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The effect of next-generation, dual-active-ingredient, long-lasting insecticidal net deployment on insecticide resistance in malaria vectors in Benin: results of a 3-year, three-arm, cluster-randomised, controlled trial

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Summary

Background Insecticide resistance among malaria vector species now occurs in 84 malaria-endemic countries and territories worldwide. Novel vector-control interventions, including long-lasting insecticidal nets (LLINs) that incorporate new active ingredients with distinct modes of action, are urgently needed to delay the evolution and spread of resistance and to alleviate reversals in malaria-control gains. We aimed to assess the longitudinal effect of two dual-active-ingredient LLINs on insecticide resistance during a cluster-randomised, controlled trial in Benin.

Methods This 3-year, three-arm, cluster-randomised, controlled trial was conducted between Oct 17, 2019, and Oct 24, 2022, in three districts in southern Benin, to compare the effects of LLINs containing chlorfenapyr–pyrethroid or pyriproxyfen–pyrethroid with LLINs containing pyrethroid only. In 19 292 mosquitoes (*Anopheles gambiae* sensu lato) collected over 36 months—3 months of baseline followed by 3 years post-intervention—we measured longitudinal phenotypic insecticide resistance profiles using bioassays and genotypic resistance profiles using quantitative, real-time, reverse transcriptase PCR of metabolic resistance genes in two clusters per trial group. The trial was registered with ClinicalTrials.gov, NCT03931473.

Findings In all three trial groups, a significant effect of LLINs on insecticide resistance selection was evident, with the median lethal dose (LD_{50}) of α-cypermethrin approximately halving between baseline and 12 months post-LLIN distribution (pyrethroid-only LLIN cluster 21: LD_{50} 78·78 μg/ml [95% CI 65·75–94·48] *vs* 35·93 [29·41–43.86] and cluster 31: 79·26 [65·40–96·44] *vs* 38·71 [30·88–48·53]; chlorfenapyr–pyrethroid LLIN cluster 43: 104·30 [82·97–133·58] *vs* 43·99 [35·30–54·86]; and pyriproxyfen–pyrethroid LLIN cluster 36: 63·76 [52·14–77·75] *vs* 37·96 [30·88–46·69] and cluster 53: 77·67 [57·63–104·56] *vs* 39·72 [29·26–53·97]). Over the subsequent 2 years, the LD_{50} of α-cypermethrin increased past baseline values in all three trial groups (year 3 pyrethroid-only LLIN cluster 21: 141·01 [111·70–181·90] and cluster 31: 115·15 [93·90–143·09]; chlorfenapyr–pyrethroid LLIN cluster 43: 97·00 [77·24–123·54] and cluster 55: 126·99 [102·34–161·26]; and pyriproxyfen–pyrethroid LLIN cluster 36: 142·29 [112·32–184·84] and cluster 53: 109·88 [79·31–157·70]). We observed minimal reductions in chlorfenapyr susceptibility and variable but significant reductions in fertility after pyriproxyfen exposure, with an overall trend of increasing susceptibility across trial years. Several metabolic genes were implicated in resistance selection, including *CYP6P4* in the pyriproxyfen–pyrethroid LLIN group, which encodes an enzyme known to metabolise pyriproxyfen in vitro, and *CYP6P3* and *CYP9K1* in the chlorfenapyr–pyrethroid LLIN group, both of which encode enzymes that are involved in pro-insecticide activation.

Interpretation After 24 months of use, chlorfenapyr–pyrethroid LLINs no longer mitigated pyrethroid resistance selection in this area of southern Benin, which has high malaria transmission dominated by highly resistant *A gambiae* sensu lato. This finding raises issues for current net-procurement schedules, which are based on an operational net lifespan of 3 years. Knowledge of the effects of next-generation LLINs on insecticide-resistance selection is crucial for the pragmatic design of prospective resistance-management strategies.

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Introduction

Malaria remains a major public health problem in 84 countries and territories. An estimated 249 million cases and 608 000 deaths were recorded globally in 2022,[1] most of which (95% of cases and 96% of deaths) were concentrated in sub-Saharan Africa.[1] Across the

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Research in context

Evidence before this study

Insecticide resistance among malaria vector species is widespread in malaria-endemic areas globally, causing reversals in disease-control gains. Novel vector-control interventions, including long-lasting insecticidal nets (LLINs) incorporating new active ingredients with distinct modes of action, are urgently needed to delay the continued escalation and spread of resistance. In 2017, WHO issued an interim recommendation for piperonyl butoxide–pyrethroid LLINs as a new malaria vector-control tool after they showed superior protection, compared with standard pyrethroid LLINs, against malaria infection in two cluster-randomised, controlled trials (RCTs) in Tanzania and Uganda. A second generation of dual-active-ingredient LLINs—combining a pyrethroid and either a pyrrole (chlorfenapyr) or an insect growth regulator (pyriproxyfen)—has also been assessed in two cluster RCTs, with chlorfenapyr–pyrethroid LLINs reducing the incidence of malaria infection by 55% in Tanzania and 46% in Benin after 2 years. The current study was nested within the cluster RCT in Benin, published in 2023, which assessed the efficacy of two types of dual-active-ingredient LLIN compared with pyrethroid-only LLINs against malaria transmission. We searched PubMed for English-language papers, published between database inception and Nov 18, 2023, using the terms "long-lasting insecticidal net" OR "insecticide-treated net" OR "bed net", "randomised controlled trial", "village trial", "community trial", "chlorfenapyr", "piperonyl butoxide", "pyriproxyfen", "insecticide resistance" OR "resistance", "malaria", "mosquito" OR "vectors", "*Anopheles gambiae*" OR "*Anopheles coluzzii*" OR "*Anopheles funestus*" OR "*Anopheles arabiensis*". We found one study that reported longitudinal phenotypic changes in insecticide resistance after the deployment of dual-active-ingredient LLINs, disaggregated by net type. This study was from the aforementioned cluster RCT in Tanzania, in which no significant increase in α-cypermethrin resistance intensity was found in the chlorfenapyr–pyrethroid LLIN group, whereas pyrethroid resistance escalation was observed in clusters that received pyriproxyfen–pyrethroid, piperonyl butoxide–pyrethroid, and pyrethroid-only LLINs.

Added value of this study

To our knowledge, this study is the first to report the influence of next-generation, dual-active-ingredient LLINs on the dynamic evolution of phenotypic and genotypic insecticide resistance in *A gambiae* sensu lato over multiple years in an area of intense insecticide resistance and high malaria transmission in west Africa. In this trial, all three LLINs were associated with a significant post-distribution effect on insecticide resistance intensity, which subsequently dissipated at varying rates over 3 years. The findings contrasted with those from the parallel cluster RCT in Tanzania, which showed a differential effect of chlorfenapyr–pyrethroid LLINs on the long-term mitigation of insecticide-resistance selection. In the cluster RCT reported here, the superior epidemiological benefit of chlorfenapyr–pyrethroid LLINs compared with pyriproxyfen–pyrethroid and pyrethroid-only LLINs was supported by a sustained effect on phenotypic insecticide resistance for 2 years, which was lost in the third trial year.

Implications of all the available evidence

The escalation of pyrethroid resistance after 24 months' use of pyrethroid-only LLINs and pyriproxyfen–pyrethroid LLINs suggests that continued widespread distribution of these nets could have potentially severe consequences for the selection of cross-resistance mechanisms among major malaria vector populations. Parallel observations from Tanzania that piperonyl butoxide–pyrethroid LLINs, which now protect more than 50% of the population in sub-Saharan Africa, exacerbate pyrethroid resistance selection to a greater extent than pyrethroid-only and pyriproxyfen–pyrethroid LLINs raise substantial concerns about their role in worsening the insecticide resistance crisis. Reliance on a single intervention (eg, LLINs combining a partner pyrethroid with a chemical from another class, such as chlorfenapyr–pyrethroid LLINs) for reactive resistance management is not recommended given the propensity of *Anopheles* vectors to rapidly evolve resistance to new active ingredients and recent reports of reduced chlorfenapyr susceptibility from other parts of west and central sub-Saharan Africa. The differential effects of LLIN types on malaria outcomes also argue for revisions to contemporary net-procurement regimens and the design of more tailored and pragmatic prospective resistance-management strategies.

subcontinent, considerable progress was made between 2000 and 2015, during which annual deaths decreased from 841 000 to 542 000.[1] This epidemiological gain was mostly attributed to the scale-up of long-lasting insecticidal nets (LLINs) and targeted indoor residual spraying.[2] However, progress stalled after 2015 owing to several biological and non-biological challenges,[3] including the spread of insecticide resistance among malaria vector populations and, more recently, the COVID-19 pandemic.[4,5]

In Benin, the most recent countrywide distribution campaign for LLINs, led by the Benin National Malaria

Control Programme, occurred in March and April, 2020. Of 14 423 998 people recorded, 13 581 637 people received 7 652 166 LLINs.[6] During this campaign, different brands of pyrethroid (deltamethrin)-containing LLIN were distributed across the country. In 2010, initial phenotypic resistance of *Anopheles gambiae* sensu lato to insecticides deployed by the National Malaria Control Programme was reported in several departments across Benin,[7–9] including moderate-to-high pyrethroid resistance intensity and minor reductions in susceptibility to carbamate and organophosphate insecticides.[10,11] Regarding implicated resistance mechanisms, the

Leu1014Phe *kdr* mutation was widespread and close to fixation, whereas the Gly119Ser *ace-1ᴿ* and Asn1575Tyr *kdr* mutations were detected at very low frequencies.[10,12,13] The cytochrome P450 genes *CYP6P3* and *CYP6M2* were also found to be overexpressed in mosquitoes from some sites.[14,15] These observations have raised substantial concerns about the potential operational failure of standard pyrethroid-only LLINs.[16]

In response to the growing threat of insecticide resistance, novel dual-active-ingredient LLINs were developed containing a pyrethroid insecticide plus a second partner chemical with a distinct mode of action. On March 14, 2023, WHO issued a new policy recommendation for the deployment of chlorfenapyr–pyrethroid LLINs after they demonstrated superior protection in two cluster-randomised, controlled trials (RCTs): compared with pyrethroid-only LLINs, Interceptor G2 (chlorfenapyr–pyrethroid) LLINs reduced malaria infection incidence by 55% in Tanzania[17] and 46% in Benin[18] over 2 trial years. Because dual-active-ingredient LLINs are now scheduled for universal distribution across multiple malaria-endemic regions, understanding their effect on the dynamic evolution of insecticide resistance throughout their operational lifetime is crucial to establish their suitability for deployment and to preserve their longer-term efficacy. During the cluster RCT in Benin, we monitored the longitudinal effect of two dual-active-ingredient LLINs—Interceptor G2 (chlorfenapyr–pyrethroid) and Royal Guard (pyriproxyfen–pyrethroid)—and standard Interceptor (pyrethroid-only) LLINs on phenotypic and genotypic resistance in *A gambiae* sensu lato, the major malaria vector across sub-Saharan Africa.[19]

Methods

Study area and trial design

This study was nested in a three-arm, superiority, single-blinded, cluster RCT conducted in the Covè, Zagnanando, and Ouinhi districts in southern Benin. Two rainy seasons occur per year, from May to July and from September to November, during which malaria transmission is intense.[19] The main malaria vector complex in the area, *A gambiae* sensu lato (consisting of a mixture of *Anopheles coluzzii* and *A gambiae* sensu stricto), is resistant to pyrethroids through several mechanisms, including the Leu1014Phe *kdr* mutation and the overexpression of mixed function oxidases.[7,9,12,14,15]

The 123 villages that formed the study area had a population of 220 000 at the start of the study. The study area was divided into 60 clusters (20 clusters per trial group; figure 1), each comprising one or more villages, with a mean of 200 households (1200 inhabitants) per cluster. To reduce contamination between trial groups, clusters consisted of a core area (minimum 100 households) surrounded by a buffer area, ensuring that core areas of neighbouring clusters were separated by at least 1000 m. The two intervention groups used either Interceptor G2
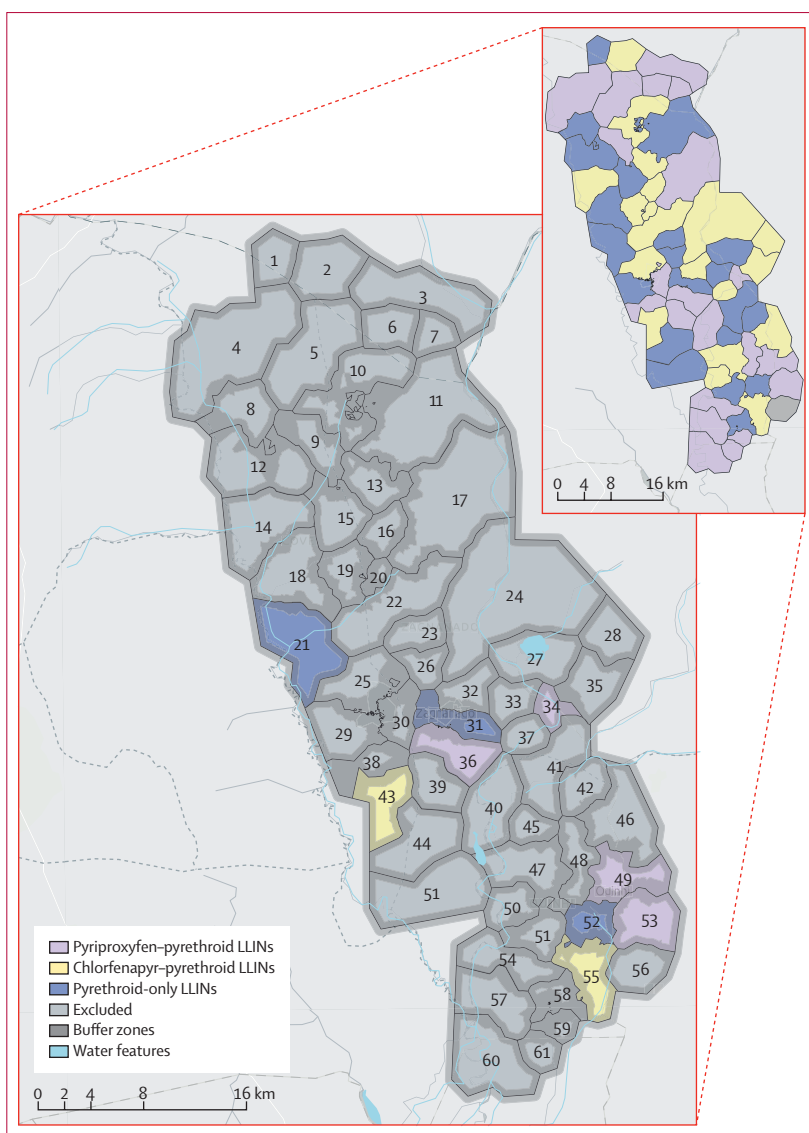
Figure 1: Map of clusters surveyed for resistance monitoring in this study
The inset shows the clusters of the full cluster-randomised, controlled trial.[18] LLINs=long-lasting insecticidal nets.

LLINs (BASF SE; Ludwigshafen, Germany), combining a pyrrole (chlorfenapyr; 4·8 g/kg) and α-cypermethrin (2·2 g/kg), henceforth chlorfenapyr–pyrethroid LLINs, or Royal Guard LLINs (Disease Control Technologies; Greer, SC, USA), combining an insect growth inhibitor (pyriproxyfen; 5·5 g/kg) and α-cypermethrin (5·5 g/kg), henceforth pyriproxyfen–pyrethroid LLINs; the control group used Interceptor LLINs (BASF SE; Ludwigshafen, Germany), containing α-cypermethrin (5·0 g/kg), henceforth pyrethroid-only LLINs. A detailed description of the study area and trial design has been reported previously.[20]

For the resistance-monitoring component of the cluster RCT, the surveyed clusters varied slightly between baseline and post-intervention periods owing to changes

in collection methods and the availability of mosquito breeding sites in the field. All households and breeding sites sampled were in the core areas of each cluster. Surveyed clusters are summarised in the appendix (p 4).

This study was part of a cluster RCT in Benin (registered with ClinicalTrials.gov on April 30, 2019; NCT03931473), which obtained ethical clearance from the Benin Ministry of Health ethics committee (6/30/MS/DC/SGM/DRFMT/CNERS/SA), the London School of Hygiene and Tropical Medicine ethics committee (16237), and the WHO Research Ethics Review Committee (ERC.0003153).[18] All study procedures were conducted in accordance with relevant guidelines and regulations.

Mosquito collections

At baseline (Oct 17–Dec 10, 2019), we collected adult mosquitoes of unknown physiological age through human landing catches, because it was challenging to identify productive mosquito breeding sites. In each cluster, four randomly selected houses were used for indoor mosquito collections. Collections were carried out by local volunteers from the community. The first collector sat in each house and collected, with a torch and manual aspirator, all mosquitoes that landed on their lower legs between 2000 h and 0200 h; they were then replaced by another collector between 0200 h and 0600 h. Three or four nights of collections took place in each cluster, with eight collectors used per cluster per collection period. The morning after each collection, all mosquitoes were transported to the field laboratory for immediate morphological identification of *A gambiae* sensu lato. Live *A gambiae* sensu lato were released into cages for a 3-h resting period before bioassay testing.[21]

Between March 19 and March 22, 2020, 115 323 LLINs were distributed to 54 030 households across the three trial groups and insecticide resistance was monitored during three post-intervention years (May 7, 2020–Oct 24, 2022). Mosquito larvae and pupae were collected from several positive breeding sites (around five to ten sites per year, depending on breeding site productivity) in two clusters per trial group. Collected larvae were reared to adulthood at 25°C (23–27°C) and 80% (70–90%) relative humidity in the field insectary. Emergent adult mosquitoes were identified to species complex level, and only *A gambiae* sensu lato were tested in bioassays.

Phenotypic insecticide-resistance monitoring

For the US Centers for Disease Control and Prevention insecticide resistance intensity bioassays, four batches of 20–25 unfed, female *A gambiae* sensu lato of either unknown age (baseline) or aged 2–5 days (post-intervention) were exposed to α-cypermethrin 1X (12·5 μg per 250 ml bottle), 2X (25 μg per 250 ml bottle), 5X (62·5 μg per 250 ml bottle), 10X (125 μg per 250 ml bottle), or 20X (250 μg per 250 ml bottle), where 1X is the diagnostic dose required to kill all susceptible mosquitoes, or to

the diagnostic dose of chlorfenapyr (100 μg per 250 mL bottle).[21–24] Each 250 mL Wheaton bottle, along with its cap, was coated with 1 mL insecticide stock solution; in each test, a parallel negative control bottle was coated with 1 ml acetone. Knockdown was recorded every 15 min during bioassays, which lasted 30 min for α-cypermethrin or 60 min for chlorfenapyr. After exposure, individual mosquitoes were aspirated into separate paper cups and provided with a 10% glucose solution. Immediate and delayed (24-h, 48-h, and 72-h post-testing) mortalities were recorded.

For pyriproxyfen testing, 70–80 randomly selected houses per trial group were surveyed in each monitoring cluster between 0700 h and 0900 h over 10–12 successive days. In each house, all mosquitoes resting on walls, roofs, earthen jars, furniture, and hung clothes were collected using mouth aspirators. Live mosquitoes were provided with a 10% glucose solution and transported to the field insectary, where blood-fed *A gambiae* sensu lato were identified and kept in cages for a 3-h resting period before exposure to pyriproxyfen (100 μg per 250 mL bottle) for 60 min. After testing, exposed and control (unexposed) mosquitoes were transferred to cages and dissected 3 days later to determine Christopher's stage of egg development (I–V).[25,26]

Genotypic and transcriptomic insecticide resistance monitoring

Before pooling specimens for RNA extraction, four to six legs from each tested mosquito were removed and genomic DNA was extracted using cetyltrimethylammonium bromide.[27] Molecular identification of species within the *A gambiae* sensu lato complex and screening for the presence of the Leu1014Phe *kdr* mutation were done by PCR.[28,29]

Individual mosquitoes that survived exposure to α-cypermethrin were selected from each study cluster for RNA extraction. Pools of five *A coluzzii* (the predominant vector species tested) of the same phenotype were homogenised using a Qiagen Tissue Lyser II (Qiagen; Hilden, Germany) with 5 mm stainless steel beads and RNA was extracted using a Qiagen RNeasy 96 Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. Approximately 2 μg of each RNA sample was treated with RQ1 RNase-free DNase (Promega; Southampton, UK). 1 μg DNase-treated RNA per mosquito pool was used to synthesise cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Loughborough, UK) according to the manufacturer's instructions.

Quantitative, real-time, reverse transcriptase PCR was used to measure the expression of eight metabolic genes (*CYP6M2*, *CYP6P3*, *CYP6P4*, *CYP6Z1*, *CYP4G16*, *CYP9K1*, *CYP6P1*, and *GSTE2*) that are commonly overexpressed in vector populations in west African countries bordering Benin.[30–32] Primer sequences and efficiencies are provided in the appendix (p 4). Standard curves of

cycle threshold (Ct) values for each gene were generated using a five-fold serial dilution of cDNA to assess PCR efficiency. Each 10 µL reaction volume contained 2 µL cDNA, 10 µM of each primer, and 5 µL 2×Roche FastStart Essential DNA Green Master mix (Roche; Welwyn Garden City, UK). Reactions were conducted in technical triplicate using a StepOnePlus real-time PCR system (Applied Biosystems; Loughborough, UK). Reaction conditions were 10 min at 95°C; 35 cycles of 10 s at 95°C, 22 s at 60°C, and 10 s at 72°C; followed by a melt curve. The fold change of each target metabolic gene from field samples, relative to a susceptible laboratory strain (*A coluzzii* N'Gousso), was calculated using the $2^{-\Delta\Delta Ct}$ method,[33] incorporating PCR efficiency. The housekeeping gene *RpS7* was used for normalisation.

Data analysis

Mortality was corrected using Abbott's formula when the mortality in control assays was between 5% and 20%.[34] The lethal doses required to kill 25%, 50%, 95%, and 99% of mosquitoes (LD_{25}, LD_{50}, LD_{95}, and LD_{99}, respectively) were estimated using a logistic model with log_{10}-transformed data. Dose–response curve estimation was based on the probability of mosquito death as a function of insecticide dose. Point estimates of lethal doses and 95% CIs were then back-transformed to their original scale to obtain the reported values; these values indicate the difference in diagnostic dose of an insecticide required to kill 25%, 50%, 95%, or 99% of tested mosquitoes. The model incorporated an automated heterogeneity factor in the calculation of CIs when appropriate to obtain unbiased interval estimates. In brief, this analysis was based on a Pearson's goodness-of-fit test; in instances in which $p < 0.15$, the model provides an offset to construct 95% CIs to ameliorate potential bias. When this occurred in our data, interpretation was based on unbiased 95% CIs to ensure that any potential heterogeneity did not affect differences between pairwise comparisons. Comparisons of LD_{50} values among clusters, years, or both, were statistically estimated using relative median potency, which was calculated as the ratio of point estimates along with simultaneous 95% CIs; 95% CIs not containing the value 1 suggest a difference at the 0·05 level. The relative median potency test is a traditional toxicological comparative ratio statistic that has been shown to align with other methods for LD_{50} comparisons.[35] To complement the logistic models, we further developed generalised linear mixed models with a logistic link function, because collections occurred over multiple timepoints and these models allow for the inclusion of clustering among the model factors—eg, random effects (such as dose or test replicate) and fixed effects (such as the study year or treatment group). To test among years in the generalised linear mixed models, Bonferroni–Holm corrections for multiple comparisons were used to reduce inflated type-1 errors. For delayed mortality

analysis, Cox regressions were used to compare hazard rate ratios, adjusted for test replications to account for changes in vector population structure across collection periods and between different insecticide doses. Immediate knockdown after exposure to insecticide was excluded. The reduction in fertility in pyriproxyfen-exposed populations was calculated as 100×(proportion of fertile control female mosquitoes – proportion of fertile pyriproxyfen-exposed female mosquitoes)/proportion of fertile control female mosquitoes.[25] The fold change in metabolic enzyme expression was compared between trial years using the Kruskal–Wallis test in GraphPad Prism version 9.5.0. Lethal dose and generalised linear mixed model analyses were conducted in IBM SPSS version 29 and Cox regression models were developed using SAS version 9.4.

The statistical analysis was extended to test whether the intensity of resistance as measured by 30-min knockdown varied by trial group or over time. A previously described binomial model with a five-parameter logistic function,[36] fit within a Bayesian framework via Markov chain Monte Carlo in the R package rstan,[37] was expanded to allow parameters to vary by both trial year and group (for full details, including model equations and priors, see appendix pp 5, 16). This framework allows greater flexibility in the shape of the relationship between insecticide dose and mosquito mortality than the relative median potency and generalised linear mixed model analyses. After an initial visual inspection of curves, three nested dose–response models were run on two separate data subsets: baseline and year 1; and years 1, 2, and 3. Model 1 fit a single dose–response curve to all data, model 2 allowed the location of the steepest part of the curve to vary linearly by trial year, and model 3 additionally allowed this time-dependent shift in the curve to vary by trial group. All models included a random effect for trial cluster. Models were compared using estimated leave-one-out cross-validation in the R package loo,[38] with higher expected log predictive density indicating a better fitting model and an expected log predictive density difference of greater than 4 between models considered significant, providing this was significantly greater than its SE (confirmed by calculating a p value from a Z score). These analyses were repeated using 72-h mortality as the outcome and analyses were done using R version 4.3.0.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report or decision to submit for publication.

Results

Between Oct 17, 2019, and Oct 24, 2022, 19 292 female *A gambiae* sensu lato were collected for resistance monitoring: 6621 at baseline (Oct 17–Dec 10, 2019), 4176 in year 1 (May 7–Oct 17, 2020), 4206 in year 2
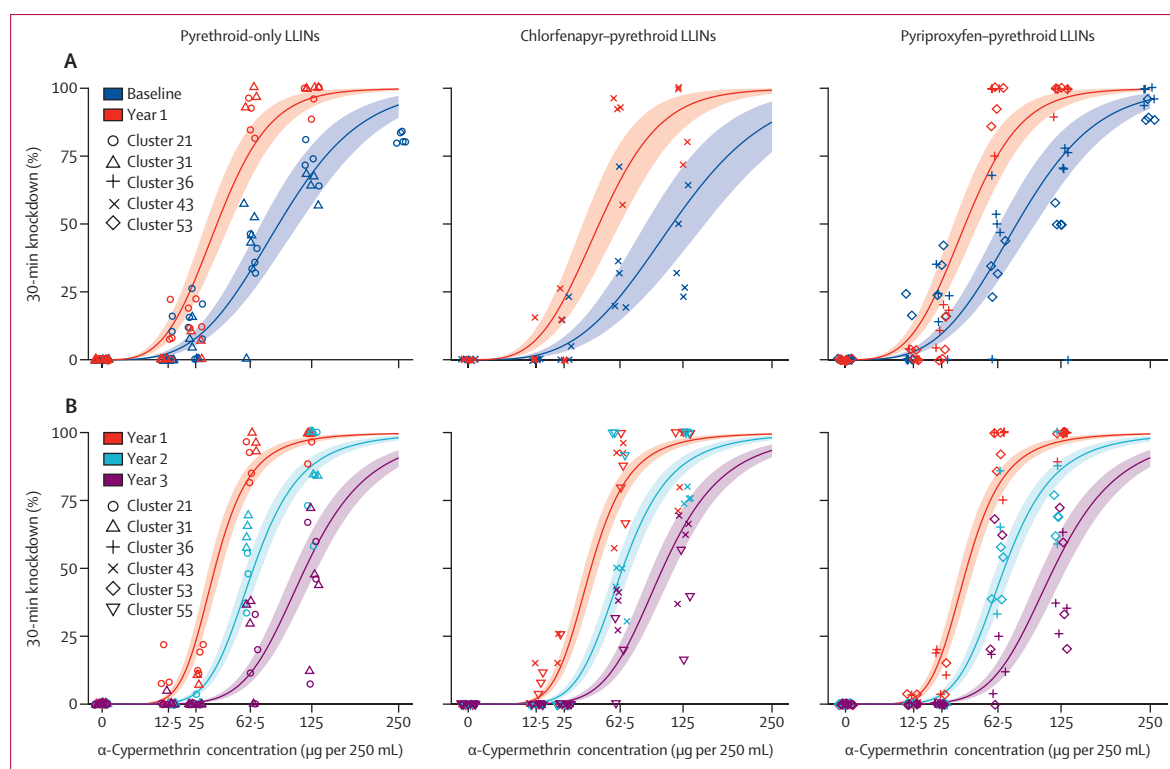
	Pyrethroid-only LLINs						Chlorfenapyr-pyrethroid LLINs						Pyriproxyfen-pyrethroid LLINs					
	Cluster number	LD$_{50}$ (µg/mL)				Relative median potency by year*	Cluster number	LD$_{50}$ (µg/mL)				Relative median potency by year*	Cluster number	LD$_{50}$ (µg/mL)				Relative median potency by year*
		Baseline	Year 1	Year 2	Year 3			Baseline	Year 1	Year 2	Year 3			Baseline	Year 1	Year 2	Year 3	
First cluster	21	78·78 (65·75–94·48), n=693	35·93 (29·41–43·86), n=484	74·61 (60·80–92·21), n=509	141·01 (111·70–181·90), n=512	Baseline vs year 1: 2·19 (1·58–3·27); baseline vs year 2: 1·06 (0·80–1·40); baseline vs year 3: 0·56 (0·40–0·75); year 1 vs year 2: 0·48 (0·33–0·66); year 1 vs year 3: 0·25 (0·15–0·39); year 2 vs year 3: 0·53 (0·37–0·73)	43	104·30 (82·97–133·58), n=474	43·99 (35·30–54·86), n=482	77·48 (62·05–97·46), n=509	97·00 (77·24–123·54), n=492	Baseline vs year 1: 2·37 (1·62–3·87); baseline vs year 2: 1·35 (0·98–1·91); baseline vs year 3: 1·08 (0·78–1·50); year 1 vs year 2: 0·57 (0·37–0·80); year 1 vs year 3: 0·45 (0·28–0·66); year 2 vs year 3: 0·8 (0·57–1·10)	36	63·76 (52·14–77·5), n=627	37·96 (30·88–46·69), n=526	65·04 (50·99–82·99), n=350	142·29 (112·32–184·84), n=491	Baseline vs year 1: 1·68 (1·22–2·48); baseline vs year 2: 0·98 (0·72–1·34); baseline vs year 3: 0·45 (0·29–0·63); year 1 vs year 2: 0·58 (0·39–0·82); year 1 vs year 3: 0·27 (0·14–0·42); year 2 vs year 3: 0·46 (0·29–0·66)
Second cluster	31	79·26 (65·40–96·44), n=495	38·71 (30·88–48·53), n=409	57·80 (47·61–70·03), n=527	115·15 (93·90–143·09), n=479	Baseline vs year 1: 2·05 (1·43–3·28); baseline vs year 2: 1·37 (1·04–1·89); baseline vs year 3: 0·69 (0·49–0·92); year 1 vs year 2: 0·67 (0·46–0·91); year 1 vs year 3: 0·34 (0·18–0·52); year 2 vs year 3: 0·50 (0·33–0·70)	55	..	41·06 (33·47–50·19), n=503	41·93 (32·15–54·78), n=280	126·99 (102·34–161·26), n=439	Year 1 vs year 2: 0·98 (0·70–1·37); year 1 vs year 3: 0·32 (0·14–0·54); year 2 vs year 3: 0·33 (0·14–0·56)	53	77·67 (57·63–104·56), n=626	39·72 (29·26–53·97), n=498	82·28 (60·34–114·52), n=524	109·88 (79·31–157·70), n=524	Baseline vs year 1: 1·96 (1·23–3·60); baseline vs year 2: 0·94 (0·61–1·47); baseline vs year 3: 0·71 (0·44–1·09); year 1 vs year 2: 0·48 (0·26–0·77); year 1 vs year 3: 0·36 (0·18–0·61); year 2 vs year 3: 0·75 (0·46–1·17)

Data are median estimate (95% CI) for LD$_{50}$ and estimate (95% CI) for relative median potency. n refers to the number of mosquitoes tested. LD$_{50}$=median lethal dose. LLINs=long-lasting insecticidal nets. *95% CIs not containing the value 1 indicate significant pairwise differences.

Table: LD$_{50}$ values of α-cypermethrin in *Anopheles gambiae* sensu lato

Figure 2: Model-derived estimates of *Anopheles gambiae* sensu lato 30-min knockdown proportions at increasing concentrations of α-cypermethrin
Raw data are shown as points, solid lines are the model-fitted dose–response curves from a five-parameter logistic function model, and shaded areas are the 95% CrIs. Results are shown for the three trial groups at baseline and trial year 1 (A) and at trial years 1, 2, and 3 (B). The results of model 3 are presented here but, during model comparison, we found no significant difference between model 3 and model 2, without the trial group variable (appendix p 18). Estimates of LD_{50} from model 3 by data subset, trial group, and year are presented in the appendix (p 11). The x-axis is shown on a square root scale and horizontal jitter has been applied to the raw data points. CrIs show uncertainty in the fitted model parameters but not sampling uncertainty from the number of mosquitoes tested. CrI=credible interval. LD_{50}=median lethal dose. LLINs=long-lasting insecticidal nets.

(April 26–Oct 2, 2021), and 4289 in year 3 (May 6–Oct 24, 2022). Results are presented for trial clusters tested across all four years; clusters tested ad hoc during the trial are presented in the appendix (pp 6–10). Species identification of a randomly sampled subset of phenotyped *A gambiae* sensu lato (n=3325) indicated that *A coluzzii* was the predominant vector species across all trial years, accounting for 3026 (91·0%) mosquitoes, with 215 (6·5%) identified as *A gambiae* sensu stricto and 84 (2·5%) as hybrid *A gambiae* sensu stricto and *A coluzzii*.

At baseline, α-cypermethrin resistance intensity was similarly high among all study clusters: between five and eight times the diagnostic dose was required to kill 50% of vector populations (table). In the pyrethroid-only LLIN group, both clusters showed a significant decrease in α-cypermethrin resistance intensity in *A gambiae* sensu lato in the first year post-intervention compared with the baseline (table, appendix p 6). By comparison, a significant increase in resistance intensity was observed in both clusters in the second year of the trial. This trend could be explained by an initial insecticidal effect elicited by brand-new LLINs that was lost as the nets began to age, allowing α-cypermethrin resistance intensity to

rebound. By the third year post-intervention, resistance intensity had increased to greater than baseline levels in both trial clusters.

Similarly, in the chlorfenapyr–pyrethroid LLIN group, α-cypermethrin resistance intensity initially declined between baseline and year 1 post-intervention (table, appendix p 6). Between years 1 and 2 post-intervention, resistance intensity increased significantly in one trial cluster. By the third year post-intervention, resistance intensity was similar to baseline in cluster 43 and had increased significantly post-intervention in cluster 55.

Results from the pyriproxyfen–pyrethroid LLIN group were consistent with those of the other trial groups, with a significant decrease in α-cypermethrin resistance intensity in the first year post-intervention compared with baseline (table, appendix p 7), followed by a significant increase in both clusters in the second year and in one cluster in the third year of the trial.

Results from the logistic generalised linear models were consistent with the relative median potency analyses presented in the table. All models were significant (likelihood ratio χ^2 test; p<0·0001). The addition of year significantly improved all models (all p<0·01). Cluster was also a significant variable in all models,
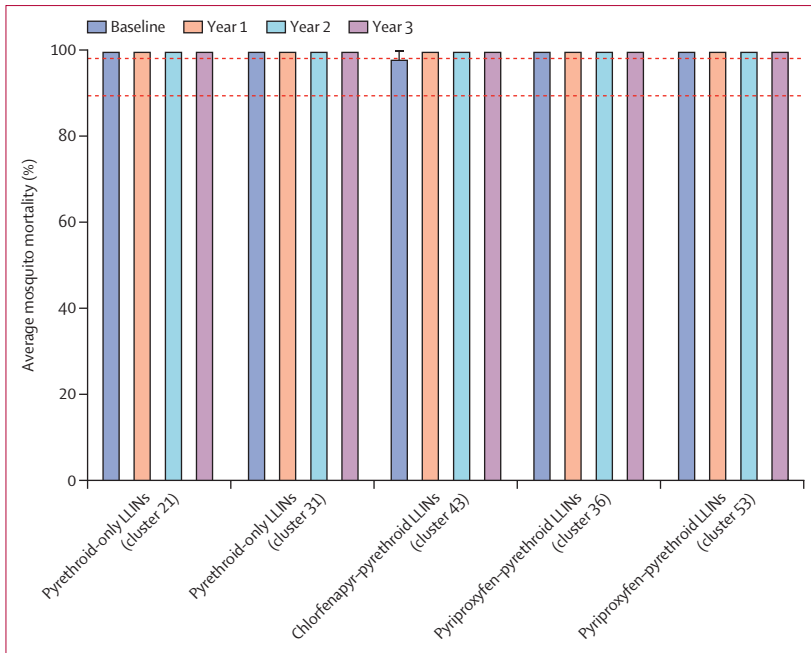
Figure 3: Susceptibility of Anopheles gambiae sensu lato to chlorfenapyr at baseline and for 3 years post-intervention
Mean mosquito mortality after 72 h is shown with 95% CIs. Mortality of less than 90% (lower red line) represents confirmed resistance at the putative diagnostic dose and less than 98% (upper red line) indicates suspected resistance at the putative diagnostic dose. LLINs=long-lasting insecticidal nets.
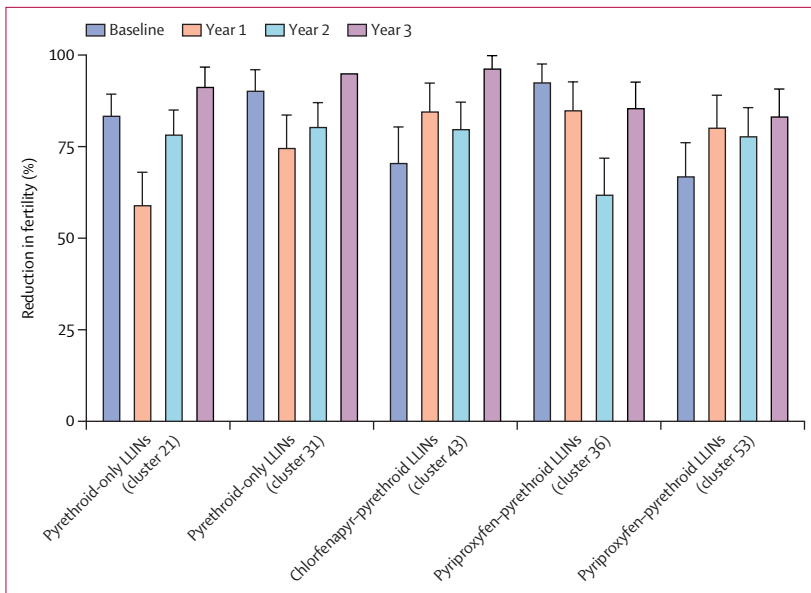

Figure 4: Effect of pyriproxyfen exposure on the fertility of Anopheles gambiae sensu lato at baseline and for 3 years post-intervention
Error bars are 95% CIs. For p values for comparisons with wild, unexposed A gambiae sensu lato, see appendix p 25. LLINs=long-lasting insecticidal nets.

a significant difference in 30-min knockdown between years (figure 2). Model 2, which included trial year, fit significantly better than model 1 for both temporal analyses; resistance intensity was significantly lower in year 1 than at baseline, but increased significantly after this point over the two subsequent years of follow-up. There was no evidence that this time-dependent change in resistance varied by trial group (the comparison between model 3, which included trial group, and model 2 was non-significant; appendix p 16).

All mosquitoes exposed to α-cypermethrin were held for 72 h to assess whether the insecticide had any delayed effects on mortality. During this holding period, we observed a significant reduction across all trial groups in the survival of *A gambiae* sensu lato exposed to intermediate-to-high concentrations of α-cypermethrin (appendix p 17). Evidence for a delayed mortality effect was absent at the diagnostic dose, as all mosquitoes survived the holding period, and at the highest concentrations tested, as all mosquitoes died on exposure to the insecticide (appendix pp 14, 15). Unlike the knockdown outcome, when measuring 72-h mortality we saw no consistent time-dependent change in resistance (appendix pp 17, 18).

Regarding resistance to new active ingredients, *A gambiae* sensu lato populations were highly susceptible to the diagnostic dose of chlorfenapyr in all study groups throughout the trial (figure 3, appendix p 12). By comparison, populations in all trial groups displayed smaller and more variable—albeit significant—reductions in fertility after exposure to the putative diagnostic dose of pyriproxyfen, with an overall trend of increasing susceptibility over successive trial years (figure 4, appendix p 12). High-to-complete sterility was observed on parallel exposure of the susceptible colony control (*A gambiae* sensu stricto Kisumu) in each trial year.

Expression levels of eight metabolic genes were monitored in PCR-confirmed *A coluzzii* in each trial group over 3 years (figure 5). The overexpression of *CYP6M2* was consistently lower in *A coluzzii* than in the susceptible colony *A coluzzii* N'Gousso (fold change <0·8). Across all trial groups, the greatest change in expression was observed for *CYP6P1*, which increased significantly by the third year post-intervention to a fold change of 3·68 (95% CI 2·14–5·39) in the pyrethroid-only LLIN group, 4·07 (2·89–10·03) in the chlorfenapyr–pyrethroid LLIN group, and 7·80 (6·05–26.86) in the pyriproxyfen–pyrethroid LLIN group. The expression of *GSTe2* also consistently increased across all trial groups by the third year, but to a lesser extent than *CYP6P1* (figure 5).

In the pyrethroid-only LLIN group (clusters 21 and 31), differential dynamic insecticide resistance selection was apparent for several metabolic genes. In cluster 31, a significant increase in the expression of *CYP6P4* and *CYP4G16* was observed between baseline and year 2, and in cluster 21, the expression of other genes (*CYP6Z1* and *CYP6P3*) decreased between baseline and year 1

except for the comparison between clusters 43 and 55 (chlorfenapyr–pyrethroid LLINs; p=0·833). For details of corrected pairwise comparisons, see appendix p 13.

Bayesian dose–response models showed results consistent with those from other analyses and indicated
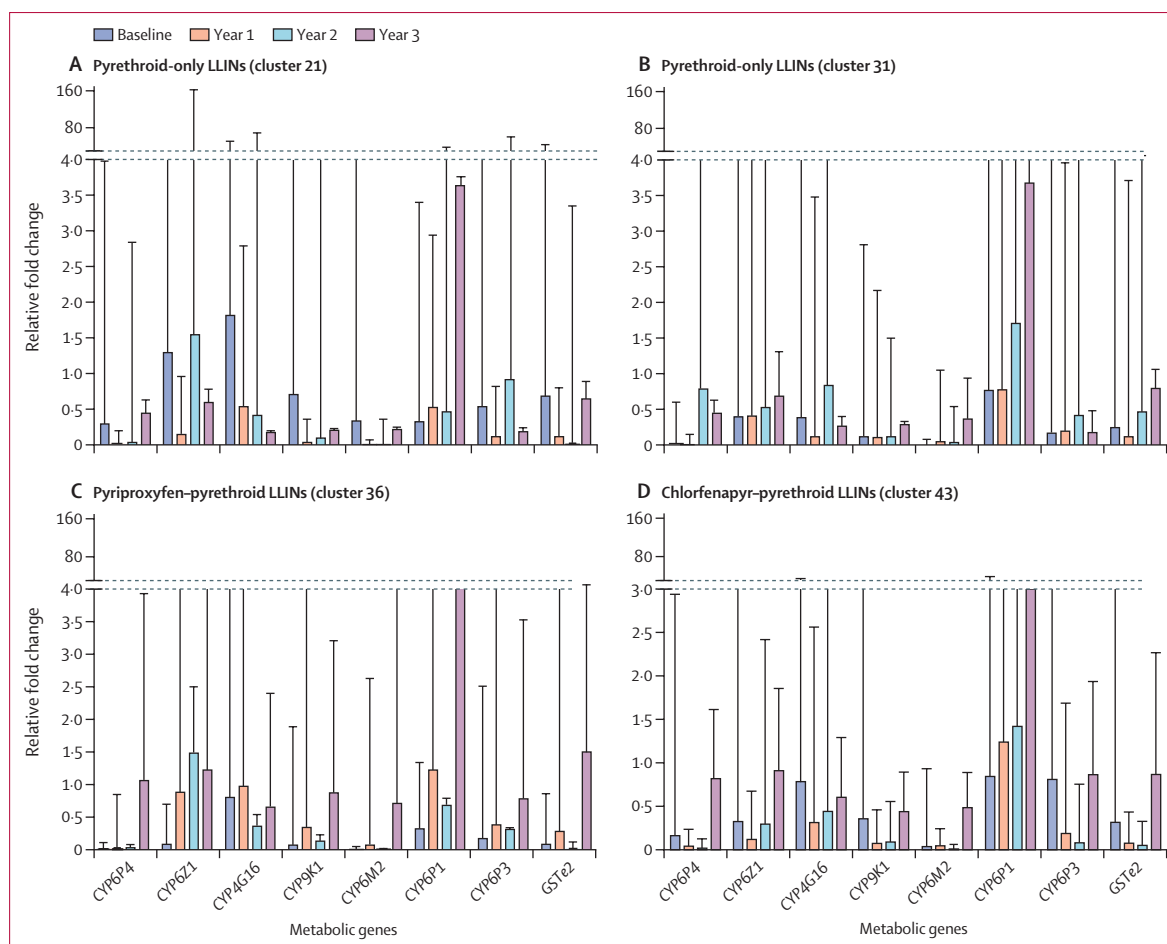
Figure 5: Changes in metabolic gene expression in *Anopheles coluzzii* relative to a susceptible colony population at baseline and for 3 years post-intervention
Changes in metabolic gene expression in PCR-confirmed *Anopheles coluzzii* relative to *A coluzzii* N'Gousso in the pyrethroid-only LLIN (A, B), pyriproxyfen–pyrethroid LLIN (C), and chlorfenapyr–pyrethroid LLIN (D) trial groups, measured by quantitative, real-time, reverse transcriptase PCR. Error bars are 95% CIs. For p values, see appendix p 26. LLINs=long-lasting insecticidal nets.

post-intervention, rebounded in year 2, only to decline again in year 3. In the pyriproxyfen–pyrethroid LLIN group (cluster 36), a significant increase in the expression of *CYP6P4, CYP9K1, CYP4G16*, and *CYP6Z1* was evident across all three trial years. Similarly, in the chlorfenapyr–pyrethroid LLIN group (cluster 43), the expression of *CYP6P4, CYP6Z1*, and *CYP9K1* significantly increased; this was the only trial group in which the expression of *CYP6P3* also significantly increased by the third year (figure 5).

The proportions of *A coluzzii* (range 71·9–87·6%) and *A gambiae* sensu stricto (81·0–95·8%) harbouring the Leu1014Phe *kdr* mutation were high, with higher mutation frequencies in *A gambiae* sensu stricto (appendix pp 19–24).

Discussion

The widespread distribution of two dual-active-ingredient LLINs and pyrethroid-only LLINs had significant effects on insecticide resistance intensity in the primary malaria

vector *A gambiae* sensu lato over 3 years in southern Benin. A clear effect was seen in all three trial groups, with significant declines in α-cypermethrin resistance intensity between baseline and year 1 post-intervention. Previously, the extent of operational failure of major vector-control tools that is directly attributable to insecticide resistance has been challenging to estimate, with concerns for the sustained community-level effectiveness of pyrethroid-only LLINs in areas of moderate-to-high resistance intensity.[39] Pyrethroid-only LLIN campaigns have persisted with the assumption that these interventions still provide personal protection as a physical barrier, by adversely affecting blood-feeding behaviours, by inducing delayed mortality of vectors after exposure, or by a combination of these effects.[39,40] In this cluster RCT, high coverage of pyrethroid-only LLINs was still able to positively affect pyrethroid resistance intensity during the first intervention year, which might be partially explained by the insecticidal activity of these LLINs and by a degree of delayed mortality, as evidenced by our bioassay data.

However, reversals in α-cypermethrin resistance selection were apparent after 12 months, cautioning against deployment of pyrethroid-only LLINs longer-term. Other studies also support the discontinuation of these tools, showing an increase in cross-resistance between type I and type II pyrethroids[41,42] and selection for alternative resistance mechanisms—including cuticular thickening[43] or salivary gland detoxification[44,45]—which impart other fitness benefits to resistant vector populations and have lasting consequences for the design and evaluation of candidate insecticides for malaria vector control.[45]

The significant reduction in α-cypermethrin resistance intensity observed in the chlorfenapyr–pyrethroid LLIN group between baseline and year 1 post-intervention strongly aligns with the epidemiological outcomes of the cluster RCT; these dual-active-ingredient LLINs provided superior protection from malaria infection compared with pyrethroid-only LLINs,[18] supported by minimal reductions in chlorfenapyr susceptibility throughout the trial. This effect on resistance selection was also evident in one of two chlorfenapyr–pyrethroid LLIN surveillance clusters during the second trial year, during which time these interventions continued to sustain significant reductions in disease burden.[18] The parallel decline in α-cypermethrin resistance intensity in the pyriproxyfen–pyrethroid trial group probably reflects some contribution from the pyrethroid component, as observed in the pyrethroid-only LLIN group, and an incomplete but variable effect of pyriproxyfen on vector sterility. In both pyriproxyfen–pyrethroid LLIN and pyrethroid-only LLIN trial groups, this initial gain in pyrethroid susceptibility was insufficient to provide significant protective efficacy from malaria in trial year 1 and was lost almost entirely by 24 months.

Molecular monitoring of key detoxification enzymes, which have been previously implicated in pyrethroid resistance in *A gambiae* sensu lato across west Africa,[30–32] revealed several important insights. Dynamic selection of several genes was evident in both the pyrethroid-only LLIN (*CYP6P4* and *CYP4G16*) and chlorfenapyr–pyrethroid and pyriproxyfen–pyrethroid LLIN (*CYP6P4*, *CYP6Z1*, and *CYP9K1*) groups. In-vitro studies have shown the metabolism of pyriproxyfen by the enzymes CYP6M2 and CYP6P4 in *A gambiae* sensu lato;[46,47] in our study, *CYP6P4* was significantly overexpressed in the pyriproxyfen–pyrethroid trial group, suggesting a potential role in resistance. By comparison, expression profiles of genes encoding metabolic enzymes displayed a significant downward trend over time in the chlorfenapyr–pyrethroid LLIN group, supported by reports of chlorfenapyr activation in *A gambiae* sensu lato by CYP6P3 and CYP9K1;[48] expression of the genes encoding both of these enzymes declined significantly over 2 years post-intervention only to increase in the third year. Overall, the overexpression of genes encoding metabolic enzymes in *A coluzzii* populations was strikingly low (average fold change <3) compared with other studies from central Africa during the rainy season, in which the same genes were reported to be upregulated 20-fold;[49] this finding supports the presence of additional, as yet undescribed cross-resistance mechanisms in our study site, which warrant investigation.

Our study findings contrast with parallel insecticide-resistance monitoring activities from the cluster RCT in Tanzania,[50] in which, over 3 years, no significant increase in α-cypermethrin resistance intensity was evident in the chlorfenapyr–pyrethroid LLIN group whereas significant resistance escalation was observed in clusters that received pyriproxyfen–pyrethroid LLINs and pyrethroid-only LLINs.[50] Possible explanations for these discordant results include differing predominant vector species complexes, characterised by distinct insecticide-resistance mechanisms and life histories (*Anopheles funestus* sensu lato in Tanzania and *A gambiae* sensu lato in Benin), and higher initial insecticide resistance intensity in Benin. These observations strongly support the need for large-scale evaluation of the effect of dual-active-ingredient LLINs on insecticide resistance selection in multiple geographies, ecologies, and malaria-transmission contexts to better understand the effect of these interventions on the insecticide-resistance crisis.

The results of this cluster RCT and an equivalent trial in Tanzania enabled WHO to issue a policy recommendation for the use of chlorfenapyr–pyrethroid LLINs to prevent malaria in areas of pyrethroid resistance.[51] Although chlorfenapyr–pyrethroid LLINs are highly promising for the control of malaria transmitted by pyrethroid-resistant vector populations, several important questions remain regarding pragmatic strategies for their prospective scale-up and deployment. Our study findings indicate that, after 24 months of use, chlorfenapyr–pyrethroid LLINs might no longer mitigate pyrethroid resistance selection in some high-disease transmission areas dominated by highly resistant *A gambiae* sensu lato; this finding raises issues for current net-procurement schedules, which are based on an operational lifespan of 3 years. Furthermore, reliance on only chlorfenapyr–pyrethroid LLINs to maintain current or greater levels of malaria control is not advisable, given the propensity of *Anopheles* to rapidly evolve resistance to new active ingredients and the reports of incipient reduced chlorfenapyr susceptibility from west and central sub-Saharan Africa.[52]

The results of our study should be interpreted considering the following limitations. *A gambiae* sensu lato populations were collected using human landing catches at the trial baseline and subsequently from larval habitats post-intervention, owing to initial challenges in identifying reliable, productive breeding sites. Convenient, non-random sampling for insecticide-resistance monitoring was used to obtain sufficient biological material for testing; although standard practice, this means that study findings might not be representative of all vector populations in the study site. Immature vectors from

multiple, productive, and varied but nearby breeding sites were pooled for bioassay testing without taking intracluster clustering into account. Post-intervention vector sampling was limited to two clusters per RCT group; two of these clusters were adjacent to one another, meaning that each observational unit might not have been entirely independent, and the estimation of inter-cluster variance was not feasible. Each cluster was separated by a buffer zone of 1000 m; however, if particular insecticide-resistance mechanisms confer a selective advantage, genetic contamination might conceivably occur over a larger geographical range. The baseline of the cluster RCT was short in duration (3 months) and the time of year differed from that of post-intervention monitoring, suggesting that vector seasonality might also contribute to resistance phenotype. Although we attribute trends in insecticide-resistance selection to different LLINs, we have not shown causation, and other, unmeasured changes could occur in vector populations over the study period—including unknown temporal or climactic factors that cannot be eliminated as confounders. Finally, adult mosquitoes tested at baseline were of unknown physiological age and, because phenotypic resistance declines over time,[53] pyrethroid resistance could have been underestimated; however, despite this caveat, the concentration of α-cypermethrin required to kill 95% of *A gambiae* sensu lato at baseline was more than 28 times the diagnostic dose.

In conclusion, our study findings raise concerns that chlorfenapyr–pyrethroid LLINs might no longer affect pyrethroid resistance selection after 2 years of community use in high malaria-transmission settings where intensely pyrethroid-resistant *A gambiae* sensu lato is the dominant vector complex, arguing for further investigation into current net-deployment regimens. This study highlights the importance of large-scale, longitudinal insecticide-resistance monitoring to inform the pragmatic design of prospective resistance-management strategies.

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