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Weekly azithromycin for 48 weeks impacts nasopharyngeal microbial prevalence and *Streptococcus pneumoniae* serotypes in children with HIV-associated chronic lung disease

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HIV-associated chronic lung disease (HCLD) accounts for over 50% of deaths in children living with HIV. Azithromycin reduces the risk of respiratory exacerbations in children with HCLD, but its impact on respiratory pathogens and *Streptococcus pneumoniae* serotypes in HCLD remains partially understood. We investigated the impact of azithromycin on the prevalence and density of respiratory microbes in children enrolled in the BREATHE randomized controlled trial. Nasopharyngeal swabs collected from 287 participants at baseline, 48 and 72 weeks were analysed using nanofluidic qPCR testing for 94 *S. pneumoniae* serotypes, 12 bacterial species, and eight respiratory viruses. No differences were observed between microbial colonisation in the azithromycin and placebo groups at baseline or 72 weeks. At 48 weeks, overall bacterial colonisation was significantly lower in the azithromycin group compared to placebo (adjusted Odd Ratio [aOR]: 0.45, 95% CI 0.25-0.82; *p*=0.008), with reduced colonisation of *S. pneumoniae* (aOR: 0.37; 95% CI: 0.24-0.71; *p*=0.003) and non-typeable *Haemophilus influenzae* (aOR: 0.29; 95% CI: 0.14-0.61; *p*=0.001). The 13-valent pneumococcal conjugate vaccine serotypes (19F and 23F) and non-vaccine type (15A/F) were most commonly observed in both groups at all time points. Findings suggest that azithromycin reduces nasopharyngeal colonisation of certain bacteria in HCLD during treatment but has no long-lasting effects after treatment cessation.

Trial registration: The BREATHE trial (ClinicalTrials.gov Identifier: NCT02426112, registered date: 24/04/2015).

Keywords *Streptococcus pneumoniae*, *Moraxella catarrhalis*, Non-typeable *Haemophilus influenzae*, Human rhinovirus, Chronic lung diseases, Azithromycin, Africa

Abbreviations

ART	Antiretroviral therapy
AZM	AZITHROMYCIN
CLD	CHronic lung diseases
HCLD	HIV-associated chronic lung disease
NTHi	Non-typeable *Haemophilus influenza*
HRV	Human rhinovirus
RSV	Respiratory syncytial virus
NVT	Non PCV 13 vaccine serotypes
VT	PCV 13 vaccine serotypes
COPD	Chronic obstructive pulmonary disease
IPD	Invasive pneumococcal disease

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Long-term therapy with macrolides is recognized as a beneficial intervention for individuals with chronic lung diseases (CLD), including asthma, cystic fibrosis (CF), and non-CF-bronchiectasis[1–6]. Azithromycin (AZM), a commonly used macrolide, has been shown to not only improve lung function and reduce the risk of acute respiratory exacerbations but also demonstrate efficacy in treating post-lung transplant bronchiolitis obliterans syndrome[7–9]. In individuals living with HIV who experience respiratory tract infections, macrolides are generally prescribed empirically and have been shown to reduce morbidity and mortality[10]. Despite these established benefits, the mechanisms by which AZM improves CLD outcomes remain partially understood; however, their dual role as both antibacterial and anti-inflammatory agents likely underpins their therapeutic effects[11].

The nasopharynx is a dynamic ecosystem composed of many respiratory microbes including viruses, fungi, and bacteria[12]. Among the bacterial residents are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and non-typeable *Haemophilus influenzae* (NTHi)[12], which have been implicated in many respiratory infections and exacerbations of CLDs, including asthma and chronic obstructive pulmonary disease (COPD)[13–15]. Studies in Zimbabwe[16], Malawi[17], and South Africa[18] have highlighted a high prevalence of obliterative bronchiolitis, a novel form of CLD reported in children living with HIV despite taking antiretroviral therapy (ART)[16]. Long-term AZM has been suggested as a potential therapeutic agent given its effectiveness in other CLDs, such as asthma[4].

To test the effectiveness of AZM treatment in preventing worsening of lung function and reducing the rate of exacerbations in children with HIV-associated chronic lung diseases (HCLD), we conducted a randomized controlled trial, BREATHE (Bronchopulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children), in Malawi and Zimbabwe between June 2016 and September 2019. We demonstrated that 48 weeks of weekly AZM treatment reduced acute respiratory exacerbations in children with HCLD[19]. We also showed that 48 weeks of AZM therapy was associated with reduced sputum and nasopharyngeal bacterial carriage of *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis*[20]. However, the parent study utilized bacterial culture, which detects viable bacteria and culturable species, limiting the scope of complex microbial analysis[20]. Additionally, the prevalence and density of respiratory viruses and other common bacteria were not studied, despite evidence that viral infections, mainly with respiratory syncytial virus and influenza, facilitate bacterial colonisation[21] and increase bacterial load, further increasing individuals' susceptibility to complications related to respiratory tract infections[22].

While the primary findings from the BREATHE study showed a decrease in *S. pneumoniae* carriage following 48 weeks of AZM treatment, serotype-specific colonization was not investigated, which is important for informing vaccine strategies[23]. The 13-valent pneumococcal conjugated vaccine (PCV13), introduced in Malawi in 2011[24] and Zimbabwe in 2012[25], targets 13 serotypes responsible for most cases of invasive pneumococcal diseases (IPD) and pneumonia globally[26]. Nevertheless, vaccine-driven serotype replacement remains a concern, as reported in studies from Malawi[27], Mozambique[28], and Tanzania[29], as well as in many Western countries. In a South African study conducted between 2012-2018, serotypes 11 A, 13, 19 F, and 6 A were linked to higher case-fatality rates in IPD among children below 15 years[30]. These findings underscore the need for continuous pneumococcal serotype surveillance in sub-Saharan Africa to inform vaccination strategies and address challenges posed by serotype replacement and antibiotic resistance, particularly in children living with HCLD.

The present study, a sub-study of the BREATHE trial, aimed to investigate the prevalence and density of 12 common bacterial species, including 94 *S. pneumoniae* serotypes, non-typeable *H. influenzae* and eight viruses in children and adolescents with HCLD.

Results

Study participants characteristics

The majority of participants were enrolled from Zimbabwe (72%; 208/287). Participants in the AZM group were younger than those in the placebo group, with the median age of 14.8 years (IQR: 12.8–16.9) compared to 16 years (IQR: 13.0–18.2, $p = 0.044$). Other differences included a higher proportion of the participants from the AZM group having a history of previous tuberculosis treatment (37%, 52/139 vs. 24%, 36/147; $p = 0.021$) compared with the placebo group. All other characteristics were similar between the two groups (Table 1).

Prevalence of nasopharyngeal microbes in participants at baseline, 48 weeks, and 72 weeks

A total of 287 nasopharyngeal samples from the 347 participants enrolled for the main trial between June 1, 2016, and September 31, 2019, were analysed at baseline (AZM; n =140, placebo: n =147; Figure 1). Participants with missing (n =21) or whose samples failed the ribonucleoprotein gene (RNP) gene testing (n=39) at baseline were excluded from analysis of this sub-study (Figure 1). At 48- and 72-weeks, sample retention was AZM: 96% [135/140] vs. Placebo: 90% [133/147], $p = 0.074$ and AZM: 76% [110/140] vs. Placebo: 65% [95/147], $p = 0.001$, respectively. The decline in retention (loss-to-follow-up (n =8), withdrawal (n =9), missing samples (n = 7) or failed RNP testing (n = 37)), was similar across groups, except for participants whose study was ended (AZM, 4% [6/140]; Placebo, 15% [22/147], p =0.004) and death (n=2) which occurred in the placebo group only (Figure 1; Table S3).

Changes in microbial prevalence over the three visits within the treatment groups are detailed in Table 2 and Figure S1. No significant differences in bacterial carriage and viruses were detected between the AZM and placebo groups at baseline or 72 weeks (Table 2). However, at 48 weeks, participants on AZM treatment were less likely to be colonized with *S. pneumoniae* (adjusted odds ratio (aOR):0.37; 95% CI: 0.24–0.71.24.71; p = 0.003) or non-typeable *H. influenzae* (aOR: 0.29; 95% CI: 0.14- 0.61; p = 0.001) compared with the placebo. The prevalence of *S. aureus* (6%, 8/135 *vs.* 5%, 7/133) and *M. catarrhalis* (11%, 14/135 vs. 18%, 24/133) was not significantly different between the groups at 48 weeks. Within the AZM group, *S. pneumoniae* (36% [51/140] vs. 19% [26/135], p = 0.002), *M. catarrhalis* (24% [33/140] vs. 11% [14/135], p = 0.009), and non-typeable *H. influenzae* (41% [58/140] vs. 21% [28/135], p <0.001) colonization decreased at 48 weeks compared to baseline (Figure S1). However, at 72 weeks, *S. pneumoniae* (19% [26/135] vs. 39% [43/110], p = 0.001) and non-typeable *H. influenzae* (21% [28/135] vs. 35% [39/110], p = 0.001) returned to baseline prevalences, while *M. catarrhalis* showed no significant changes (18% [24/140] vs 15% [17/110], p = 0.735). *B. pertussis/holmesii*, Influenza B, Human metapneumoviruses, and Human parainfluenza types 1 and 3 were not detected at any of the study visits, while some bacteria (*K. pneumoniae*, *N. meningitidis*, *A. baumanii, S. pyogenes)* and viruses (Influenza A, RSVA, and RSVB) were infrequently identified on either study visit (Table S2).

Characteristics		AZM [% (n/N)]	Placebo [% (n/N)]	*p*-value
Sociodemographic				
Site	Zimbabwe	74% (104/140)	71% (104/147)	0.512
	Malawi	26% (36/140)	29% (43/147)	
Sex	Male	53% (74/140)	50% (73/147)	0.637
	Female	47% (66/140)	50% (74/147)	
Age (years)	Median (IQR)	14.8 (12.8 - 16.9)	16 (13–18.2.2)	**0.044**
Anthropometric				
BMI for age Z-score	Median (IQR)	−1.87 (−1.14, −0.33)	−1.76 (−0.95, −0.23)	0.302
Height for age Z-score	<− 2 (Stunted)	51% (71/140)	50% (69/147)	0.556
Weight for age Z-score	<−2 (Underweight)	54% (76/140)	49% (72/147)	0.409
Current medications				
Taking cotrimoxazole prophylaxis		92% (127/138)	90% (132/147)	0.544
Antiretroviral regimen[m]	NNRT-base-1st line[a]	72% (101/140)	77% (113/147)	0.416
	PI-base 2nd line[b]	28% (39/140)	23% (34/147)	
HIV clinical parameters				
Age at ART initiation (years)	Median (IQR)	8.2 (5.1 - 12.1)	8.8 (6.6 −11.6)	0.164
Duration on ART (years)[m]	Median (IQR)	6.2 (3.7 - 9.0)	6.4 (4.3 - 8.5)	0.672
CD4 count categories (Cells/mm)	< 200	11% (15/140)	12% (18/147)	0.226
	200–500	25% (35/140)	33% (49/147)	
	>500	64% (90/140)	55% (80/147)	
Viral load suppression	VL < 1000 copies/mL	56% (79/140)	50% (74/147)	0.166
Respiratory status				
Hospitalization for chest problems in the last 48 weeks		2% (3/140)	2% (3/147)	1.000
Previously treated for tuberculosis		37% (52/139)	24% (36/147)	**0.021**
Has asthma		4% (6/139)	2% (3/147)	0.324
FEV1 z-score	Median (IQR)	−2.0 (−2.5, −1.5)	−1.9 (−2.4, −1.5)	0.759
ARE experienced during the 48 weeks		9% (12/133)	16% (20/126)	0.094
Adherence to protocol		76% (107/140)	69% (102/147)	0.180

Table 1. Participants' characteristics in the AZM and placebo trial arms. Abbreviations: AZM, Azithromycin; FEV1, forced expiratory volume in 1 second; IQR, interquartile range; ARE, acute respiratory exacerbations; ART, antiretroviral therapy[a]; Nonnucleoside Reverse Transcriptase Inhibitor[b], Protease inhibitor[m]; Participants with missing responses are excluded from that variable: Duration on ART (3 patients in the AZM group and 6 in the placebo group) and Antiretroviral regimen (1 patient in the placebo group).

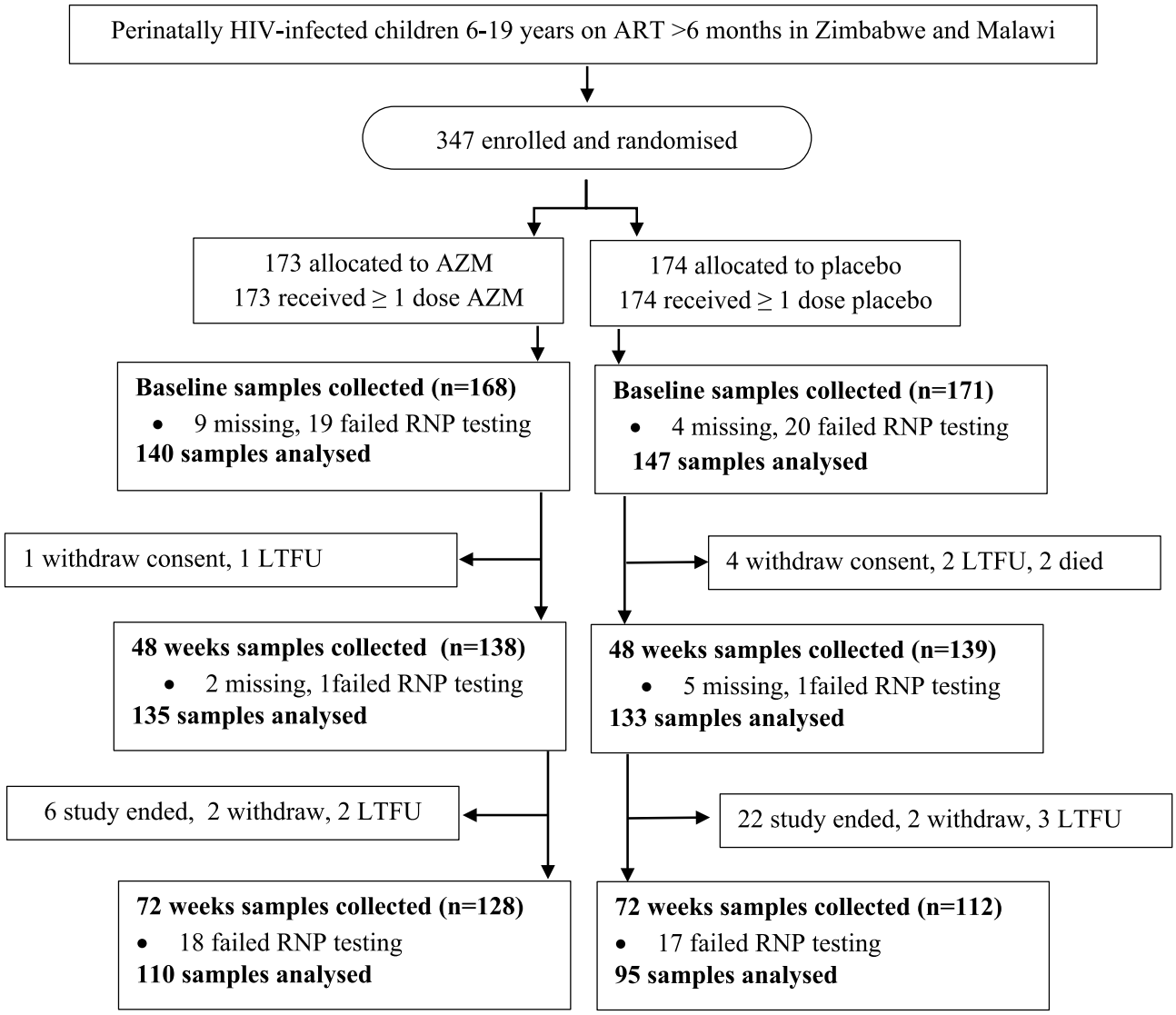


Fig. 1. Study profile. Abbreviations: LTFU, lost to follow-up; AZM, Azithromycin; RNP, Ribonucleoprotein; ART, Antiretroviral therapy; HIV, Human immunodeficiency virus; n, total number of samples collected at each sampling timepoint. A subset of samples from the AZM treatment group (n = 5) and placebo group (n = 3) were not collected at baseline because participants refused sampling; these participants and their subsequent follow-up samples were excluded from the analysis. Follow-up samples included at 48 and 72 weeks are only from participants with baseline samples analysed (AZM, n = 140; placebo, n = 147).

Microbial densities of selected nasopharyngeal microbes among study participants

There was no significant difference in the microbial densities of the tested microbes between the AZM and placebo groups at baseline or 72 weeks (Table 3). Although there was no difference in prevalence of *M. catarrhalis* at 48 weeks between the treatment groups, the microbial density of *M. catarrhalis* was significantly lower in the AZM (2.84×10^2 genomic equivalents [GE/ml]) group compared with the placebo (7.9×10^2 GE/ml), $p = 0.004$.

S. pneumoniae serotypes distribution and density in participants at baseline, 48- and 72 weeks

Overall, a total of 288 *S. pneumoniae* positive serotypes (from the 94 serotypes tested) were detected from trial arm participants across study visits, excluding the 29 non-typable *S. pneumoniae* reported (Figure S2). Overall, there was a higher detection of PCV13 non-vaccine type (NVT) than the PCV13 vaccine type (VT) serotypes (73% [209/288] vs 27% [79/288]) in both groups at all time points. Comparison between groups showed a significantly lower prevalence of VT serotype in the AZM group [9% (13/140)] compared to placebo [19% (28/147)], p =0.019 at baseline (Figure 2). No difference was observed between groups at 48 and 72 weeks. Within the placebo group, the prevalence of VT serotypes decreased significantly overall across the three visits (baseline [19%], 48 weeks [11%], and 72 weeks [6%], p=0.01). Pairwise comparisons within the placebo group showed a marginally significant reduction between 48 and 72 weeks (p = 0.06), whereas the decrease from

Microbe	Baseline			48 weeks				72 weeks			
	AZM (n = 140)	Placebo (n = 147)	P^a	AZM (n = 135)	Placebo (n =133)	aOR (95% CI)	P^b	AZM (n = 110)	Placebo (n = 95)	aOR[b] (95% CI)	P^b
Bacterial carriage (any)	57% (80)	65% (95)	0.194	36% (48)	53% (70)	0.45 (0.25–0.82.25.82)	**0.008**	54% (59)	62% (59)	0.47 (0.11–1.97.11.97)	0.301
Non-typeable *H. influenzae*	41% (58)	45% (66)	0.634	21% (28)	40% (53)	0.29 (0.14 –0.61)	**0.001**	35% (39)	42% (40)	0.87 (0.18 –4.08)	0.856
S. pneumoniae	36% (51)	44% (65)	0.188	19% (26)	36% (48)	0.37 (0.24–0.71.24.71)	**0.003**	39% (43)	45% (43)	0.44 (0.11 –1.77)	0.254
M. catarrhalis	24% (33)	29% (42)	0.350	11% (14)	18% (24)	0.58 (0.28–1.22.28.22)	0.152	15% (17)	18% (17)	2.07 (0.39 –11.0)	0.392
S. aureus	7% (10)	5% (7)	0.459	6% (8)	5% (7)	0.97 (0.33–2.83.33.83)	0.961	5% (5)	4% (4)	-	>0.999
S. oralis	2% (3)	3% (5)	0.723	2% (3)	5% (6)	-	0.333	3% (3)	3% (3)	-	>0.999
N. lactamica	3% (4)	1.4% (2)	0.438	0% (0)	1% (1)	-	0.496	0% (0)	0% (0)	-	-
HRV	8% (11)	7% (10)	0.822	2% (3)	5% (6)	0.31 (0.05- 1.7)	0.181	7% (8)	7% (7)	1.08(0.11 –10.9)	0.945

Table 2. Prevalence of nasopharyngeal microbes among participants at baseline, 48 weeks, and 72 weeks. Abbreviations: AZM, Azithromycin treatment group; HRV, human rhinovirus; aOR, adjusted Odds Ratio; *P*, p-value; [a]Fisher's exact test; [b] logistic mixed effect modelling method using the child as a random effect adjusted for age, sex, site, visit and viral load, with aOR for 72 weeks indicating baseline as the comparator; -, Could not calculate the aOR due to a few variables; *K. pneumoniae*, *N. meningitidis*, *A. baumanii, Streptococcus pyogens)* and viruses (Influenza A, RSVA, and RSVB) were only detected at either one or two visits. *B. pertussis/holmesii*, Influenza B, Human metapneumoviruses, and Human parainfluenza types 1 and 3 were not detected at any of the study visits (Results presented in Table S2).

Microorganism	Baseline, (GE/ml)			48 weeks (GE/ml)			72 weeks (GE/ml)		
	AZM	Placebo	p^a	AZM	Placebo	p^b	AZM	Placebo	p^b
Non-typeable *H. influenzae*	1.1×10^4	1.8×10^4	0.610	4.8×10^3	1.4×10^3	0.587	2.7×10^3	1.1×10^3	0.585
S. pneumoniae	8.3×10^3	1.2×10^5	0.638	1.1×10^4	7.7×10^3	0.799	1.1×10^4	5.0×10^3	0.255
M. catarrhalis	8.3×10^3	1.2×10^4	0.370	2.8×10^2	7.9×10^2	**0.004**	1.2×10^3	8.1×10^2	0.919
S. aureus	1.5×10^3	2.7×10^2	0.079	4.7×10^2	6.3×10^1	0.921	1.1×10^2	1.1×10^2	0.875
S. oralis	2.8×10^3	4.1×10^2	0.655	2.3×10^2	4.2×10^2	0.819	2.3×10^2	2.4×10^2	0.938
N. lactamica	1.5×10^3	1.8×10^3	0.129	-	1.4×10^3	-	-	-	-
HRV	1.4×10^4	8.3×10^3	0.481	7.4×10^3	4.2×10^3	0.945	1.1×10^4	2.8×10^3	0.969

Table 3. Microbial densities of selected nasopharyngeal microbes among study participants. Abbreviations: HRV, human rhinovirus; AZM, azithromycin treatment group; GE, Genomic Equivalents; *P*, p-value; [a] Mann-Whitney U test; b linear mixed effect modelling method using the child as a random effect adjusted for age, sex, site, visit, and viral load.; -, Could not calculate the aOR due to a few variables; *K. pneumoniae*, *N. meningitidis*, *A. baumanii, Streptococcus pyogens)* and viruses (Influenza A, RSVA, and RSVB) were only detected at either one or two visits. *B. pertussis/holmesii*, Influenza B, Human metapneumoviruses, and Human parainfluenza types 1 and 3 were not detected at any of the study visits.

baseline to 48 weeks was statistically significant ($p = 0.02$) (Figure S3). Similarly, within the AZM group, carriage of NVT serotypes decreased significantly at 48 weeks [11% (16/140)] compared to baseline [35% (49/140)], p=0.003, but rebounded by 72 weeks [23% (32/140)], p=0.025 relative to 48 weeks. The median VT and NVT log10 densities in the AZM and placebo groups were similar at all three timepoints (Figures 3 and 4).

Multiple serotypes were detected in 14% of participants at baseline (AZM: 7%; placebo: 7.5%), 6% at 48 weeks (AZM: 3%; placebo: 9%) and 6% at 72 weeks (AZM: 6%; placebo: 5.5%), with single serotype carriage predominating (Table S4). There was no significant difference in individual serotype prevalence (both in VT and NVT) between the AZM and placebo group at any time point (Table S5). However, the most common VT serotypes that differed slightly between groups at baseline: in the AZM group, 23 F was the most frequent (2.9%), whereas in the placebo group, 19 A (4.1%), 4 (3.4%), and 19 F (3.4%) were the most common (Figure 5A). Among the NVT, serotypes 16 F (2.9%), 15A/F (2.1%), and 34 (2.1%) were most prevalent in the AZM group, whereas serotypes 21 (3.4%) and 13 (2.7%) predominated in the placebo group. Over time, 19 F and 23 F remained the most frequently detected VT serotypes in both groups at 48 and 72 weeks (Figure 5B and 5 C). Notably, though not statistically significant, serotype 19 F prevalence increased in the AZM group from 2.2% at 48 weeks to 4.5% at 72 weeks, mirroring a similar trend in the placebo group (3% to 4.5%). NVT serotype 15A/F was observed at all timepoints in both groups, though most prevalent only in the placebo group at 48 weeks (3%), while 16 F and 11A/D were equally common (3.2% each) at 72 weeks.

Discussion

The main findings of our study indicate that AZM treatment significantly reduced nasopharyngeal bacterial colonisation while on treatment, but this is not sustained through 24 weeks after stopping treatment. Participants

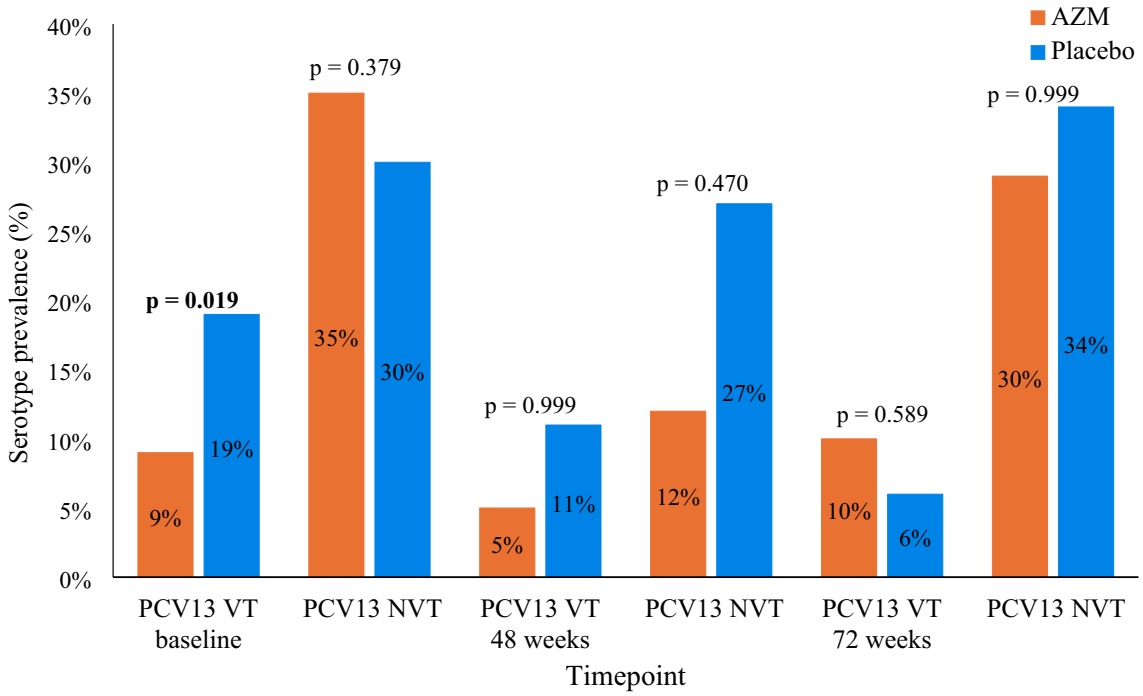


Fig. 2. Prevalence of *S. pneumoniae* 13-valent pneumococcal conjugated vaccine type (PCV13 VT) and PCV13 non-vaccine type (PCV13 NVT) serotypes at baseline, 48 weeks and 72 weeks among children living with HIV-associated CLD receiving azithromycin (AZM) vs placebo. Fisher's exact test was used to compare 2 time points at baseline; logistic mixed effect modelling method using the child as a random effect adjusted for age, sex, site, visit and viral load for comparing 48- and 72-week time points.

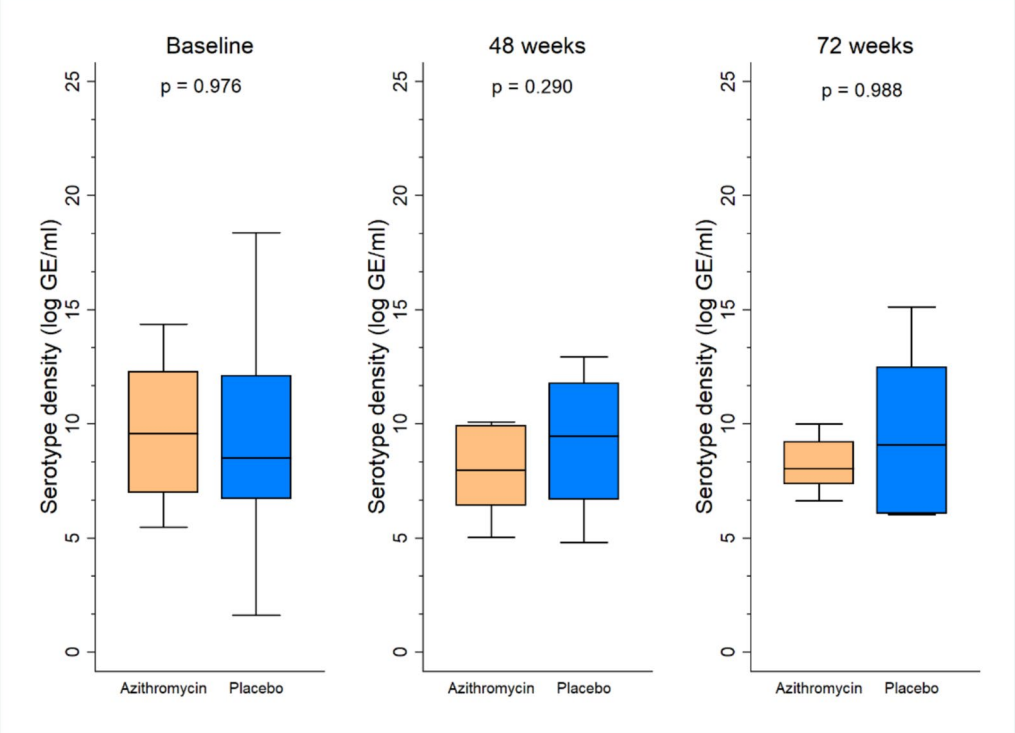


Fig. 3. Density of PCV13 vaccine-type serotypes at baseline, 48 weeks, and 72 weeks in children living with HIV-associated chronic lung disease. P-values for baseline comparisons are from the Mann–Whitney U test. Differences at 48 and 72 weeks were assessed using linear mixed-effects models with child as a random effect, adjusted for age, sex, study site, visit, and viral load.

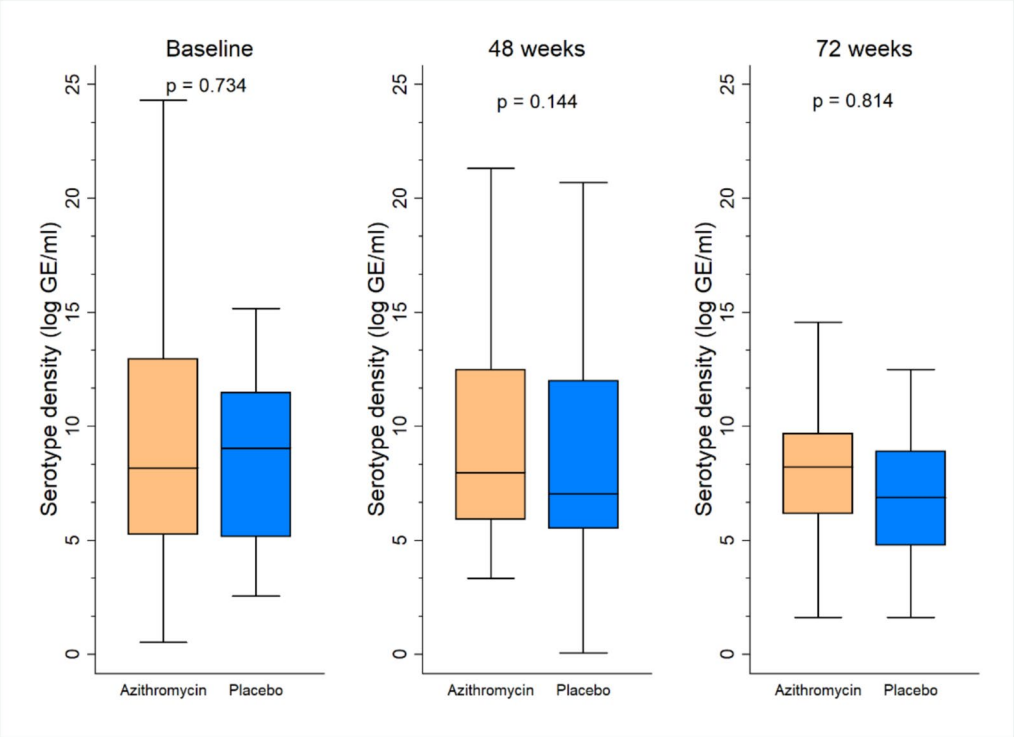


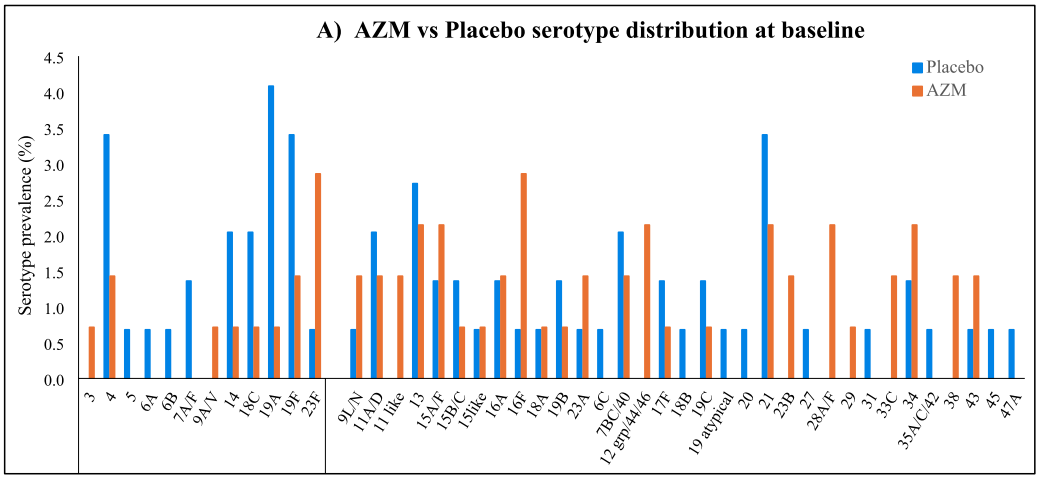
Fig. 4. Density of PCV13 non-vaccine type serotypes at baseline, 48 weeks, and 72 weeks in children living with HIV-associated chronic lung diseases; P-values for baseline comparisons are from the Mann–Whitney U test. Differences at 48 and 72 weeks were assessed using linear mixed-effects models with child as a random effect, adjusted for age, sex, study site, visit, and viral load.

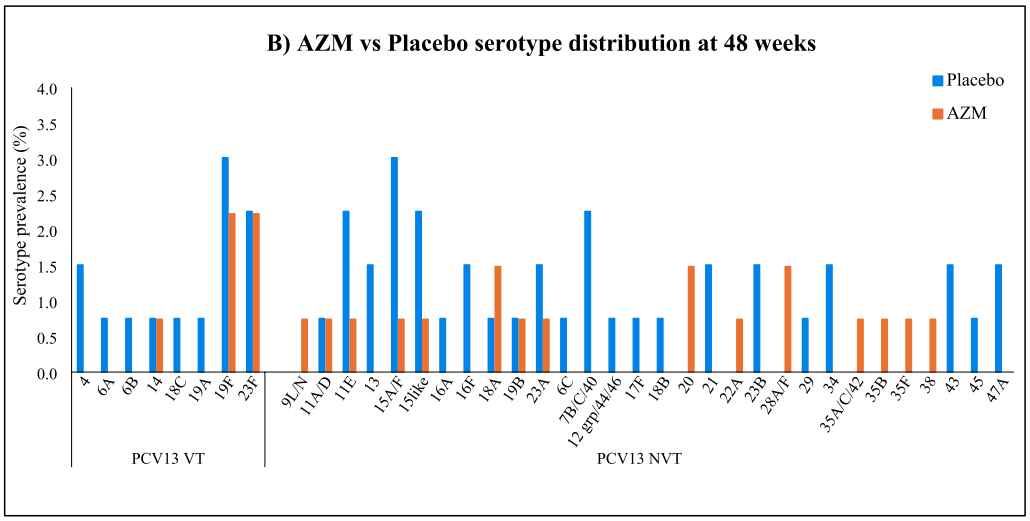
on AZM treatment were less likely to carry *S. pneumoniae* or non-typeable *H. influenzae*. Within the AZM group, colonisation by *S. pneumoniae*, non-typeable *H. influenzae*, and *M. catarrhalis* decreased significantly at 48 weeks relative to baseline, followed by a partial recovery of *S. pneumoniae* and non-typeable *H. influenzae* to baseline levels by 72 weeks. Despite the prevalence of *M. catarrhalis* colonisation remaining comparable between groups across all time points, its bacterial density was lower in the AZM group at 48 weeks. At baseline, VT serotypes were less prevalent in the AZM group compared to the placebo group, with no differences at subsequent time points. VT serotypes detected in both groups at all time points were serotypes 19 F and 23 F, while the NVT serotypes were 15A/F. Overall, VT and NVT serotype median densities were similar between groups at all time points.

AZM has broad-spectrum activity against Gram-positive and Gram-negative microorganisms, including *S. pneumoniae*, *S. aureus*, *M. catarrhalis* and *H. influenzae*[31]. Our findings within the AZM group indicate a significant reduction in non-typeable *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* carriage in response to AZM treatment. These findings are consistent with those of Abotsi *et al*[20]., who reported reduced sputum colonization of *S. pneumoniae*, *S. aureus*, *M. catarrhalis* and *H. influenzae* in AZM-treated participants. Long-term AZM therapy is often prescribed to suppress bacterial load and reduce airway inflammation[50], thus reducing the risk of respiratory exacerbations and improving quality of life[32]. The reduced carriage of nasopharyngeal respiratory bacterial pathogens at 48 weeks may be one of the factors that contributed to the reduction of respiratory exacerbations in the AZM treatment group, as reported by Ferrand *et al*[19].

We noted a significant increase in the prevalence of *S. pneumoniae* and non-typeable *H. influenzae* at 72 weeks compared to 48 weeks in the AZM treatment group, with no difference noted in the placebo group. The rebound of *S. pneumoniae* and non-typeable *H. influenzae* at 72 weeks in the AZM group suggests that the effect of AZM therapy may not persist long after treatment completion, which is an important consideration for the management of bacterial colonization in children with HCLD[20]. This finding is consistent with the results observed by Hare *et al*[33]., which reported a rebound of *S. pneumoniae* prevalence at 12 months post-therapy in children aged 1–8 years with chronic suppurative lung diseases on AZM[33]. The observed post-treatment increase in bacterial colonization suggests that extending intermittent prophylactic AZM treatment beyond one year may benefit children with HCLD. This is supported by the British Thoracic Society guidelines[34], which recommend long-term macrolide therapy for adults over 16 years of age with CLDs, highlighting its potential role in managing persistent bacterial colonization and reduction in respiratory exacerbation rate.

The polysaccharide capsule is key for *S. pneumoniae* pathogenesis, facilitating immune evasion[35,36] and its ability to colonize the nasopharynx[37–39]. Widespread introduction of PCVs has influenced the pneumococcal serotypes now responsible for IPD. Our study found a higher PCV13 NVT than PCV13 VT serotype prevalence at all timepoint visits in both groups. This mirrors findings from England and Wales, where PCV13 NVT serotypes





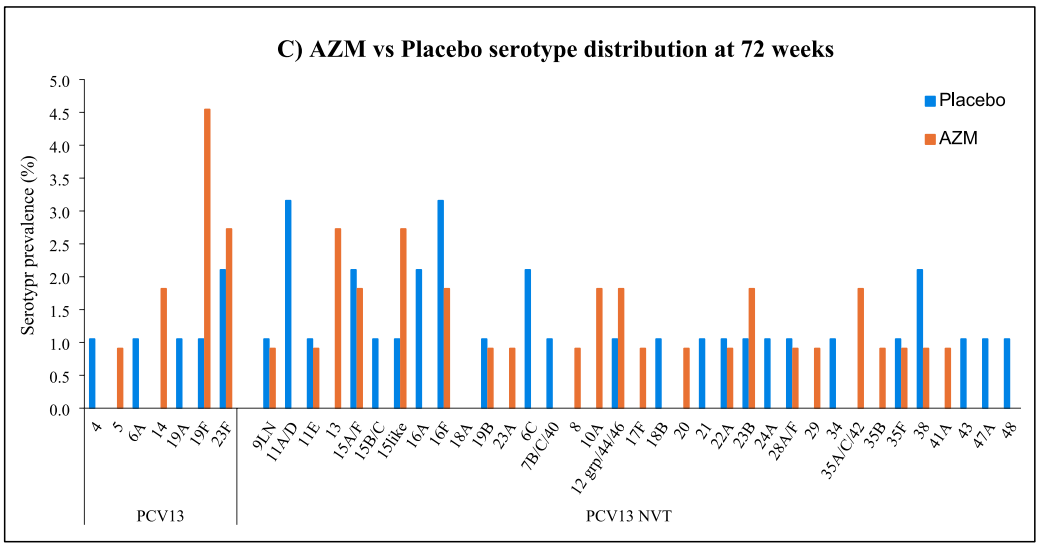


Fig. 5. Serotype distribution of *S. pneumoniae* among children and adolescents living with HIV-associated CLD at each visit. **(A)** Baseline with participants from the azithromycin (AZM) group (n =140) and Placebo (n = 147); **(B)** at 48 weeks from AZM group (n = 135) and Placebo (n =133) and **(C)** 72 weeks from AZM (n = 110) and Placebo (n =95) stratified into PCV13 VT serotype and PCV13 NVT serotype. The denominator for prevalence is the total number of participants for each visit, grouped into the AZM and Placebo groups. No significant difference was observed between recovered serotypes at any time point.

(especially serotypes 8, 12 and 9 N) were more common in children (5 to 15 years) with CLD and linked to IPD and death[40]. Similarly, a survey by Lukhuleni *et al*[41]. conducted between 1999 and 2022 in South Africa reported an increased burden of IPD caused by NVTs in children during the post-PCV13 era, with serotypes 8 (18%), 12 F (6%), 15B/C (5%), and 16 F (5%) being the most prominent. Though we did not have data on vaccination status in our participants, we assume community herd immunity could have significantly reduced the VT serotypes, given the WHO reported PCV13 coverage in 2019 was 90% in Zimbabwe[42] and 95% in Malawi[43].

While AZM treatment is known to suppress bacterial colonization, its impact on serotype distribution remains complex. The persistence of PCV13 VT serotypes, such as 19 A and 19 F, in the AZM group is consistent with the Ethiopian study, where AZM did not alter VT serotypes in children (1 −10 years) with trachoma[44]. Similarly, a study in The Gambia found no difference in PCV7 VT serotype distribution between villages that received three mass AZM distributions and those with a single distribution, suggesting that serotypes with higher antibiotic resistance potentially persist despite treatment[45]. This resistance is often mediated by the upregulation of the efflux pumps[46,47], enabling VT serotypes to survive antibiotic pressure. Conversely, the decline in VT carriage in the placebo treatment group (baseline [19%], 48 weeks [11%] and 72 weeks [6%]) may reflect the natural declining of PCV13 VT strains post-PCV13 introduction as reported in population-based surveillance studies[10,24,48] and may also be associated with the frequent use of amoxicillin-clavulanate antibiotics within this group to treat acute exacerbations.

Although no significant difference was observed between treatment groups in overall PCV13 NVT serotype prevalence, within-group analysis of the AZM arm revealed notable dynamics. PCV13 NVT serotype prevalence was lower at the end of treatment compared with baseline but rebounded by 72 weeks, consistent with transient suppression rather than permanent clearance. Similar patterns have been reported in other mass drug administration studies, where suppressed pneumococcal serotypes re-emerged after treatment cessation[49,50]. These findings suggest that the temporary reduction in pneumococcal serotypes may reflect short-term AZM effects, although unmeasured factors such as seasonality and other transient ecological shifts could also have contributed[51].

The predominance of PCV13 NVT serotypes such as 15A/F in both groups at later time points highlights the potential ongoing serotype replacement phenomenon. This observation is consistent with global trends observed post-PCV13 introductions, where NVT serotypes progressively occupy the ecological niche left by VT serotypes. Prolonged AZM use at subtherapeutic serum concentrations has been linked to the emergence of co-resistance to other macrolides, beta-lactams, and cotrimoxazole[52]. Of particular concern is serotype 15 A, which has been frequently associated with multiple drug resistance[53], potentially explaining its persistence in the AZM group despite antibiotic exposure. Furthermore, *S. pneumoniae* strains carrying both *erm*(B) and *mef*(A) genes have been shown to exhibit high-level resistance to multiple antimicrobial classes[54]. These findings raise important concerns regarding the long-term efficacy of AZM and the potential for resistant NVTs to undermine treatment outcomes in children with HCLD.

The absence of significant differences in individual serotype prevalence between groups suggests that antibiotic exposure alone is not the primary driver of *S. pneumoniae* serotype distribution, consistent with findings from other African studies[55,56]. Instead, complex host-pathogen interactions, herd immunity and antibiotic selection pressure collectively shape serotype distribution, particularly emerging NVT serotypes with resistance traits[24,46].

The carriage density of potentially pathogenic bacteria such as *S. pneumoniae* correlates with pathogenesis, transmission, and disease severity[35,57]. Our study found no significant difference in *S. pneumoniae* density across visits or between treatment groups, possibly due to prior antibiotic use, including current cotrimoxazole use, which may have affected baseline microbial carriage density[58]. Additionally, the persistent carriage of resistant strains may have masked transient changes or the acquisition of new strains[59]. Unlike *S. pneumoniae*, *M. catarrhalis* density significantly declined at 48 weeks in the AZM group, likely due to *M. catarrhalis* almost universal susceptibility to AZM, previously observed in our cohort[20,60].

Asymptomatic carriage of respiratory viruses is common, especially human rhinovirus (HRV) particularly prevalent among asymptomatic children and adolescents[61]. Both HRV and adenoviruses have been implicated in otitis media and lower respiratory tract infections and are frequently associated with hospitalisation in children living with HIV[62,63]. Moreover, HRV has been the most frequently detected virus in studies of children with pneumonia in Asia, Africa and South America[64–66]. Our study showed no significant difference in viral detection between treatment groups. This finding could be due to azithromycin's limited antiviral effects[67–69]. Although viral presence is known to enhance bacterial adherence, we observed no association between the viruses and bacteria detected at any time point.

Our study has several strengths, including being among the few that offer insights into nasopharyngeal microbial colonization and microbial density among children with HCLD receiving long-term AZM therapy. It provides valuable insights into long-term trends in bacterial carriage and density in sub-Saharan Africa. Importantly, our study provides serotype-specific data, which were not previously described in the HCLD cohort, offering a broader understanding of pneumococcal dynamics following AZM therapy. We also included an assessment of respiratory viruses and other common bacteria, providing a broader view of the nasopharyngeal microbial system; however, the low numbers of these organisms limit the strength of the conclusions.

Our study limitations include the lack of genomic investigation of nasopharyngeal samples and infrequent sampling, which may have overlooked transient colonization patterns. Vaccination status was not available, limiting the interpretation of *S. pneumoniae* data, and the low number of viruses detected restricted conclusions. Additionally, our mixed-effects logistic regression analyses assumed data were missing at random; however, retention differed slightly between study arms, with all deaths and a higher number of participants whose study ended occurring in the placebo group, suggesting that missingness may not have been entirely random and could be related to underlying clinical status. Future research should also incorporate whole-genome sequencing and more frequent sampling to capture and describe complex microbial dynamics.

In conclusion, 48 weeks of once-weekly AZM reduced the *S. pneumoniae* and non-typeable *H. influenzae* carriage, along with *M. catarrhalis* microbial density in children receiving AZM treatment. Within-group analyses further support these findings, showing a reduction in *M. catarrhalis,* non-typeable *H. influenzae*, *S. pneumoniae* and NVT serotypes associated with AZM treatment. However, a rebound of non-typeable *H. influenzae*, *S. pneumoniae* and NVT after cessation of therapy suggests that AZM exerts a treatment-specific but not long-lasting effect. Nevertheless, these findings are clinically significant and may contribute to improved outcomes in HCLD participants during treatment. Modulating the upper respiratory microbiome and reducing the carriage rate of disease-associated pathogens could be a promising strategy to reduce respiratory complications in HCLD, supporting the clinical benefits of AZM therapy. There is a need for future research to investigate AZM-associated dysbiosis and resistome dynamics. Such studies will help advance understanding of respiratory microbiome management and continuously guide safe and effective long-term treatment strategies in HCLD.

Methods and materials

BREATHE (ClinicalTrials.gov Identifier: NCT02426112, registered date: 24/04/2015); a double-blinded, randomised, placebo-controlled trial of weekly weight-adjusted AZM therapy, which investigated whether AZM could improve lung function and reduce the risk of acute respiratory exacerbations among children with HCLD, is described elsewhere[70–72]. Briefly, children and adolescents with HCLD, aged 6–19 years of age and taking ART for at least six months, were enrolled between June 1, 2016, and September 31, 2019, from the HIV outpatient clinics in Harare, Zimbabwe, and Blantyre, Malawi. Participants with active tuberculosis or any acute respiratory infection were excluded from the study. HCLD was defined as forced expiratory volume in 1 second (FEV1) z-score less than −1 with no reversibility [<12% improvement in FEV1 after 200 µg of salbutamol inhaled using a spacer] measured by spirometry. This was after the protocol was changed in January 2017, to increase generalizability from <−1.64 (original protocol) to <−1.0 to improve generalizability. Participants with FEV_1 z-scores between −1.0 and −1.64 screened before the change were invited for rescreening.

Participants were individually randomized to receive weight-based dosing of AZM (10 to 19.9 kg, 250 mg; 20 to 29.9 kg, 500 mg; 30 to 39.9 kg, 750 mg; and 40 kg or more, 1250 mg) or placebo once-weekly for 48 weeks, with a further follow-up visit at 24-week post-intervention. Randomization was conducted by an independent statistician using variable block sizes ranging from 2 to 6 and was stratified by country. Participants, study personnel (including laboratory personnel and pharmacists) and staff performing outcome assessments were blinded to treatment allocation. The intervention and comparator, including full details of implementation and most prespecified primary and secondary outcomes of the study, are described elsewhere[19,70]. All study participants completed a detailed questionnaire regarding demographic, socio-economic and clinical history information. However, none of the participants or the public were involved in the design or reporting of this study.

The study was approved by the following: London School of Hygiene and Tropical Medicine, Harare Central Hospital Ethics Committee, Medical Research Council of Zimbabwe, College of Medicine Research Ethics Committee Malawi, Regional Committee for Medical and Health Research Ethics Northern Norway and the Human Research and Ethics Committee of the University of Cape Town -UCT HREC (HREC/REF: 092/2019). All participants and/or legal guardians gave written informed consent to participate in the study.

Nasopharyngeal samples were collected from all participants at baseline, 48- and 72-week visits using sterile flocked flexible nylon swabs and stored in PrimeStore MTM® (Longhorn Diagnostics, Bethesda, MD). Details on sample collection, handling and transportation, microbial detection and quantification are described elsewhere[73]. Briefly, the total nucleic acid extraction was conducted using a two-step system. First, mechanical lysis was performed in ZR BashingBead Lysis tubes (catalog no. ZR S6002–50, Zymo Research Corp., Irvine, CA, United States), followed by nucleic acid extraction using the QIAsymphony® DSP Virus/Pathogen Kit (Qiagen GmbH, Hilden, Germany) on the QIAsymphony SP/AS instrument (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The extracted nucleic acids were analyzed for 94 pneumococcal serotypes, 12 bacterial species (*S. pneumoniae*, *H. influenzae* including NTHi, *M. catarrhalis*, *S. aureus*, *Neisseria lactamiae*, *Neisseria meningitidis*, *Streptococcus pyogenes*, *Bordetella pertussis*, *Bordetella holmesii, Klebsiella pneumoniae*, *Acinetobacter baumanii,* and *Streptococcus oralis*), and eight viruses (respiratory syncytial virus [RSV] A and B, human rhinovirus, influenza A and B, human parainfluenza 1 and 3, and human metapneumovirus) using nanofluidic qPCR as previously described[74,75] (Table S1). Positive samples were defined as those with a cycle of quantification (Cq) value ≤36 for both the ribonucleoprotein gene (RNP) and their respective reference genes. Furthermore, *S. pneumoniae*-positive samples (positive for both *lyt*A and *pia*B pneumococcal reference genes) were further tested for each serotype-specific qPCR target. The method, however, could not specifically distinguish all serotypes, including serotypes 15 A and 15 F, as well as 11 A and 11D, which are therefore reported as serotypes 15A/F and 11A/D, respectively or as serogroups (Table S1).

Outcomes

The primary outcome of this study was the difference in the prevalence and density of key nasopharyngeal species including 94 Streptococcus pneumoniae serotypes, non-typeable Haemophilus influenzae, and eight viruses in children with HCLD between trial arms at AZM cessation (48 weeks). Secondary outcomes included comparisons of microbial prevalence and density between the trial arms at baseline and 72 weeks (i.e., 24 weeks post-treatment cessation). Additionally, we examined within-group changes over time (baseline, 48 weeks, and 72 weeks) in both AZM and placebo groups, focusing on the same bacterial and viral species to assess temporal trends and potential rebound effects following discontinuation of AZM.

Statistical analysis

Statistical analyses were conducted using Stata version 13 (StataCorp, College Station, TX). Fisher's exact and Mann-Whitney U tests were used to compare proportions and continuous variables as appropriate. Weight-for-age and height-for-age Z-scores (WAZ and HAZ, respectively) were calculated using the British 1990 growth reference curves[76]. Those with Z-scores lower than two were characterized as being underweight and stunted, respectively. Viral load suppression was defined as viral load (VL) <1000 copies/ml. All randomized participants were included in the analysis. Comparisons of bacterial carriage between trial arms and within each treatment group at all visits were conducted using a mixed-effects logistic regression model or a mixed-effects linear model, including a random effect for participants to account for repeated measures. These models inherently accommodated missing data under the missing at random assumption, as mixed-effect models provide valid estimates in the presence of incomplete data when the missing at random assumption holds[77]. No imputation was performed. All models for prespecified and subgroup analysis were adjusted for trial arm, study site, sex, age category, and HIV viral load at enrolment and visit. Adjusted odds ratios were reported with 95% confidence intervals (CI), and a *p*-value of less than 0.05 was considered statistically significant.

Data availability

The datasets used and analysed during the current study are available from Felix Dube (sizwe.dube@uct.ac.za) on reasonable request and ethical approval.

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Author contributions

FSD conceived the study. PKM conducted the laboratory experiments and data analysis and wrote the first draft of the manuscript, supervised by REA and FSD. CO and SM supervised the Fluidigm assay. JOO, MN and RAF conceived and led the parent BREATHE study. JOO secured funding from GLOBVAC on behalf of the consortium. All authors contributed to, read, and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The parent study (BREATHE) was approved by the Human Research and Ethics Committee of the University of Cape Town - UCT HREC (HREC/REF:754/2015), the London School of Hygiene and Tropical Medicine Ethics Committee (reference 8818), the Harare Central Hospital Ethics Committee and Medical Research Council of Zimbabwe (reference MRCZ/A/1946), the College of Medicine Research Ethics Committee Malawi (reference P.04/15/1719) and the Regional Committee for Medical and Health Research Ethics, Northern Norway (reference 2015/1650). The University of Oxford waived approval. Additional ethical approval was received for this sub-study from the UCT HREC (HREC/REF: 092/2019). No additional data were collected other than those approved in the parent study. Written informed consent and assent were given by guardians and participants, respectively. Participants who were 18 years old and above consented independently at the time of enrolment. All data obtained and generated during the study were kept confidential. This research was

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Additional information

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