48		✓	✓					
<i>Streptococcus parasanguinis</i>	7	48		✓	✓					
<i>Streptococcus salivarius</i>	7	48		✓	✓					
<i>Streptococcus sanguinis</i>	10	48		✓	✓					

*Defined by EUCAST Clinical Breakpoints v.8.1. Where breakpoints are not defined, the epidemiological cut-offs were used

^a Sputum sample collection week during azithromycin therapy

^b Position numbers for L4 based on *H. parainfluenzae* numbering system

Table E8: Fold change in bacterial load and diversity between weeks 0 and 48 in Placebo and Azithromycin groups

	Placebo median fold change (IQR)	Azithromycin median fold change (IQR)	P value
Total bacterial load	0.636 (0.315-1.56)	0.711 (0.297-2.04)	0.925
Faith's phylogenetic diversity	0.979 (0.797-1.12)	0.871 (0.728-1.03)	0.303
<i>Haemophilus influenzae</i> load	1.00 (0.0987-3.57)	0.249 (0.00695-1.00)	0.0304
<i>Streptococcus pneumoniae</i> load	0.861 (0.116-20.4)	0.635 (0.123-1.63)	0.632
<i>Staphylococcus aureus</i> load	1.00 (0.806-1.00)	1.00 (1.00-1.00)	0.103
<i>Pseudomonas aeruginosa</i> load	1.00 (1.00-1.00)	1.00 (1.00-1.00)	0.865
<i>Moraxella catarrhalis</i> load	1.00 (1.00-1.00)	1.00 (1.00-1.00)	0.788

P values determined by Mann-Whitney test

Table E9: Fold change in antibiotic resistance gene carriage between weeks 0 and 48 in Placebo and Azithromycin groups

	Placebo median fold change (IQR)	Azithromycin median fold change (IQR)	P value
<i>erm</i> (B) normalised levels	1.51 (0.119-3.31)	6.98 (2.56-38.0)	<0.0001
<i>mef</i> normalised levels	0.865 (0.461-1.61)	2.30 (1.47-5.18)	<0.0001
<i>mel</i> normalised levels	1.05 (0.307-2.62)	2.05 (0.908-5.33)	0.0597
<i>erm</i> (F) normalised levels	1.15 (0.321-5.79)	19.7 (1.00-222)	0.0021
<i>tet</i> (W) normalised levels	0.885 (0.317-2.12)	2.48 (0.669-15.5)	0.0100
<i>tet</i> (M) normalised levels	1.28 (0.279-2.91)	1.705 (0.799-5.92)	0.282
<i>msr</i> (E) normalised levels	1.03 (0.167-4.32)	22.2 (1.93-143)	0.0003

P values determined by Mann-Whitney test

References

1. Jervis-Bardy J, Leong LE, Marri S, Smith RJ, Choo JM, Smith-Vaughan HC, Nosworthy E, Morris PS, O'Leary S, Rogers GB, Marsh RL. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome* 2015; 3: 19.
2. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology* 2014; 12: 1-12.
3. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Burr LD, Wesselingh S, Rogers GB. Understanding the impact of antibiotic therapies on the respiratory tract resistome: a novel pooled-template metagenomic sequencing strategy. *Multidiscip Respir Med* 2018; 13: 9-14.
4. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; 30: 2114-2120.
5. Bushnell B. BBMap short read aligner. *University of California, Berkeley, California URL* <http://sourceforgenet/projects/bbmap> 2016.
6. Peng Y, Leung HC, Yiu SM, Chin FY. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 2012; 28: 1420-1428.
7. Zhu WH, Lomsadze A, Borodovsky M. Ab initio gene identification in metagenomic sequences. *Nucleic acids research* 2010; 38.
8. Li WZ, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 2006; 22: 1658-1659.
9. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FS, Wright GD, McArthur AG. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic acids research* 2017; 45: D566-D573.
10. Lakin SM, Dean C, Noyes NR, Dettenwanger A, Ross AS, Doster E, Rovira P, Abdo Z, Jones KL, Ruiz J, Belk KE, Morley PS, Boucher C. MEGARes: an antimicrobial resistance database for high throughput sequencing. *Nucleic acids research* 2017; 45: D574-D580.
11. Li R, Yu C, Li Y, Lam TW, Yiu SM, Kristiansen K, Wang J. SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* 2009; 25: 1966-1967.
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.
13. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection* 2014; 20: O255-O266.
14. Seemann T, Goncalves da Silva A, Bulach DM, Schultz MB, Kwong JC, Howden BP. Nullarbor. 2017. Available from: <https://github.com/tseemann/nullarbor>.
15. Zhang L, Kinkelaar D, Huang Y, Li Y, Li X, Wang HH. Acquired Antibiotic Resistance: Are We Born with It? *Applied and environmental microbiology* 2011; 77: 7134-7141.
16. Chung WO, Werckenthin C, Schwarz S, Roberts MC. Host range of the ermF rRNA methylase gene in bacteria of human and animal origin. *The Journal of antimicrobial chemotherapy* 1999; 43: 5-14.
17. Choo JM, Abell GCJ, Thomson R, Morgan L, Waterer G, Gordon DL, Taylor SL, Leong LEX, Wesselingh SL, Burr LD, Rogers GB. Impact of Long-Term Erythromycin Therapy on the Oropharyngeal Microbiome and Resistance Gene Reservoir in Non-Cystic Fibrosis Bronchiectasis. *Msphere* 2018; 3.
18. Srinivasan V, du Plessis M, Beall BW, McGee L. Quadriplex real-time polymerase chain reaction (lytA, mef, erm, pbp2b(wt)) for pneumococcal detection and assessment of antibiotic susceptibility. *Diagnostic microbiology and infectious disease* 2011; 71: 453-456.

19. Ambrose KD, Nisbet R, Stephens DS. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (mel and mef) and is erythromycin inducible. *Antimicrobial agents and chemotherapy* 2005; 49: 4203-4209.
20. Florez AB, Alegria A, Rossi F, Delgado S, Felis GE, Torriani S, Mayo B. Molecular Identification and Quantification of Tetracycline and Erythromycin Resistance Genes in Spanish and Italian Retail Cheeses. *BioMed research international* 2014.
21. Tao CW, Hsu BM, Ji WT, Hsu TK, Kao PM, Hsu CP, Shen SM, Shen TY, Wan TJ, Huang YL. Evaluation of five antibiotic resistance genes in wastewater treatment systems of swine farms by real-time PCR. *Sci Total Environ* 2014; 496: 116-121.
22. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002; 148: 257-266.
23. Corris PA, Ryan VA, Small T, Lordan J, Fisher AJ, Meachery G, Johnson G, Ward C. A randomised controlled trial of azithromycin therapy in bronchiolitis obliterans syndrome (BOS) post lung transplantation. *Thorax* 2015; 70: 442-U150.
24. Sistek V, Boissinot M, Boudreau DK, Huletsky A, Picard FJ, Bergeron MG. Development of a real-time PCR assay for the specific detection and identification of *Streptococcus pseudopneumoniae* using the *recA* gene. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2012; 18: 1089-1096.
25. Thomas LC, Gidding HF, Ginn AN, Olma T, Iredell J. Development of a real-time *Staphylococcus aureus* and MRSA (SAM-) PCR for routine blood culture. *Journal of microbiological methods* 2007; 68: 296-302.
26. Feizabadi MM, Majnooni A, Nomanpour B, Fatolahzadeh B, Raji N, Delfani S, Habibi M, Asadi S, Parvin M. Direct detection of *Pseudomonas aeruginosa* from patients with healthcare associated pneumonia by real time PCR. *Infect Genet Evol* 2010; 10: 1247-1251.
27. Greiner O, Day PJR, Altwegg M, Nadal D. Quantitative detection of *Moraxella catarrhalis* in nasopharyngeal secretions by real-time PCR. *Journal of clinical microbiology* 2003; 41: 1386-1390.

Supplementary Figure Legends

Figure E1: Effect of placebo on individual taxa. Linear discriminant analysis effect size (LEfSe) of taxa that significantly changed in relative abundance before and after trial in placebo group. Red indicates taxa that were lower, and green indicates taxa that were higher.

Figure E2: Effect of azithromycin on other pathogenic species. Copy number of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Moraxella catarrhalis* before and after either placebo (left) or azithromycin (right).

Figure E3: Effect of azithromycin on antibiotic resistance genes from pooled-template shotgun metagenomic screening. Counts per million total reads (CPM) of reads that matched to antibiotic resistance genes from the CARD database. Change in CPM (Δ CPM) between pooled samples before and after azithromycin or placebo, where red indicates higher in pooled-sample post azithromycin or placebo and blue indicates lower in pooled-sample post azithromycin or placebo. Resistance genes are grouped by function (as defined by CARD) where: red = aminoglycoside resistance genes, blue = beta-lactam resistance genes, green = efflux pump resistance genes, orange = fosfomycin resistance genes, purple = glycopeptide resistance genes, beige = macrolide resistance genes, pink = polymyxin resistance genes, light green = tetracycline resistance genes, and brown = other resistance genes. Asterisks (*) indicate transmissible genes that were increased in the azithromycin group but not in the placebo group.

Figure E4: Levels of screened antibiotic resistance genes in placebo. Levels of the seven antibiotic resistance genes before and after trial in placebo group. Genes identified by pooled-template shotgun metagenomic sequencing were quantified by qPCR and normalized to total bacteria.

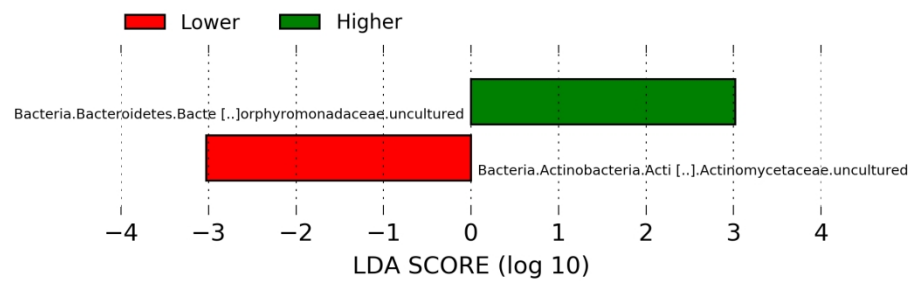


Figure E1

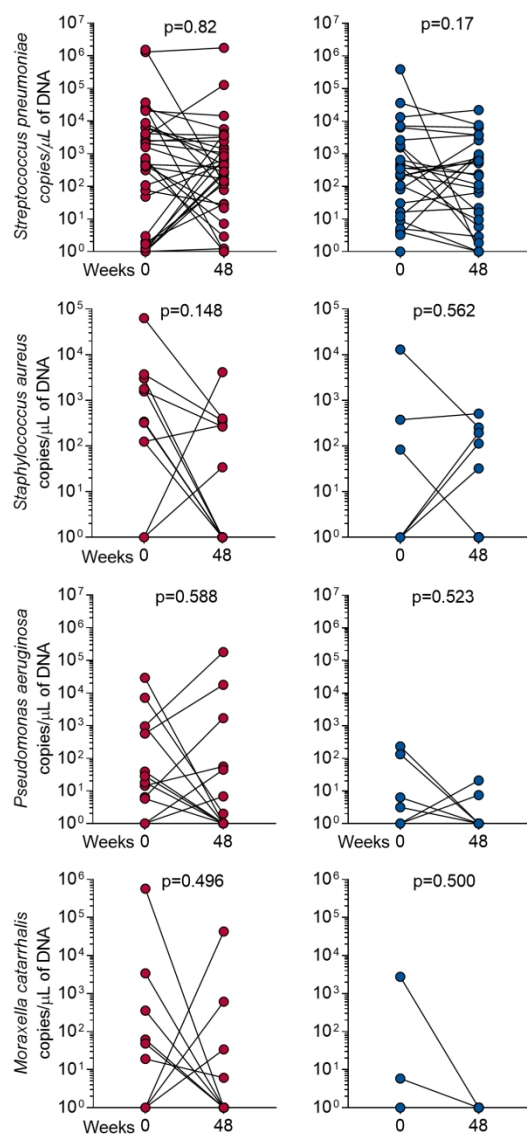


Figure E2

121x231mm (300 x 300 DPI)

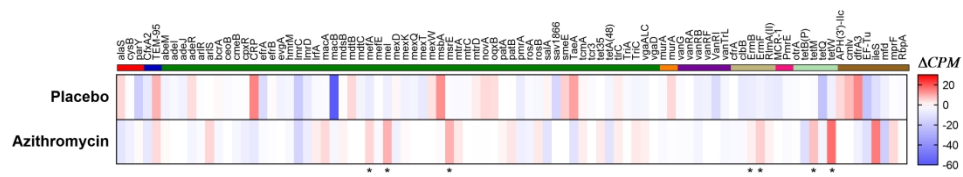


Figure E3

261x57mm (300 x 300 DPI)

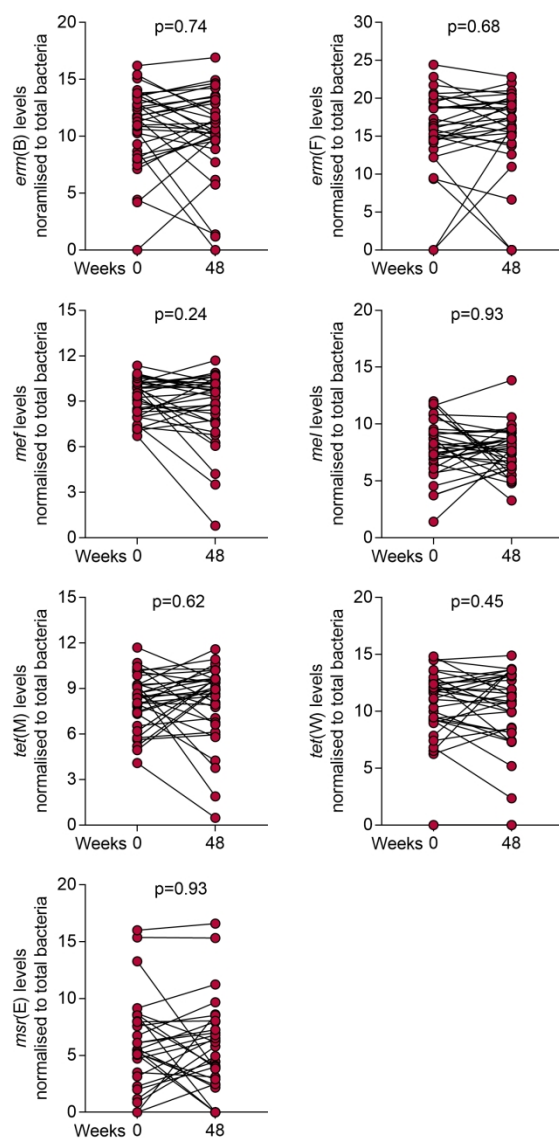


Figure E4

146x267mm (300 x 300 DPI)