Long-term effects of azithromycin mass administration to reduce childhood mortality on *Streptococcus pneumoniae* antimicrobial resistance: a population-based, cross-sectional, follow-up carriage survey

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Summary

Background Mass drug administration (MDA) programmes with the macrolide antibiotic azithromycin to reduce childhood mortality are expanding in Africa; however, concerns remain about the long-term effects of these programmes on antimicrobial resistance (AMR). We aimed to evaluate the persistence and spread of *Streptococcus pneumoniae* AMR following a community-randomised MDA trial.

Methods This population-based, cross-sectional, pneumococcal carriage survey was conducted in Mangochi, Malawi, 3·5 years after the MORDOR trial, in which communities received twice-yearly azithromycin or placebo for 2 years. Eligible participants in this carriage survey were children aged 4–9 years who lived in an azithromycin-treated or placebo-treated cluster during the MORDOR trial, and children aged 1–3 years who were resident in a cluster but born after the MORDOR trial ended. Nasopharyngeal swabs were collected from participants and analysed by whole genome sequencing; pneumococcal genomes obtained from a distant site in Malawi, in which MDA had not been conducted, were used as reference genomes. The primary outcome was the prevalence of *S pneumoniae* macrolide resistance, comparing placebo-treated and azithromycin-treated clusters at baseline, 6 months post-MDA, and 3·5 years post-MDA.

Findings Between April 8 and May 14, 2021, 924 children aged 1–9 years were screened, of whom 19 were excluded and 905 were recruited to the follow-up carriage survey: 452 from azithromycin-treated clusters and 453 from placebo-treated clusters of the MORDOR trial. We assessed 426 isolates from these participants (190 from azithromycin-treated clusters and 236 from placebo-treated clusters), as well as samples from the baseline of the MORDOR trial (164 isolates; 83 from azithromycin-treated clusters and 81 from placebo-treated clusters) and from 6 months post-MDA (223 isolates; 119 from azithromycin-treated clusters and 104 from placebo-treated clusters). In azithromycin-treated clusters, macrolide resistance increased from 21·7% (95% CI 14·2–31·7; 18 of 83 isolates) at baseline to 31·9% (24·2–40·8; 38 of 119 isolates) 6 months post-MDA and to 32·1% (25·9–39·0; 61 of 190 isolates) 3·5 years post-MDA. In placebo-treated clusters, resistance increased from 21·0% (13·5–31·1; 17 of 81 isolates) at baseline to 25·0% (17·7–34·1; 26 of 104 isolates) 6 months post-MDA and to 30·9% (25·4–37·1; 73 of 236 isolates) 3·5 years post-MDA. No significant differences were observed in odds ratios between treatment groups across the survey timepoints: 0·97 (95% CI 0·36–2·55) at baseline, 1·46 (0·67–3·17) at 6 months post-MDA, and 1·12 (0·66–1·91) at 3·5 years post-MDA. Macrolide resistance in the non-MDA site remained stable: 16·9% (95% CI 12·8–21·8; 45 of 267 isolates) at baseline, 16·5% (13·3–20·3; 70 of 424 isolates) at 6 months, and 16·5% (12·5–21·4; 44 of 267 isolates) at 2·5 years. Among children born into azithromycin-treated clusters after MDA, macrolide resistance was 36·0% (27·7–45·1; 41 of 114 children). Multidrug resistance to at least three antibiotic classes was significantly higher in azithromycin-treated (p=0·0015) and placebo-treated (p<0·0001) clusters than in the comparator population at 3·5 years post-MDA and was associated with integrative conjugative elements.

Interpretation Azithromycin MDA is associated with macrolide resistance that persists and potentially spreads to untreated populations. The co-existence of multidrug resistance and transmissible resistance on integrative conjugative elements in these populations is a public health concern. Careful monitoring of AMR is essential in areas where MDA is implemented.

Funding The Gates Foundation, the National Institute for Health and Care Research, and the Wellcome Trust.

Lancet Infect Dis 2025

Published **Online** June 2, 2025 https://doi.org/10.1016/S1473-3099(25)00212-9

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Research in context

Evidence before this study

We searched PubMed from database inception to Nov 6, 2024, with no language restrictions, using the search terms "*Streptococcus pneumoniae*", "mass drug administration", "azithromycin", and "antibiotic resistance". Two systematic reviews of antimicrobial resistance (AMR) following mass drug administration (MDA) for trachoma showed that *S pneumoniae* macrolide resistance frequently emerges, with evidence of resolution over time. Our search identified 13 other articles focused on AMR following MDA. 11 articles reported phenotypic resistance testing and one article reported the use of molecular methods to identify resistance genes. Among the studies that reported phenotypic resistance testing was the MORDOR trial, which investigated the effect of azithromycin on all-cause childhood mortality in three countries in sub-Saharan Africa. Communities in Malawi, Niger, and Tanzania received twice-yearly azithromycin or placebo for 2 years (between December, 2014 and February, 2017) and phenotypic pneumococcal macrolide resistance was evaluated at baseline and up to 0·5 years post-MDA. Only one study applied whole-genome sequencing to assess changes in pneumococcal population structure and AMR over time; however, this assessment was limited to 0·5 years post-MDA for trachoma. We could not find any publications describing pneumococcal AMR, population diversity, or transmissible integrative conjugative elements beyond 0·5 years after the completion of MDA.

Added value of this study

To our knowledge, this study is the first long-term evaluation of *S pneumoniae* AMR, population structure, and transmissible integrative conjugative elements following MDA in either a trial or an implementation setting. Our results show that, 3·5 years after completion of the MORDOR trial, pneumococcal AMR has not returned to pre-MDA levels in the trial regions in Malawi. In addition, pneumococcal AMR has spread to children living in the placebo-treated clusters adjacent to the MORDOR clusters in which MDA was implemented. Macrolide resistance was similarly high among children who were born after the MORDOR trial was completed and who were therefore not directly exposed to MDA. We show that macrolide-resistant and multidrug-resistant *S pneumoniae* lineage variants, some carrying mobile integrative conjugative elements, could still be driving AMR long after exposure to MDA. Together, these findings suggest that AMR resulting from MDA could persist and spread through a population, facilitated by emergent AMR pneumococcal lineages and transmissible elements.

Implications of all the available evidence

MDA evaluation and implementation programmes are expanding in parts of sub-Saharan Africa where infant mortality is high. Our findings highlight that, contrary to evidence from some population-based studies in high-income settings and some trachoma MDA studies from low-income settings, pneumococcal resistance does not necessarily revert to pre-exposure levels following the withdrawal of macrolide treatment from a population. Furthermore, the spread of macrolide-resistant and multidrug-resistant lineages through a population, facilitated by resistant pneumococcal lineages and transmissible elements, poses a substantial regional and global health threat, particularly if left unmonitored. Our data emphasise the added value of whole-genome sequencing beyond conventional antibiotic susceptibility testing in the surveillance of this emerging threat.

Introduction

Mass drug administration (MDA) with the macrolide antibiotic azithromycin is a well established intervention for the control of trachoma. The Macrolides Oraux pour Réduire les Décès avec un Oeil sur la Résistance (MORDOR) community-randomised trial, conducted in Malawi, Niger, and Tanzania, assessed the effect of MDA with azithromycin on childhood mortality.[1] The trial showed a 13·5% reduction in all-cause mortality among children aged 1–59 months following MDA.[1] As a result, WHO conditionally recommended MDA for children aged 1–11 months in high-mortality regions.[2] MDA programmes have expanded across sub-Saharan Africa, including in Burkina Faso and Mali.[3] However, widespread MDA raises concerns about antimicrobial resistance (AMR) among pathogens with a high disease burden, such as *Streptococcus pneumoniae*, potentially outweighing the benefits on mortality.[4]

S pneumoniae is a leading cause of pneumonia, sepsis, and meningitis and is responsible for approximately 3·7 million severe infections and 294 000 deaths annually among children under the age of 5 years, primarily in sub-Saharan Africa.[5] *S pneumoniae* is one of the six leading pathogens associated with deaths from AMR.[6] Nasopharyngeal carriage precedes transmission and invasive disease,[7] and follow-up studies after MDA show increased carriage of macrolide-resistant pneumococci.[8] Although resistance might decline over time,[4] evidence from Europe and Israel links the use of macrolides to the emergence of penicillin-resistant and multidrug-resistant (MDR) pneumococci.[9] In The Gambia, MDA led to emergent MDR pneumococcal lineages carrying integrative conjugative elements,[10] which facilitate the transfer of AMR genes. We therefore hypothesised that widespread MDA could promote the long-term expansion and spread of AMR, potentially via integrative conjugative elements. Such resistance could lead to clinical treatment failure.

To assess the long-term effect of MDA on AMR, we used whole-genome sequencing to analyse pneumococcal isolates obtained from children living at a site of the

MORDOR trial in Malawi, collected at the trial baseline, 6 months after MDA,[8] and 3·5 years after MDA.

Methods

Study design

This population-based, cross-sectional, follow-up pneumococcal carriage survey was conducted from April 8 to May 14, 2021, in Mangochi, Malawi. The survey followed the MORDOR trial, conducted between December, 2014, and February, 2017, which investigated the effect of azithromycin on all-cause childhood mortality in Malawi, Niger, and Tanzania.[1] In Malawi, the MORDOR trial comprised 30 clusters, which were based on Health Surveillance Assistant catchment areas and located over three sites, including Mangochi. Within clusters, children aged 1–59 months were randomly assigned (1:1) to receive twice-yearly oral azithromycin (around 20 mg/kg) or placebo for 2 years.[1] MDA for trachoma prevention was not conducted in Mangochi during the trial period. In September–October, 2017, 6 months after the final post-MDA survey of the MORDOR trial, all eligible children in placebo-treated clusters received one round of azithromycin MDA.[8] In this follow-up carriage survey, conducted 3·5 years after this round of MDA, we assessed *S pneumoniae* AMR among children living in Mangochi. Ethical approval was granted by the College of Medicine Research Ethics Committee (COMREC ref P.05/20/3061) and the University College London Research Ethics Committee (18331/001).

Participants

Eligibility for this follow-up survey required permanent residence in Mangochi within a cluster defined by the MORDOR trial.[1] Eligible participants were children aged 4–9 years who lived in an azithromycin-treated or placebo-treated cluster during the MORDOR trial (termed MORDOR-exposed) and children aged 1–3 years who lived in an azithromycin-treated or placebo-treated cluster but were born after the MORDOR trial ended (termed MORDOR-unexposed). Electronic data capture devices determined the eligibility of participants on the basis of their birth dates. PCV13 vaccination status was not considered owing to high national coverage (>90%) with this vaccine.[11] Exclusion criteria were antibiotic use or hospitalisation within the previous 28 days (self-reported or recorded in the patient-retained health passport), diagnosed terminal illness, lack of consent from a parent or guardian, or lack of assent (for children aged >8 years). Written informed consent was obtained from the parent or guardian of each child, and assent was obtained from children older than 8 years.

12 randomly selected clusters were surveyed with the intention of selecting six azithromycin-treated and six placebo-treated clusters. However, owing to access challenges in some clusters, we recruited equal numbers of children from five azithromycin-treated clusters and seven placebo-treated clusters. Additionally, owing to COVID-19 restrictions, door-to-door recruitment of participants was not feasible. Health Surveillance Assistants created age-specific registers for each cluster and simple random sampling was applied to reduce selection bias. Selected children and their guardians were invited to a central location for screening and to provide consent.

Procedures

Demographic data on participants, including sex and age, were either obtained from medical health passport books or reported by guardians and were recorded electronically. Nasopharyngeal swabs were collected from participants, processed, and analysed by resistance phenotyping, DNA extraction, whole genome sequencing, and bioinformatics (appendix pp 4–6). As reference genomes (termed non-MDA), we used pneumococcal genomes obtained from participants of surveys carried out from June 19, 2015, to Aug 9, 2019, in Blantyre,[12] an urban site approximately 200 km south of Mangochi where MDA had not been conducted (appendix pp 41–66).

See Online for appendix

Outcomes

The primary outcome was the prevalence of pneumococcal macrolide resistance, comparing placebo-treated and azithromycin-treated clusters in MORDOR-exposed populations at baseline, 6 months post-MDA, and 3·5 years post-MDA. Secondary outcomes were the prevalence of macrolide resistance in MORDOR-unexposed cohorts (3·5 years after the trial) as well as changes in pneumococcal populations and AMR genotypes across all three survey timepoints (baseline, 6 months post-MDA, and 3·5 years post-MDA). All primary and secondary outcomes were reported. Post-hoc exploratory analyses of integrative conjugative elements, which are key macrolide resistance carriers, were conducted in the context of pneumococcal population changes.

Statistical analysis

We did not calculate a sample size a priori for predefined power for the study. Instead, for the May–July 2015 (baseline; pre-MDA) and March–June 2017 (post-MDA) cross-sectional surveys, sample size was based on the number of available pneumococcal isolates from the MORDOR trial.[8] For the follow-up survey, 3·5 years after MDA, we aimed to compare 300 pneumococcal isolates: 150 from children in azithromycin-treated clusters and 150 from children in placebo-treated clusters. Assuming a cluster effect modification coefficient (ρ) of 0·2, this sample would provide 80% power at a 5% significance level to detect a 25% difference in macrolide resistance if baseline resistance was 20%. The same difference can be shown at 70% power if ρ is 0·3. As a comparator, we included all available sequenced pneumococcal genomes

isolated from PCV13-vaccinated children (aged 1–3 years and 4–9 years) participating in surveys in Blantyre during June–December, 2015, and January–December, 2017 (ie, similar periods to the MORDOR trial and the follow-up survey, respectively); however, the follow-up carriage survey in Mangochi took place around 1 year after the final Blantyre survey. Further details of the comparator are in the appendix (pp 41–66).

Statistical tests and associated diagrams were generated in R (version 3.6.0) and edited in Inkscape (version 1.0.0). Variables that were not distributed normally—genome size distribution, antibiotic minimum inhibitory concentrations (MICs), and Shannon's and Simpson's indices—were summarised by median and IQR. Genotypic antibiotic susceptibility and multidrug resistance were summarised by mean and 95% CI. The Kruskal–Wallis test was used to compare median MICs among isolates in each survey. The Wilcoxon test was used to compare the Shannon's and Simpson's indices of azithromycin and placebo-treated clusters. Genotypic resistance, PCV13, and PCV20 serotypes were expressed as binomial proportions with bar plots and 95% CIs using the binom package. The Wilcoxon test in the rstatix package was used to compare the pairwise proportions between azithromycin-treated clusters, placebo-treated clusters, and the comparator at baseline, 6 months post-MDA, and 3·5 years post-MDA. For point estimates of effect size, 95% CIs were used and p values adjusted for multiple comparisons were calculated using default parameters. Odds ratios and 95% CIs for macrolide resistance between azithromycin-treated and placebo-treated groups were calculated. We accounted for clustering by community for macrolide and penicillin resistance using a generalised linear mixed model fit by maximum likelihood in which resistance, treatment exposure, and age were fixed effects and cluster was a

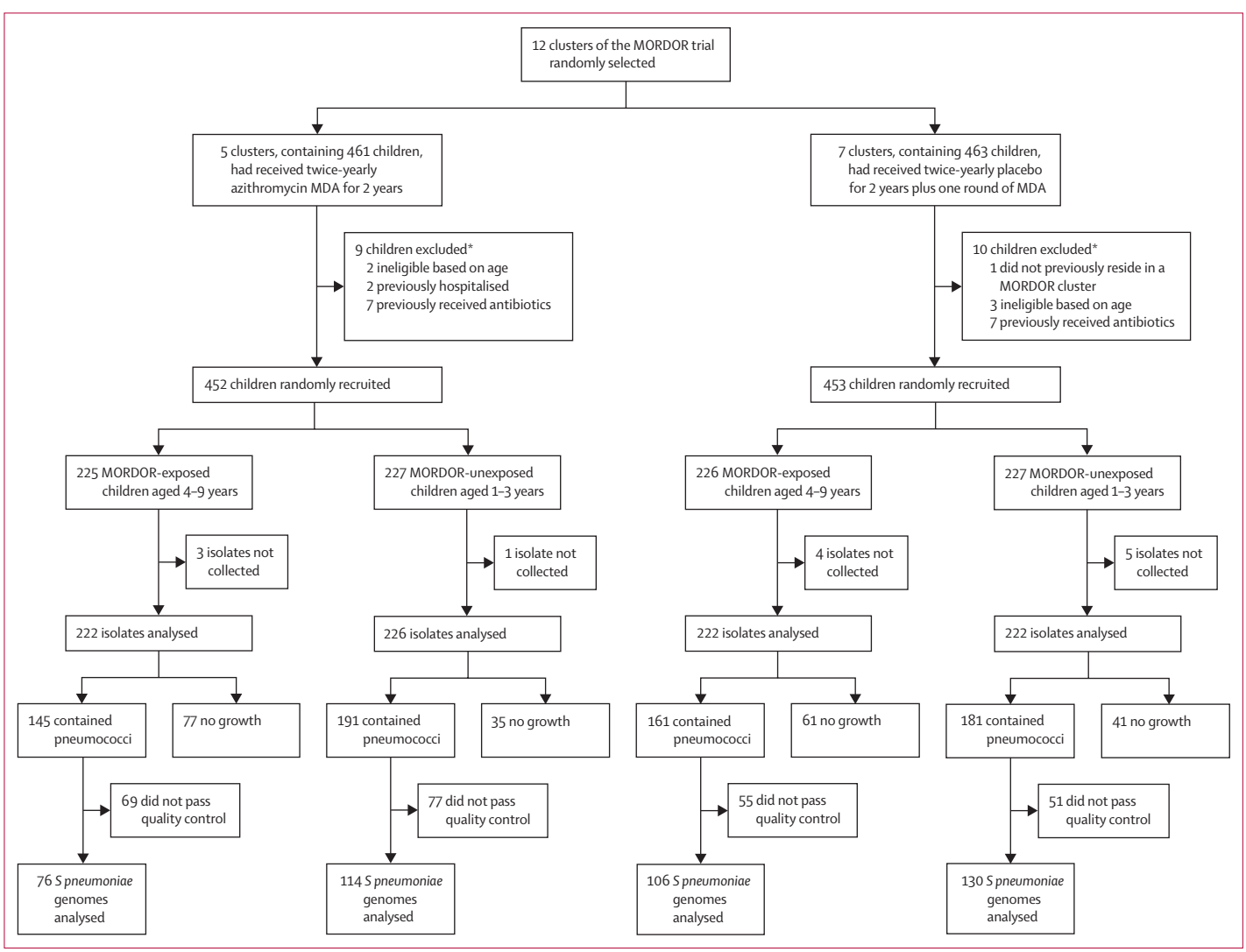


Figure 1: **Study design**
MDA=mass drug administration. *Some children were excluded for more than one reason.

random effect; the dependent variable was resistance. Fisher's exact test was used to assess changes in pneumococcal serotype over time, and multiple testing correction was done using the Benjamini–Hochberg false discovery rate of 5%. A p value of less than 0·05 was considered statistically significant.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between April 8 and May 14, 2021, we screened 924 children aged 1–9 years, of whom 19 were excluded. 905 children were recruited to the follow-up carriage survey: 452 from azithromycin-treated clusters and 453 from placebo-treated clusters of the MORDOR trial (figure 1). 451 (50%) of 905 children had been living in a MORDOR-defined cluster during the MORDOR trial[1] (MORDOR-exposed; aged 4–9 years) and 454 (50%) children were resident in a cluster but born after the end of the MORDOR trial (MORDOR-unexposed; aged 1–3 years). Characteristics of the participants are shown in the table. Original vaccination records were available for only 179 (20%) of 905 children; however, of these 179 children, 168 (94%) had received a complete PCV13 3+0 schedule, reflecting national PCV13 coverage rates.[11] The prevalence of pneumococcal carriage by culture in MORDOR-exposed children was 65% (145 of 222 children) in azithromycin-treated clusters and 73% (161 of 222 children) in placebo-treated clusters; among MORDOR-unexposed children, pneumococcal carriage prevalences were 85% (191 of 226 children) in azithromycin-treated clusters and 82% (181 of 222 children) in placebo-treated clusters.

678 pneumococci were identified by culture and biochemical testing. However, 252 (37%) of these 678 genomes were excluded from further analysis on the basis of genome size, fragmentation, assignment as a non-pneumococcal species, and genome contamination with different strains of the same species or with other species (appendix pp 7, 74). Quality control analysis of genomes obtained during the MORDOR trial is shown in the appendix (p 75). We assessed 426 isolates from the 905 participants (190 from azithromycin-treated clusters and 236 from placebo-treated clusters), as well as samples from the baseline of the MORDOR trial (164 isolates; 83 from azithromycin-treated clusters and 81 from placebo-treated clusters) and from 6 months post-MDA (223 isolates; 119 from azithromycin-treated clusters and 104 from placebo-treated clusters). We also identified and validated 958 pneumococcal genomes from cross-sectional studies conducted in Blantyre in 2015 (June 22–Dec 22), 2017 (Jan 3–Dec 20), and 2019 (Jan 17–Aug 9; table, appendix pp 41–66).[12]

	Mangochi, Malawi (MORDOR trial site)						Blantyre, Malawi (non-MDA site)		
	2015 (baseline)		2017 (6 months post-MDA)		2021 (3·5 years post-MDA)		2015 (n=267)	2017 (n=424)	2019 (n=267)
	Placebo clusters (n=81)	Azithromycin clusters (n=83)	Placebo clusters (n=104)	Azithromycin clusters (n=119)	Placebo clusters (n=236)	Azithromycin clusters (n=190)			
Sex									
Male	38 (47%)	39 (47%)	40 (38%)	56 (47%)	115 (49%)	83 (44%)	132 (49%)	201 (47%)	139 (52%)
Female	43 (53%)	44 (53%)	64 (62%)	63 (53%)	121 (51%)	107 (56%)	135 (51%)	223 (53%)	128 (48%)
Exposure									
MORDOR-exposed*	81 (100%)	83 (100%)	104 (100%)	119 (100%)	106 (45%)	76 (40%)	0	0	0
MORDOR-unexposed†	N/A	N/A	N/A	N/A	130 (55%)	114 (60%)	267 (100%)	424 (100%)	267 (100%)
Age group, months									
0–11	5 (6%)	1 (1%)	11 (11%)	28 (24%)	0	0	0	0	0
12–23	7 (9%)	4 (5%)	21 (20%)	17 (14%)	42 (18%)	40 (21%)	0	0	0
24–35	22 (27%)	13 (16%)	25 (24%)	25 (21%)	47 (20%)	39 (21%)	0	1 (<1%)	39 (15%)
36–47	16 (20%)	15 (18%)	20 (19%)	24 (20%)	38 (16%)	35 (18%)	154 (58%)	182 (43%)	82 (31%)
48–59	15 (19%)	15 (18%)	27 (26%)	23 (19%)	13 (6%)	17 (9%)	111 (42%)	179 (42%)	74 (28%)
60–71	7 (9%)	20 (24%)	0	2 (2%)	23 (10%)	14 (7%)	2 (1%)	54 (13%)	37 (14%)
72–83	8 (10%)	13 (16%)	0	0	17 (7%)	13 (7%)	0	8 (2%)	12 (4%)
84–95	1 (1%)	2 (2%)	0	0	30 (13%)	15 (8%)	0	0	19 (7%)
96–107	0	0	0	0	16 (7%)	14 (7%)	0	0	4 (1%)
108–119	0	0	0	0	7 (3%)	3 (2%)	0	0	0
Unknown	0	0	0	0	3 (1%)	0	0	0	0

Data are n (%). MDA=mass drug administration. MORDOR=Macrolides Oraux pour Réduire les Décès avec un Oeil sur la Résistance. *MORDOR-exposed children were resident in a MORDOR-defined cluster, had lived in a MORDOR-defined cluster during the MORDOR trial, and were aged 4–9 years. †MORDOR-unexposed children were resident in a MORDOR-defined cluster, were born after the MORDOR trial ended, and were aged 1–3 years.

Table: **Characteristics of study population**

In azithromycin-treated clusters, genotypic macrolide resistance increased from 21·7% (95% CI 14·2–31·7; 18 of 83 isolates) at baseline to 31·9% (24·2–40·8; 38 of 119 isolates) 6 months post-MDA and to 32·1% (25·9–39·0; 61 of 190 isolates) 3·5 years post-MDA (figure 2A, appendix p 8). In placebo-treated clusters, resistance increased from 21·0% (13·5–31·1; 17 of 81 isolates) at baseline to 25·0% (17·7–34·1; 26 of 104 isolates) 6 months post-MDA and to 30·9% (25·4–37·1; 73 of 236 isolates) 3·5 years post-MDA. Mixed-effects logistic regression, adjusting for community clustering, showed no significant differences in odds ratios between azithromycin-treated clusters and placebo-treated clusters across all survey timepoints (baseline 0·97 [95% CI 0·36–2·55], 6 months post-MDA 1·46 [0·67–3·17], and 3·5 years post-MDA 1·12 [0·66–1·91]). However, macrolide resistance was significantly higher in the azithromycin-treated clusters than at the non-MDA site in Blantyre at 6 months post-MDA (38 [31·9%] of 119 isolates *vs* 70 [16·5%] of 424 isolates; p=0·0098) and at the longer follow-up (3·5 years post-MDA at the MORDOR site and 2·5 years at Blantyre; 61 [32·1%] of 190 isolates *vs* 44 [16·5%] of 267 isolates; p=0·0049). Macrolide resistance was also significantly higher in the placebo-treated clusters than at the non-MDA site at 2·5–3·5 years post-MDA (73 [30·9%] of 236 isolates *vs* 44 [16·5%] of 267 isolates; p=0·0065). Macrolide resistance at the non-MDA site remained stable: 16·9% (12·8–21·8; 45 of 267 isolates) at baseline, 16·5% (13·3–20·3; 70 of 424 isolates) at 6 months, and 16·5% (12·5–21·4; 44 of 267 isolates) at 2·5 years. Among MORDOR-unexposed children in the azithromycin-treated clusters, macrolide resistance was highest at 36·0% (27·7–45·1; 41 of 114 isolates; figure 2B), significantly higher than among children from the non-MDA site (41 [36·0%] of 114 isolates *vs* 38 [29·2%] of 130 isolates; p=0·0007). Genotypic and phenotypic macrolide resistance concordance was 91% (appendix p 11). After adjusting for clustering, treatment exposure, and age, no significant predictors of resistance were found in the long-term survey (appendix p 12). Overall, macrolide resistance did not return to baseline levels in the azithromycin-treated clusters, spilled over into the placebo-treated clusters, and further increased after a round of MDA in the placebo clusters after the MORDOR trial.

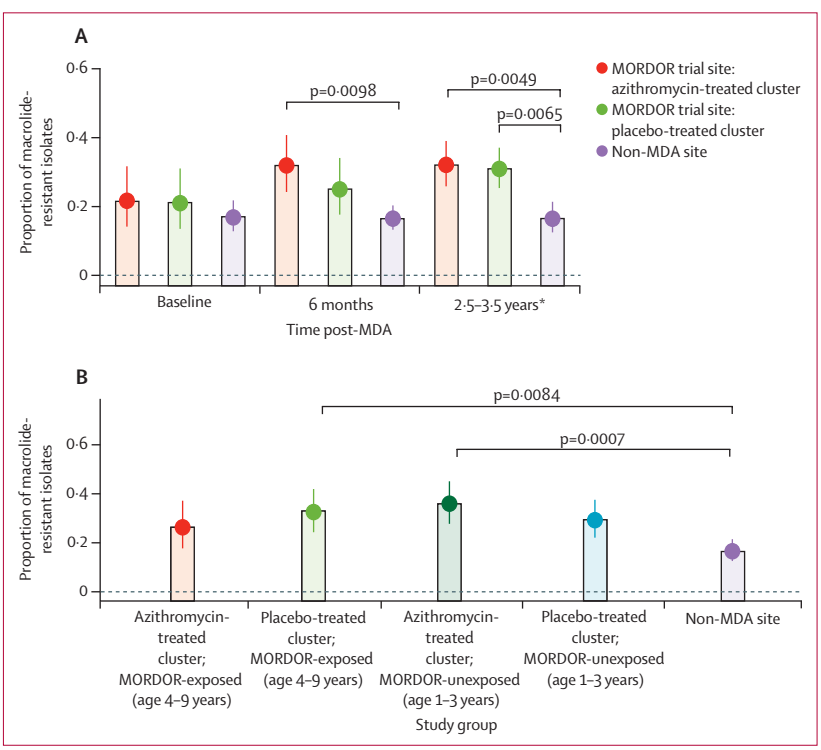


Figure 2: **Macrolide resistance in pneumococcal carriage isolates**
(A) Proportion of macrolide-resistant isolates among children from the MORDOR trial site and the non-MDA site at baseline, 6 months post-MDA, and 2·5–3·5 years post-MDA. (B) Proportion of macrolide-resistant isolates among children who were living in the trial clusters during the MDA trial (MORDOR-exposed, aged 4–9 years), children living in the trial clusters who were born after the MDA trial (MORDOR-unexposed, aged 1–3 years), and children living in a non-MDA trial site (aged 1–9 years). Data are mean (95% CI). *The prospective survey at the MDA trial site was conducted 3·5 years after MDA, whereas the most recent survey conducted at the non-MDA site was at 2·5 years after the baseline survey. MDA=mass drug administration.

We investigated the effect of MDA on the co-selection of other resistance determinants (figure 3A–F, appendix p 8). Tetracycline resistance increased in the azithromycin-treated clusters at 6 months post-MDA compared with the baseline and was significantly higher than at the non-MDA site (59 [49·6%] of 119 isolates *vs* 127 [30·0%] of 424 isolates; p=0·0035), with spillover into the placebo-treated clusters. At the 2·5–3·5-year follow-up, tetracycline resistance remained significantly higher in both azithromycin-treated clusters (84 [44·2%] of 190 isolates *vs* 77 [29·1%] of 267 isolates; p=0·029) and placebo-treated clusters (110 [46·6%] of 236 *vs* 77 [29·1%] of 267; p=0·0020) than at the non-MDA site. Across all survey timepoints, erythromycin and tetracycline resistance were strongly associated (p<0·0001). Tetracycline resistance remained stable in the non-MDA site: 28·9% (95% CI 23·7–34·5; 77 of 267 isolates) at baseline, 30·0% (25·8–34·5; 127 of 424) at 6 months post-MDA, and 29·1% (23·7–34·5; 77 of 267) at 3·5 years post-MDA. Multidrug resistance, defined as resistance to at least three antibiotic classes, was significantly higher in azithromycin-treated clusters (78 [41·1%] of 190 isolates *vs* 61 [22·8%] of 267 isolates; p=0·0015) and placebo-treated clusters (102 [43·2%] of 236 isolates *vs* 61 [22·8%] of 267 isolates; p<0·0001) at 3·5 years post-MDA than at the non-MDA site at 2·5 years. MDR remained stable in the non-MDA site, with prevalences of 22·5% (17·9–27·8, 60 of 267 isolates) at baseline, 26·2% (22·2–30·6, 111 of 424 isolates) at 6 months, and 22·8% (18·2–28·2, 61 of 267 isolates) at 2·5 years.

The proportion of penicillin non-susceptible pneumococci was similar across azithromycin-treated and placebo-treated clusters at baseline and at 6 months post-MDA (figure 3F). However, by 3·5 years post-MDA, the proportion of penicillin non-susceptible pneumococci exceeding the Clinical and Laboratory Standards Institute (CLSI) meningitis breakpoint (MIC 0·12 µg/mL) in both the azithromycin-treated (113 [59%] of 190 isolates

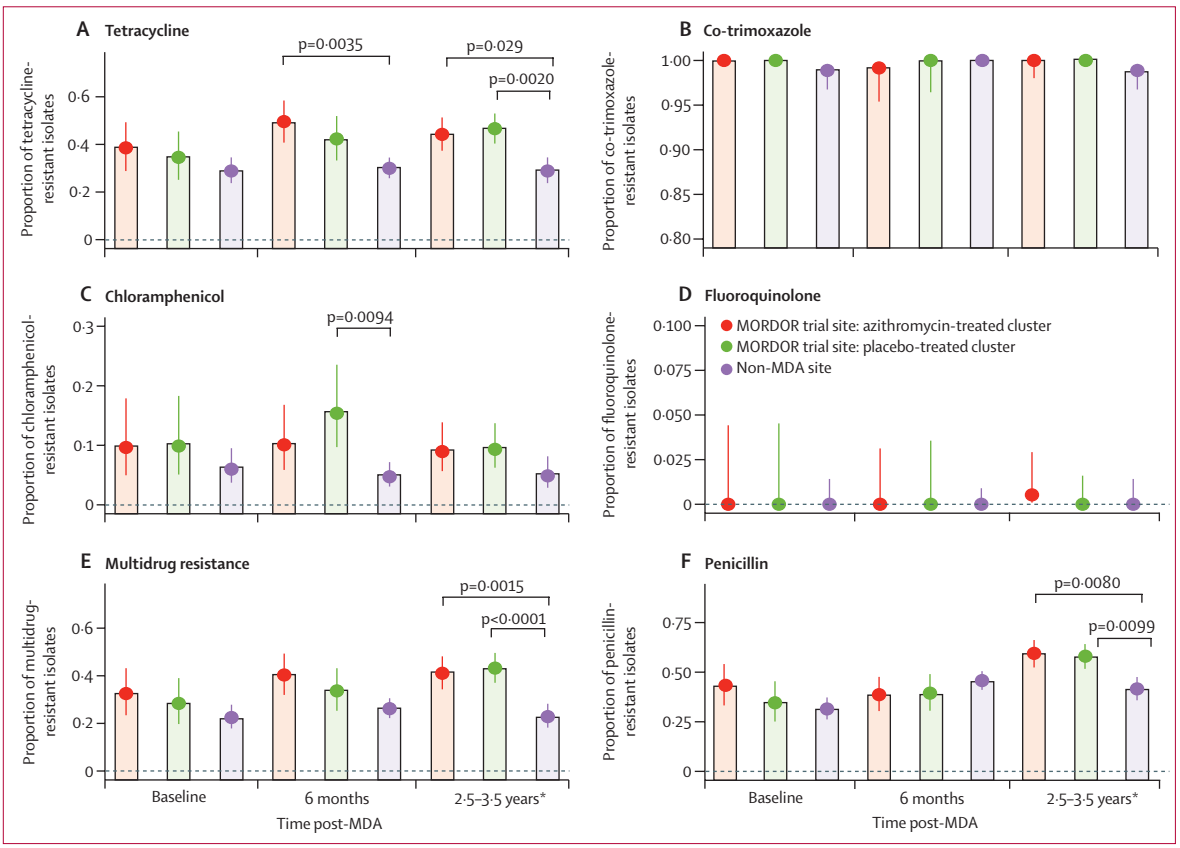


Figure 3: **Antibiotic susceptibility and multidrug resistance**
Proportion of pneumococcal carriage isolates resistant to tetracycline (A), co-trimoxazole (B), chloramphenicol (C), fluoroquinolone (D), at least three antibiotic classes (multidrug-resistant; E), and penicillin (CLSI meningitis breakpoint; F) at the MORDOR trial site and the non-MDA site at baseline, 6 months post-MDA, and 2·5–3·5 years post-MDA. Data are mean (95% CI). CLSI=Clinical and Laboratory Standards Institute. MDA=mass drug administration. *The prospective survey at the MDA trial site was conducted 3·5 years following MDA, whereas the most recent survey conducted in the non-MDA site was at 2·5 years after the baseline survey.

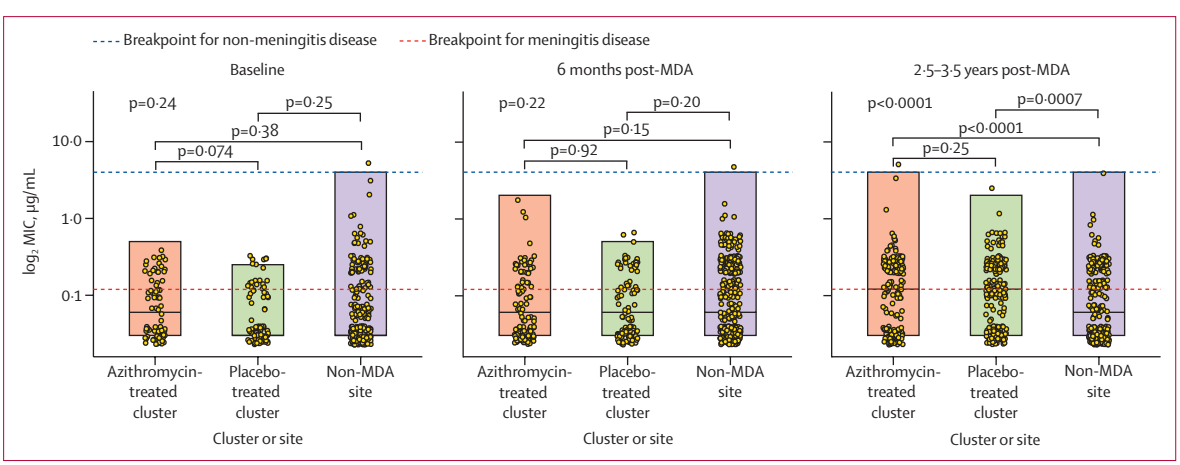


Figure 4: **Distribution of genotypically predicted penicillin MIC values**
Penicillin MICs for pneumococcal isolates from the MORDOR trial site and non-MDA site at baseline, 6 months post-MDA, and 2·5–3·5 years post-MDA surveys. The blue horizontal dotted lines indicate CLSI clinical breakpoints for non-meningitis disease, in which pneumococci with MIC values below the line are considered susceptible and those above the line are non-susceptible. The red horizontal dotted lines represent CLSI clinical breakpoints for meningitis disease, with MIC values below the line indicating susceptibility and values above indicating non-susceptibility. For each group, the thick black horizontal line indicates the median MIC, and the shaded rectangles show the range of MIC values. The yellow dots are individual data points, which were spread with the jitter plotting feature (ggplot) in R. CLSI=Clinical and Laboratory Standards Institute. MDA=mass drug administration. MIC=minimum inhibitory concentration. *The prospective survey at the MDA trial site was conducted 3·5 years following MDA, whereas the most recent survey conducted at the non-MDA site was at 2·5 years after the baseline survey.

vs 111 [42%] of 267 isolates; p=0·0080) and placebo-treated (137 [58%] of 236 isolates *vs* 111 [42%] of 267 isolates; p=0·0099) clusters at the MORDOR trial site was significantly higher than at the non-MDA site. Penicillin resistance was significantly associated with erythromycin resistance at both 6 months post-MDA (p<0·0001) and 3·5 years post-MDA (p=0·0005). At baseline, median penicillin MICs were below the clinical breakpoints for meningitis and non-meningitis diseases (figure 4). By 3·5 years post-MDA, both the range of MICs and median MIC increased, reaching the meningitis breakpoint in the azithromycin-treated clusters (median 0·12 µg/mL, IQR 0·03–0·25; p<0·0001) and the placebo-treated clusters (0·12, 0·03–0·25; p=0·0007)—significantly higher than at the non-MDA site (0·06, 0·03–0·25). MORDOR-unexposed children in the azithromycin-treated clusters had the highest MICs among all age groups (appendix p 77). Accounting for clustering by community using penicillin MIC, treatment exposure, and age as fixed effects and cluster as a random effect, we found significant associations with age (appendix p 13). Overall, our findings suggest a gradual increase in pneumococcal penicillin MICs following MDA.

We hypothesised that the emergence of pneumococcal AMR after MDA could be due to the expansion of macrolide-resistant and MDR lineages. However, no significant differences in the proportion of PCV13 or PCV20 serotypes were observed between treatment clusters and across the survey timepoints (appendix p 78). There was no effect of MDA on pneumococcal genetic diversity at the level of sequence types, and the accessory genome composition remained stable across all survey timepoints and between exposures (appendix pp 79–81). The frequencies of global pneumococcal sequence clusters (GPSCs), such as GPSC5 (non-PCV13 serotypes), increased over time in both azithromycin-treated and placebo-treated clusters, but decreased at the non-MDA site (figure 5A, B). Macrolide-resistant GPSC5 ST10599 and ST361 strains were observed 3·5 years post-MDA at the trial site but were largely absent at the non-MDA site (figure 5C, D). These strains exhibited a mixed AMR profile, with the dominant strain being resistant to penicillin and co-trimoxazole and other strains showing variable resistance to erythromycin, tetracycline, and chloramphenicol (figure 5C, D).

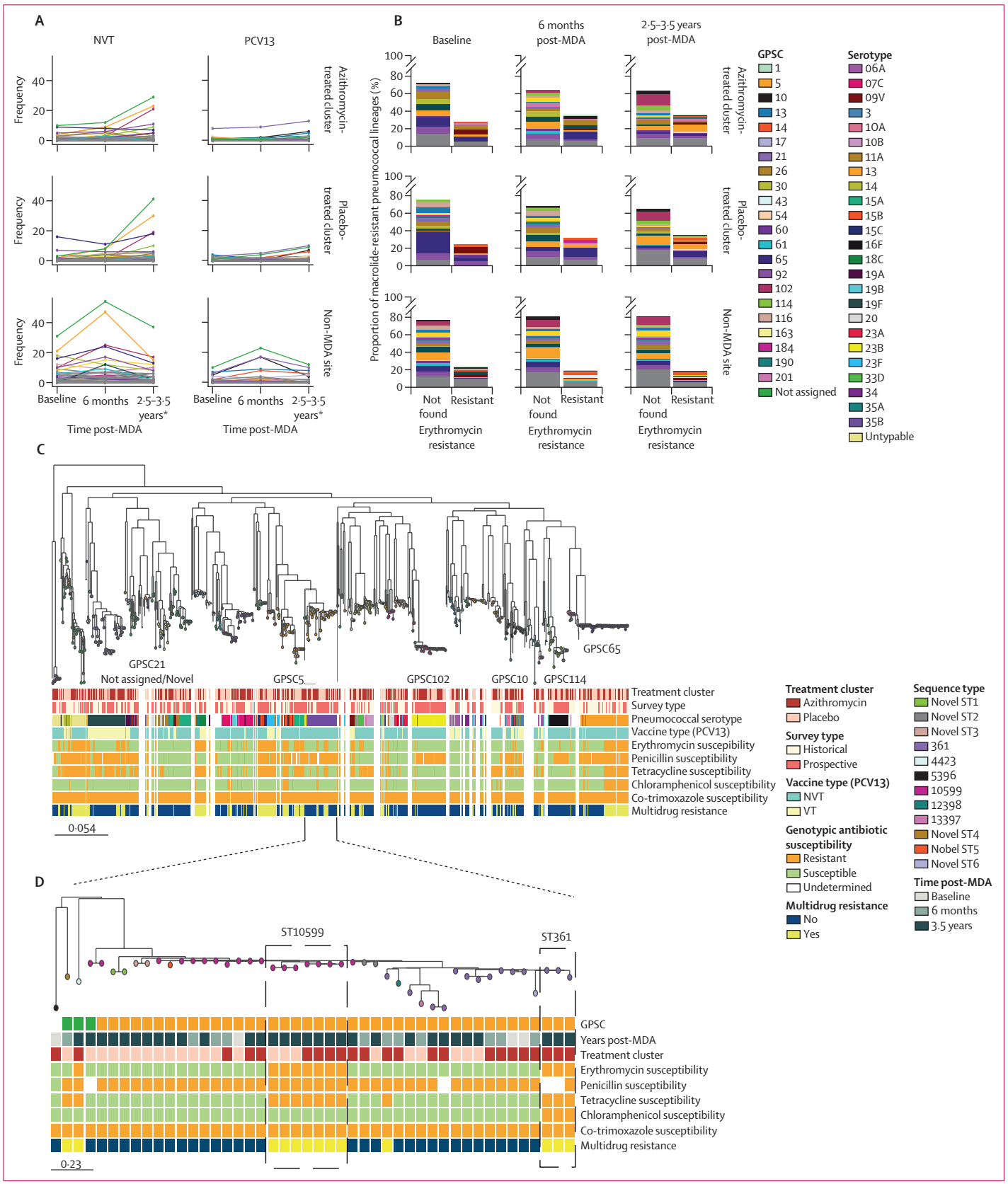
S pneumoniae is highly transformable, enabling the acquisition of AMR through integrative conjugative elements.[13] To investigate whether the emerging GPSC5 ST361 and ST10599 MDR variants acquired integrative conjugative elements at the MDA trial site, we compared the strains isolated from this site with global datasets (appendix pp 67–72). Integrative conjugative elements for GPSC5 ST361 strains were highly fragmented owing to challenges in resolving repetitive DNA repeat sequences. However, we identified a complete integrative conjugative element of 23 032 bp in the GPSC5 ST10599 variants, which contained 20 open reading frames (appendix p 17). Using the nucleotide Basic Local Alignment Search Tool, we found that this integrative conjugative element belongs to the Tn916 family, previously identified in *Streptococcus mitis*, *Streptococcus oralis*, and other pneumococci. The data suggest that the MDR ST10599 variant, carrying the macrolide and tetracycline resistance-associated integrative conjugative elements, probably emerged or expanded after the MDA trial, with a source that could be other commensal *Streptococci*.

Discussion

MDA has been effective in reducing childhood mortality,[1] particularly in countries where the implementation of other public health interventions is challenging.[3] However, our findings from Malawi, where the MORDOR trial found no significant benefit of MDA on mortality,[1] reveal the expansion and persistence of *S pneumoniae* macrolide resistance after MDA. Resistance spread to children who had not yet been born while the MORDOR trial was in progress and who had therefore not been directly treated. AMR levels at the site of the MORDOR trial 3·5 years after MDA remained higher than before MDA. Although macrolides are not the first-line therapy for pneumonia in many countries in sub-Saharan Africa, they remain an important treatment option.[14] By integrating epidemiological data with high-resolution genomics, our approach shows that pneumococcal resistance coexists with the emergence of MDR and the clonal expansion of lineages with transmissible resistance on integrative conjugative elements. Pneumococcal clonal expansion after MDA has been observed elsewhere,[10,15] but whether the long-term use of MDA will facilitate the transmission of resistant clones to surrounding non-trial sites remains to be established. Antimicrobial stewardship, particularly in the context of MDA programmes, is essential to balance

Figure 5: **Emerging and expanding macrolide-resistant lineages following azithromycin MDA at baseline, 6 months post-MDA, and 2·5–3·5 years post-MDA**

(A) Frequency changes of GPSC lineages (PCV13 and NVTs) associated with macrolide resistance across surveys conducted at the MORDOR trial sites and non-MDA sites. (B) Proportion of GPSC lineages associated with macrolide resistance across surveys conducted at the MORDOR trial sites and non-MDA sites. (C) Maximum likelihood phylogenetic tree of 813 *Streptococcus pneumoniae* isolates (142 042 single-nucleotide polymorphisms from 1 424 324 core nucleotides), with tips coloured by GPSC. Metadata are treatment group, survey type, serotype, vaccine type (PCV13), genotypic susceptibility, and multidrug resistance. The expanding GPSC5 lineage associated with macrolide resistance is highlighted by the dotted rectangle. (D) Maximum likelihood phylogenetic tree of 46 *S pneumoniae* serotype 35B isolates (36 807 single-nucleotide polymorphisms from 1 533 065 core nucleotides), with tips coloured by sequence type. Metadata are GPSC, years post-MDA, treatment group, genotypic susceptibility, and multidrug resistance. The dotted rectangles highlight lineages with acquired mobile genetic elements linked to macrolide and tetracycline resistance. GPSC=Global Pneumococcal Sequence Cluster. MDA=mass drug administration. NVT=non-vaccine type. VT=vaccine type. *The prospective survey in the MDA trial site was conducted 3·5 years following MDA, whereas the most recent survey conducted in the non-MDA site was at 2·5 years after the baseline survey.


A
NVT
PCV13
Frequency
Azithromycin-treated cluster
Placebo-treated cluster
Non-MDA site
Baseline
6 months
2·5–3·5 years*
Time post-MDA
B
Baseline
6 months post-MDA
2·5–3·5 years post-MDA
Proportion of macrolide-resistant pneumococcal lineages (%)
Not found
Resistant
Erythromycin resistance
GPSC
1
5
10
13
14
17
21
26
30
43
54
60
61
65
92
102
114
116
163
184
190
201
Not assigned
Serotype
06A
07C
09V
3
10A
10B
11A
13
14
15A
15B
15C
16F
18C
19A
19B
19F
20
23A
23B
23F
33D
34
35A
35B
Untypable
C
GPSC21
Not assigned/Novel
GPSC5
GPSC102
GPSC10
GPSC114
GPSC65
Treatment cluster
Survey type
Pneumococcal serotype
Vaccine type (PCV13)
Erythromycin susceptibility
Penicillin susceptibility
Tetracycline susceptibility
Chloramphenicol susceptibility
Co-trimoxazole susceptibility
Multidrug resistance
0·054
Treatment cluster
Azithromycin
Placebo
Survey type
Historical
Prospective
Vaccine type (PCV13)
NVT
VT
Genotypic antibiotic susceptibility
Resistant
Susceptible
Undetermined
Multidrug resistance
No
Yes
Sequence type
Novel ST1
Novel ST2
Novel ST3
361
4423
5396
10599
12398
13397
Novel ST4
Nobel ST5
Novel ST6
Time post-MDA
Baseline
6 months
3.5 years
D
ST10599
ST361
GPSC
Years post-MDA
Treatment cluster
Erythromycin susceptibility
Penicillin susceptibility
Tetracycline susceptibility
Chloramphenicol susceptibility
Co-trimoxazole susceptibility
Multidrug resistance
0·23

the life-saving but potentially short-term benefits of MDA against the long-term risks of AMR. Macrolide resistance has not reverted to pre-exposure levels, emphasising the need for ongoing surveillance to establish whether this resistance has become permanently fixed in the population.

After the introduction of the pneumococcal conjugate vaccine PCV13, we identified shifts in the pneumococcal population structure in Malawi, with the emergence of genotypes with virulence and AMR profiles that confer a competitive advantage.[16] We have also seen the emergence of pneumococcal capsule locus variants that retain their serotype.[17] Although MDA has different and more subtle effects on the population dynamics, these empirical data—together with stochastic dynamic models of pneumococcal carriage—suggest that population-level antibiotic pressure results in the emergence of new resistant types.[18] The combined effects of vaccine and antibiotics might therefore accelerate genetic shifts within these populations.[19]

The persistence of macrolide resistance after MDA in our study contradicts earlier findings that suggest resistance dissipates after antibiotic pressure is removed,[20] indicating a complex relationship between antimicrobial exposure and resistance. We also show AMR spillover into untreated clusters at 6 months post-MDA, consistent with previous phenotypic data from Malawi,[8] and further increases in resistance following an additional round of MDA in the placebo-treated clusters after the MORDOR trial. However, no spillover was observed in Blantyre, an area where MDA was not conducted and where AMR levels remained stable. Similar AMR spillover has been reported in placebo-treated clusters following MDA in Niger,[21] and geospatial analyses in The Gambia suggest frequent sharing of pneumococcal strains between neighbouring villages.[22] In Malawi, frequent movement of individuals between fishing communities[23] might further facilitate the spread of resistant strains.

The widespread use of MDA raises concerns about vaccine escape, particularly through the co-selection of resistance to non-macrolide antibiotics, including β-lactams.[24] Although some short-term studies have found no effect of MDA on pneumococcal resistance to penicillin,[8] at 3·5 years post-MDA we show the emergence and expansion of lineages with increased MICs of penicillin in both azithromycin-treated and placebo-treated clusters. Median MICs approached the meningitis breakpoint, raising concerns about treatment failure.

In 2020, WHO recommended MDA for children aged 1–11 months to reduce childhood mortality in regions of sub-Saharan Africa where infant mortality is greater than 60 per 1000 live births or under-five mortality is greater than 80 per 1000 live births, provided that AMR is monitored and child survival interventions are strengthened.[2] However, MDA could have a wider effect on AMR for several pathogens. For example, a longitudinal study in Papua New Guinea, evaluating WHO's yaws eradication strategy, found that MDA facilitated the emergence and spread of azithromycin-resistant *Treponema pallidum* subspecies *pertenue*.[25] Our findings, together with evidence of MDA selecting for both macrolide and non-macrolide resistance among gut pathogens,[24] underscore the importance of robust surveillance for AMR in resource-limited settings after MDA. Although surveillance for invasive bacterial infections is crucial, it requires sustainable clinical and laboratory infrastructure.[26] Carriage surveys might be less resource-intensive; however, although mathematical models can fill some data gaps,[27] estimating the disease risk from AMR pathogens without supporting data on invasive disease remains open to bias.

Whole-genome sequencing underestimates macrolide resistance.[28] Resistance to macrolides in *S pneumoniae* is conferred by the expression of the genes *ermB* (encoding an enzyme that modifies 23S rRNA) or *mefA* (encoding macrolide efflux protein A);[29] however, some phenotypically resistant isolates in which these genes are absent have been shown to have mutations in 23S rRNA or genes encoding ribosomal proteins.[28] We might therefore have underestimated the long-term effect of MDA.

This study has several limitations. Genome contamination, probably due to the carriage of multiple pneumococcal strains, restricted the number of genomes analysed and limited our ability to detect emerging macrolide-resistant genotypes at low frequencies. However, our genotypic resistance findings were consistent with phenotypic data from baseline and 6 months post-MDA.[8] Children in the placebo clusters of the MORDOR trial received a round of MDA in September–October, 2017 (ie, after the 6-month follow-up), resulting in an increase in macrolide resistance at 3·5 years post-MDA. Although resistance was already increasing at the 6-month follow-up before this MDA,[8] the placebo-treated and azithromycin-treated clusters could therefore not be directly compared at this final timepoint. Nonetheless, increasing resistance was seen across the population and resistance did not revert to pre-exposure levels by 3·5 years post-MDA. These data underscore the potential cumulative effect of MDA exposure in populations in which AMR is already increasing.

Our comparator population was surveyed in the same years as the baseline and 6-month post-MDA surveys of the MORDOR trial; however, the most recent survey of the comparator population was in 2019 (Jan 17–Aug 9), compared with 2021 (April 8–May 14) for the MDA site in the MORDOR trial. As such, for the long-term carriage survey, direct comparison of the populations in the same year was not possible. Additionally, although we could not confirm the vaccination status of all children in our prospective survey owing to missing health records, PCV13 coverage among those with records aligned with the high national coverage rates.[11]

We did not directly assess antimicrobial use within the clusters; however, although informal or cross-border

acquisition of macrolides cannot be ruled out, urban and periurban data from Malawi suggest that erythromycin is used in less than 1% of households.[30] The COVID-19 pandemic restricted our ability to collect detailed geographical data, limiting strain-sharing analyses. We did not assess the mobility of the local population. However, fishing communities in Mangochi, where mobility is high due to the search for better markets,[23] could facilitate the transmission of resistant strains. Finally, some integrative conjugative elements linked to macrolide resistance could not be fully resolved owing to their large, repetitive sequences, which are challenging to reconstruct using short-read sequencing.

In conclusion, analysis of *S pneumoniae* up to 3·5 years post-MDA reveals the persistence and spread of macrolide-resistant and MDR lineages associated with integrative conjugative elements. These findings highlight the need for ongoing genomic surveillance to monitor AMR dynamics in MDA programmes. Without timely detection and intervention, these trends could become difficult to reverse.

Contributors

This study was designed by AK, ABI-P, NF, TDS, and RSH. AK, ABI-P, KK, FB, and DVB coordinated the fieldwork. CB, JM, DC, LS, HM, and JC coordinated and conducted laboratory work. RB, JDH, and AC provided the historical isolates and dataset. AK, CC, RC, and JMC analysed the data and all authors contributed to data interpretation. AK, JMC, CC, and RC accessed and verified the underlying data. AK wrote the manuscript, which was revised and approved by all authors. All authors had full access to all data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

NF declares funding from The Gates Foundation, paid to their institution. JMC received salary support from UK Research and Innovation Medical Research Council.

Data sharing

Genomes sequenced in this study have been deposited in the US National Center for Biotechnology Information database under BioProject accession code PRJNA1206800. Other publicly available genomes from Malawi used in this project are available under BioProject accession code PRJNA1011974. The accession codes for all genomes used in this study are listed in the appendix (pp 18–72). The study protocol and all individual-participant data collected during the trial, after de-identification, will be made available after publication and on receipt of a well documented and substantiated request made to the Malawi Liverpool Wellcome Programme via the corresponding author. Requests will be assessed by an independent review committee and requestors will need to sign a data access agreement.

Acknowledgments

This publication is based on research funded, at least in part, by the Gates Foundation. The findings and conclusions contained within are those of the authors and do not necessarily reflect the positions or policies of the Gates Foundation. RSH is a National Institute for Health and Care Research Senior Investigator. We thank the District Health Office in Mangochi, Malawi, and the Blantyre Institute for Community Outreach in Blantyre, Malawi, for their support of the fieldwork; and the laboratory and field staff at the Kamuzu University of Health Sciences in Mangochi and Blantyre. Views expressed in this publication are those of the authors and not necessarily those of the funders.

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