# Effects of next-generation, dual-active-ingredient, long-lasting insecticidal net deployment on insecticide resistance in malaria vectors in Tanzania: an analysis of a 3-year, cluster-randomised controlled trial

*Louisa A Messenger, Nancy S Matowo, Chad L Cross, Mohamed Jumanne, Natalie M Portwood, Jackline Martin, Eliud Lukole, Elizabeth Mallya, Jacklin F Mosha, Robert Kaaya, Oliva Moshi, Bethanie Pelloquin, Katherine Fullerton, Alphaxard Manjurano, Franklin W Mosha, Thomas Walker, Mark Rowland, Manisha A Kulkarni, Natacha Protopopoff*

## Summary

**Background** Insecticide resistance among malaria-vector species is a pervasive problem that might jeopardise global disease-control efforts. Novel vector-control tools with different modes of action, including long-lasting insecticidal nets (LLINs) incorporating new active ingredients, are urgently needed to delay the evolution and spread of insecticide resistance. We aimed to measure phenotypic and genotypic insecticide-resistance profiles among wild *Anopheles* collected over 3 years to assess the longitudinal effects of dual-active-ingredient LLINs on insecticide resistance.

**Methods** For this analysis, data nested in a 3-year, four parallel-arm, superiority cluster-randomised controlled trial (cRCT) in Tanzania, collected from 84 clusters (39 307 households) formed of 72 villages in the Misungwi district, were used to measure insecticide-resistance profiles among female *Anopheles* mosquitoes via insecticide-resistance bioassays and quantitative RT-PCR of metabolic-resistance genes. Wild, blood-fed, indoor-resting mosquitoes were collected annually during the rainy seasons from house walls in clusters from all four trial groups. Mosquitoes were morphologically identified as *An gambiae* sensu lato (SL) or *An funestus* SL before separate bioassay testing. The primary outcomes were lethal-dose values for α-cypermethrin, permethrin, and piperonyl butoxide pre-exposure plus permethrin-resistance intensity bioassays, mortality 72 h after insecticidal exposure for chlorfenapyr bioassays, fertility reduction 72 h after insecticidal exposure for pyriproxyfen bioassays, and fold change in metabolic-enzyme expression relative to an insecticide-susceptible laboratory strain. All primary outcomes were measured in *An funestus* SL 1 year, 2 years, and 3 years after LLIN distribution. Primary outcomes were also assessed in *An gambiae* SL if enough mosquitoes were collected. The cRCT is registered with ClinicalTrials.gov (NCT03554616).

**Findings** Between May 24, 2019, and Oct 25, 2021, 47 224 female *Anopheles* were collected for resistance monitoring. In the pyrethroid (PY)-LLIN group, there were significant increases in α-cypermethrin-resistance intensity (year 1 LD50=9·52 *vs* year 2 76·20, p<0·0001) and permethrin-resistance intensity (year 1 13·27 *vs* year 2 35·83, p=0·0019) in *An funestus* SL. In the pyriproxyfen PY-LLIN group, there was similar increase in α-cypermethrin-resistance intensity (year 1 0·71 *vs* year 2 81·56, p<0·0001) and permethrin-resistance intensity (year 1 5·68 *vs* year 2 50·14, p<0·0001). In the piperonyl butoxide PY-LLIN group, α-cypermethrin-resistance intensity (year 1 33·26 *vs* year 3 70·22, p=0·0071) and permethrin-resistance intensity (year 1 47·09 *vs* year 3 2635·29, p<0·0001) also increased over time. In the chlorfenapyr PY-LLIN group, there were no effects on α-cypermethrin-resistance intensity (year 1 0·42 *vs* year 3 0·99, p=0·54) or permethrin-resistance intensity (data were not estimable due to nearly 100% mortality). There were also minimal reductions in chlorfenapyr susceptibility. However, in the chlorfenapyr PY-LLIN group, a significant decline in piperonyl-butoxide synergy was seen by year 3 (year 1 0·02 *vs* year 3 0·26, p=0·020). Highly over-expressed detoxification enzymes showed dynamic patterns of selection throughout the trial.

**Interpretation** Our phenotypic data supports trial epidemiological findings; chlorfenapyr PY-LLINs provided superior protection from malaria across multiple transmission seasons, with few effects on insecticide-resistance selection. Rapid pyrethroid-resistance intensification in the piperonyl butoxide PY-LLIN group and pre-existing tolerance of pyriproxyfen in vector populations might explain the poorer performance of these two interventions regarding malaria outcomes. Further work is required to elucidate the potential mechanisms driving cross-resistance between pyrethroids and novel active ingredients to better inform the design of pre-emptive resistance-management strategies.

**Funding** UK Department for International Development; UK Medical Research Council; Wellcome Trust; UK Department of Health and Social Care; UK Foreign, Commonwealth and Development Office; and The Bill and Melinda Gates Foundation via the Innovative Vector Control Consortium.



*Lancet Planet Health* 2023; 7: e673–83

**Department of Disease Control, London School of Hygiene & Tropical Medicine, London, UK** (L A Messenger PhD, N S Matowo PhD, N M Portwood MSc, J Martin MSc, E Lukole MSc, B Pelloquin MSc, K Fullerton MSc, T Walker PhD, Prof M Rowland PhD, N Protopopoff PhD); **Department of Environmental and Occupational Health** (L A Messenger), **Parasitology and Vector Biology Laboratory** (L A Messenger, C L Cross PhD), **Department of Epidemiology and Biostatistics** (C L Cross), **School of Public Health, University of Nevada, Las Vegas, NV, USA; National Institute for Medical Research, Mwanza Medical Research Centre, Mwanza, Tanzania** (M Jumanne BSc, J Martin, E Lukole, E Mallya BSc, J F Mosha PhD, A Manjurano PhD); **Parasitology Unit, Universitätsklinikum Heidelberg, Heidelberg, Germany** (N M Portwood); **Kilimanjaro Christian Medical University College, Moshi, Tanzania** (J Martin, R Kaaya MSc, O Moshi BSc, Prof F W Mosha PhD); **School of Tropical Medicine and Global Health, Nagasaki University, Nagasaki, Japan** (B Pelloquin); **School of Life Sciences, University of Warwick, Coventry, UK** (T Walker); **School of Epidemiology and Public Health, University of Ottawa, Ottawa, ON, Canada** (M A Kulkarni PhD)

Correspondence to:
Dr Louisa A Messenger, Department of Environmental and Occupational Health, School of Public Health, University of Nevada, Las Vegas, NV 89119, USA
**louisa.messenger@unlv.edu**

## Research in context

### Evidence before this study

Insecticide resistance among malaria-vector species is now a universal occurrence, affecting malaria-endemic areas worldwide. Novel vector control interventions, including long-lasting insecticidal nets (LLINs) incorporating new active ingredients with distinct methods of action, are urgently needed to delay the evolution and spread of insecticide resistance. Piperonyl butoxide pyrethroid (PY)-LLINs received an interim WHO recommendation as a new malaria-vector control tool after showing superior protection from malaria infection and transmission compared with standard PY-LLINs in two cluster-randomised controlled trials (cRCTs) 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) have also been assessed in two cRCTs, with the chlorfenapyr PY-LLIN reducing malaria infection prevalence by 55% after 2 years in Tanzania and by 46% after 2 years in Benin. The current analysis uses data nested within the cRCT in Tanzania, which assessed the effectiveness and cost-effectiveness of three types of dual-active-ingredient LLINs against malaria burden compared with PY-LLINs. We searched for papers published in English in PubMed from database inception to Jan 13, 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*" and found no studies that reported longitudinal changes in insecticide resistance after deployment of dual-active-ingredient LLINs disaggregated by net type.

### Added value of this study

This is the first analysis to report data on the influence of next-generation, dual-active-ingredient LLINs on the dynamic evolution of phenotypic and genotypic insecticide resistance in *An funestus* sensu lato during multiple years. Chlorfenapyr PY-LLINs showed no significant selection for phenotypic pyrethroid resistance and vector populations remained susceptible to the partner active ingredient throughout the analysis; however, piperonyl butoxide PY-LLINs, pyriproxyfen PY-LLINs, and PY-LLINs all intensified pyrethroid-insecticide resistance, which, in turn, adversely affected piperonyl-butoxide synergy.

### Implications of all the available evidence

Multiple cRCTs have previously reported the improved effectiveness of dual-active-ingredient LLINs compared with standard PY-LLINs on malaria outcomes and key entomological indicators. However, little attention has been given to the long-term implications of this next generation of LLINs on the evolution of insecticide resistance among malaria-vector populations. Escalation of pyrethroid resistance by PY-LLINs is not surprising, but continued widespread distribution of these nets has potential to drive the selection of cross-resistance mechanisms. Ongoing deployment of PY-LLINs might also exacerbate the evolution of novel insecticide-resistance mechanisms, which will further complicate the design of new insecticidal interventions for malaria-vector control. Chlorfenapyr PY-LLINs elicited no observable intensification of pyrethroid-resistance; however, reliance on a single intervention for reactive resistance management is entirely inadvisable due to the propensity of *Anopheles* vectors to rapidly evolve resistance to new active ingredients and reports of incipient-reduced chlorfenapyr susceptibility from west and central sub-Saharan Africa. At the molecular level, this analysis revealed the focal nature of specific insecticide-resistance mechanisms among vectors with distinct life histories and argues for further in-depth genetic characterisation of diverse mosquito populations across different ecologies, transmission intensities, and malaria-endemic areas to better inform pre-emptive resistance monitoring and intervention development.

## Introduction

Malaria remains a major global health problem, with nearly half of the world's population living in transmission areas across 85 countries and territories.[1] Previously, substantial progress in disease control was accomplished by scaling-up the provision of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and other key diagnostic and treatment measures.[2] In the past two decades, WHO estimated that 1·5 billion malaria cases and 7·6 million malaria deaths were averted, with LLINs accounting for 68% of these achievements and IRS accounting for 10% of these achievements. However, rates of malaria decline have stalled, coinciding with the emergence of several biological and non-biological challenges, of which the rapid spread of insecticide resistance across *Anopheles* mosquito populations now seriously threatens vector-control efforts worldwide.[3–5]

In response to pervasive insecticide resistance, substantial investments have been made in the development of new insecticides and chemical classes with distinct methods of action, which can be used to improve mosquito control and potentially mitigate further selection for insecticide resistance. The first new class of dual-active-ingredient LLINs were treated with a mixture of a pyrethroid (PY) and a synergist, piperonyl butoxide, which enhances insecticide toxicity by inhibiting the activity of metabolic enzymes that are commonly overexpressed in resistant vector populations. Piperonyl butoxide PY-LLINs received a WHO recommendation after successful evaluation in two cluster-randomised controlled trials (cRCTs) after 2 years of community use.[6] In these cRCTs, piperonyl butoxide PY-LLINs reduced malaria infection prevalence by 44% in Tanzania[7] and by 27% in Uganda[8] compared with standard PY-LLINs in

areas of intense pyrethroid resistance. A second generation of LLINs, combining a pyrethroid and either a pyrrole (chlorfenapyr) or insect growth regulator (pyriproxyfen), have also been assessed in two cRCTs, with chlorfenapyr reducing malaria infection prevalence by 55% after 2 years in Tanzania[9] and 46% after 2 years in Benin.[10]

Effective insecticide-resistance management strategies are predicated on a clear understanding of the specificity of resistance mechanisms to individual insecticides and the likelihood of selecting for cross-resistance in genetically diverse vector species with distinct life histories.[11] As dual-active-ingredient LLINs are scheduled for universal distribution across multiple malaria-endemic regions, understanding their effects on the dynamic evolution of insecticide resistance is crucial, particularly throughout the operational lifetime of these interventions.

Using data nested within the cRCT in Tanzania,[9] which evaluated the effectiveness of three dual-active-ingredient LLINs compared with PY-LLINs, we aimed to measure phenotypic and genotypic insecticide-resistance profiles among wild *Anopheles* collected over 3 years to assess the longitudinal effects of dual-active-ingredient LLINs on insecticide resistance.

## Methods

### Study design and participants

Data for this analysis was nested in a 3-year, four parallel-arm, superiority cRCT conducted in 84 clusters (39 307 households) formed of 72 villages in the Misungwi district, Mwanza region, northwest Tanzania.[9] The major malaria-vector species were *Anopheles arabiensis*, *Anopheles gambiae* sensu stricto (SS), and *Anopheles funestus* sensu lato (SL), with *An funestus* SS being responsible for more than 90% of ongoing malaria transmission during trial baseline.[12] Most LLINs in the Misungwi district before the cRCT were Olyset (Sumitomo Chemical, Tokyo, Japan) permethrin-only LLINs, which were distributed during the previous universal-coverage campaign in 2015 and through antenatal care clinics. PermaNet 2.0 (Vestergaard, Lausanne, Switzerland) deltamethrin-only LLINs accounted for 33% of LLINs, which were distributed via the annual school net campaign.

The cRCT study groups were Royal Guard (Disease Control Technologies, Greer, SC, USA), combining the insect growth inhibitor pyriproxyfen (5·5 g/kg) and the pyrethroid α-cypermethrin (5·5 g/kg), henceforth referred to as pyriproxyfen PY-LLINs; Interceptor G2 (Badische Anilin und Sodafabrik, Ludwigshafan, Germany), combining the pyrrole chlorfenapyr (4·8 g/kg) and α-cypermethrin (2·2 g/kg), henceforth referred to as chlorfenapyr PY-LLINs; Olyset Plus (Sumitomo Chemical, Tokyo, Japan), combining the synergist piperonyl butoxide (10·0 g/kg) and the pyrethroid permethrin (20·0 g/kg), henceforth referred to as piperonyl butoxide PY-LLINs; and the reference group Interceptor (Badische Anilin und Sodafabrik, Ludwigshafan, Germany), containing α-cypermethrin (5·0 g/kg), henceforth referred to as PY-LLINs. Between Jan 26 and Jan 28, 2019, 147 230 LLINs were distributed among the four study groups and monitored for 3 years. Community members and the field team were masked to group allocation. The trial design has been reported previously.[13]

The cRCT, registered with ClinicalTrials.gov (NCT03554616), obtained ethical approval from the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol. IX/2743), Kilimanjaro Christian Medical University College (2267), the University of Ottawa (H-05–19–4411), and the London School of Tropical Medicine and Hygiene (14952). The use of guinea pigs to feed colony mosquitoes received approval from the Animal Welfare and Ethical Review Board of the London School of Tropical Medicine and Hygiene (2019–14). All study procedures were conducted in accordance with relevant guidelines and regulations. Before study initiation, community consent was obtained from village leaders and written informed consent was obtained from the heads of all households that were selected for participation. Study information, including the study purpose, risks, and benefits, was provided to participants in Swahili.

### Procedures

Between May 24, 2019, and Oct 25, 2021, wild, blood-fed, indoor-resting mosquitoes were collected annually during the rainy seasons from house walls in clusters from all four trial groups with Prokopack (John W Hock Company, Gainesville, FL, USA) and manual aspirators and were held for 3 days to allow for blood-meal digestion. Mosquitoes were sampled every year from Isesa and Lubili villages (both of which were using piperonyl butoxide PY-LLINs), Mbarika village (using chlorfenapyr PY-LLINs), Ihelele, Ilujamate hamlet (using PY-LLINs), and Nyangh'omango, Ilujamate hamlet (using pyriproxyfen PY-LLINs; appendix p 6). From July 23 to Sept 17, 2020, mosquito collections were also done in Igokelo village (using pyriproxyfen PY-LLINs). All mosquitoes were provided with 10% glucose solution and maintained at 26°C (±3) and 60–80% relative humidity with 12 h cycles of light and dark. Mosquitoes were morphologically identified as *An gambiae* SL or *An funestus* SL[14] before separate bioassay testing.

See Online for appendix

US Centers for Disease Control and Prevention (CDC) insecticide-resistance intensity bioassays for α-cypermethrin and permethrin were conducted according to published guidelines.[15] Chlorfenapyr bioassay testing was done with a diagnostic dose of 100 µg per bottle.[16] Piperonyl butoxide pre-exposures used 4% impregnated piperonyl-butoxide papers in WHO tube assays.[16] Per bioassay, approximately, 20–25 wild-caught, adult, female *An gambiae* SL or *An funestus* SL were exposed to pyrethroid insecticides for 30 min or to chlorfenapyr for 60 min. Surviving mosquitoes were held and scored for delayed mortality after 24 h, 48 h, and 72 h.

For pyriproxyfen testing, 20–25 freshly collected, blood-fed, adult, female *An gambiae* SL or *An funestus* SL were exposed to 100 µg per bottle or 1000 µg per bottle pyriproxyfen for 60 min. Furthermore, the laboratory, pyrethroid-susceptible *An gambiae* SS Kisumu strain was assayed at the same time as the field mosquitoes were being bioassayed; mosquitoes aged 2–5 days were blood-fed on guinea pigs before testing. After insecticide exposure, surviving mosquitoes were held for 72 h, after which gravid mosquitoes underwent ovarian dissection with a light microscope. Each mosquito was scored for fertility status and Christopher's egg stage of development.[17] At the end of each exposure and dissection period, all mosquitoes were stored at –80°C in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA).

Because of the predominance of *An funestus* SL in the study area,[12] this vector species was the focus of subsequent molecular characterisation. Unexposed individual mosquitoes were homogenised with Qiagen Tissue Lyser II (Qiagen, Hilden, Germany) with 5 mm stainless-steel beads and RNA was extracted via Qiagen RNeasy 96 kit according to the manufacturer's instructions. Approximately 2 µg of each RNA sample were treated with RQ1 rNase-free DNase (Promega, Southampton, UK). 1 µg of DNase-treated RNA per individual mosquito was used to synthesise cDNA with a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Molecular identification of *An funestus* SL was done according to described protocols.[18] Quantitative RT-PCR (qRT-PCR) was used to measure expression of nine metabolic genes that had been previously implicated in pyrethroid resistance in *An funestus* SL populations across sub-Saharan Africa (appendix p 5).[19,20] Standard curves of cycle threshold values for each gene were generated with a five-fold serial dilution of cDNA to assess PCR efficiency. Reactions were conducted in technical duplicate with a Stratagene Mx3005P Real-Time PCR system (Agilent, Santa Clara, CA, USA) with FastStart Essential DNA Green Master mix (Roche, Basel, Switzerland). Reaction conditions were denaturation for 10 min at 95°C, then 40 cycles of 10 s at 95°C, 22 s at 55°C (*CYP4H17*, *CYP6M7*, *CYP6Z1*, *CYP9K1*, *GSTE2*, *CYP6P9a*, and *CYP6P9b*), or 60°C (*CYP6M1* and *CYP6N1*), then 10 s at 72°C. The fold change of each target gene in field populations, relative to the susceptible laboratory strain (*An funestus* SS FANG colony strain), was calculated with the $2^{-\Delta\Delta CT}$ method, incorporating PCR efficiency; ribosomal protein S7 and actin were used for normalisation.

### Outcomes

The primary outcomes were lethal-dose values (ie, lethal dose [LD]25, LD50, LD95, and LD99) calculated at 30 min after insecticidal exposure to α-cypermethrin, permethrin, and piperonyl butoxide pre-exposure plus permethrin-resistance intensity bioassays; mortality 72 h after insecticidal exposure to chlorfenapyr; fertility reduction 72 h after insecticidal exposure to pyriproxyfen, assessed by ovarian dissection; and fold change in metabolic-enzyme expression relative to an insecticide-susceptible laboratory strain. All primary outcomes were measured in *An funestus* SL, the dominant vector-species complex, 1 year, 2 years, and 3 years after LLIN distribution and were compared between intervention and reference groups. Primary outcomes were also assessed in *An gambiae* SL if enough mosquitoes were collected. The secondary outcome was the effect of pyrethroid-insecticide exposure on delayed mortality of *An funestus* SL. We also conducted secondary analyses to assess differences in pyrethroid phenotypic-resistance intensity in multiple clusters from the same trial group.

### Statistical analysis

Mortality of 5–20% in control assays was corrected with Abbott's formula. LD25, LD50, LD95, and LD99 were estimated with a probit model with $\log_{10}$-transformed data. Curve estimation was based on the probability of mosquito death as a function of the total number of mosquitoes and insecticide dose. Point estimates of LDs and 95% CI 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 (appendix pp 9–12). The model incorporated an automated-heterogeneity factor in the CI calculations to obtain unbiased interval estimates. If the sample size or death probabilities were insufficient to derive stable estimates, LD point estimates were replaced with a value less than the lowest value tested to indicate that the estimate was less than the lowest tested concentration. Comparisons of LD50 values among clusters or years were statistically estimated with relative median potency (RMP), which was calculated as the ratio of point estimates with simultaneous 95% CIs. Comparisons of potency in this analysis are median lethal concentrations or doses. A ratio of 1 was not significant; it would mean that the LD50 was equal among comparison groups. The RMP test is a traditional analysis in probit models and has been shown to align with other methods for comparing LD50 values.[21] To complement the probit models, we also developed generalised linear mixed models (GLMMs) with a logistic link function to model potential differences in survival probability among collections occurring in multiple treatment groups, study years, test replicates, and testing dates. For delayed-mortality analysis, Cox regression was used to compare hazard rate ratios (HRRs), adjusted for test replicate to account for changes in vector-population structure across collection periods, between mosquitoes exposed to or not exposed to pyrethroid or piperonyl butoxide then pyrethroid (or piperonyl butoxide then acetone), and between different insecticide doses. A ratio of 1 was not significant; it would mean that the HRR was equivalent among comparison groups. Therefore, 95% CIs not containing the value 1 were interpreted as being statistically

| | PY-LLIN (Interceptor) | | | | Chlorfenapyr PY-LLIN (Interceptor G2) | | | |
|---|---|---|---|---|---|---|---|---|
| | Year 1 | Year 2 | Year 3 | Relative median potency by year* | Year 1 | Year 2 | Year 3 | Relative median potency by year* |
| α-cypermethrin | 9·52 (3·68–16·96); 670 mosquitoes tested | 76·20 (45·05–155·03); 695 mosquitoes tested | 59·16 (32·52–119·83); 552 mosquitoes tested | Year 1 *vs* year 2† 0·12 (0·03–0·31, p<0·0001); year 1 *vs* year 3† 0·16 (0·04–0·40, p=0·0019); year 2 *vs* year 3 1·29 (0·57–3·15, p=0·57) | 0·42 (0·00–3·13); 404 mosquitoes tested | 1·55 (0·00–7·34); 870 mosquitoes tested | 0·99 (0·00–5·68); 743 mosquitoes tested | Year 1 *vs* year 2 0·27 (0·00–1·43, p=0·31); year 1 *vs* year 3 0·42 (0·00–2·34, p=0·54); year 2 *vs* year 3 1·56 (0·27–36·44, p=0·74) |
| Permethrin | 13·27 (7·70–19·49); 981 mosquitoes tested | 35·83 (23·34–51·16); 734 mosquitoes tested | 168·79 (114·33–272·65); 644 mosquitoes tested | Year 1 *vs* year 2† 0·37 (0·18–0·63, p=0·0019); year 1 *vs* year 3† 0·08 (0·02–0·18, p<0·0001); year 2 *vs* year 3† 0·21 (0·09–0·40, p<0·0001) | <21·5‡ | <21·5‡ | <21·5‡ | NE‡ |
| Piperonyl butoxide and permethrin | 0·28 (0·01–3·13); 194 mosquitoes tested | 5·54 (1·86–10·51); 760 mosquitoes tested | 43·06 (29·66–58·57); 957 mosquitoes tested | Year 1 *vs* year 2† 0·05 (0·00–0·56, p=0·036); year 1 *vs* year 3† 0·01 (0·00–0·08, p<0·0001); year 2 *vs* year 3† 0·13 (0·04–0·27, p<0·0001) | 0·02 (0·00–0·82); 362 mosquitoes tested | 0·10 (0·00–2·53); 594 mosquitoes tested | 0·26 (0·00–3·95); 883 mosquitoes tested | Year 1 *vs* year 2 0·17 (0·00–2·28, p=0·20); year 1 *vs* year 3† 0·07 (0·00–0·91, p=0·020); year 2 *vs* year 3 0·40 (0·00–2·69, p=0·53) |

Data are LD50 (95% CI) or RMP (95% CI, p value). RMP 95% CIs provide estimates to compare LD50 among years. LD50=lethal dose 50. NE=not estimable. PY-LLIN=pyrethroid long-lasting insecticidal nets. RMP=relative median potency. *RMP ratio 95% CIs not containing 1 are statistically different (p<0·05). †Significant pairwise difference. ‡NE due to nearly 100% mortality; LD50 values have been replaced with the lowest dose tested.

***Table 1:*** **Summary statistics of LD50 values for *Anopheles funestus* sensu lato collected per trial group during 3 years**

different. Fertility reduction in pyriproxyfen-exposed populations was calculated as 100×(the proportion of fertile control female mosquitoes−the proportion of fertile pyriproxyfen-exposed female mosquitoes)/the proportion of fertile control female mosquitoes. Metabolic-enzyme fold change was compared between trial years with the Kruskal-Wallis test in Prism version 9.5.0 (GraphPad, Boston, MA, USA). Probit analyses were conducted in SPSS version 28, GLMMs were conducted in SPSS version 29, and Cox regression models were developed with SAS version 9.4.

### Role of the funding source

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

## Results

Between May 24, 2019, and Oct 25, 2021, 47224 female *Anopheles* were collected in the Misungwi district for resistance monitoring. 8942 (18·9%) were collected in year 1 (7847 [87·8%] *An funestus* SL and 1095 [12·2%] *An gambiae* SL), 17413 (36·9%) were collected in year 2 (13251 [76·1%] *An funestus* SL and 4162 [23·9%] *An gambiae* SL), and 20869 (44·2%) were collected in year 3 (13290 [63·7%] *An funestus* SL and 7579 [36·3%] *An gambiae* SL). A summary of mosquitoes collected and tested per insecticide and study groups is available in the appendix (pp 6–8). In all study years, *An funestus* SL was the dominant vector-species complex, although its proportions declined steadily during the trial from 7847 (87·8%) of 8942 in year 1 to 13290 (63·7%) of 20869 in year 3. CDC indoor light-trap collections after intervention indicated that most *An funestus* SL were *An funestus* SS (81·7%, 95% CI 75·7–86·5) and that most *An gambiae* SL were *An arabiensis* (93·8%, 86·3–97·3).[22] Species identification of a subset of 1440 phenotyped *An funestus* SL, collected manually from house walls, were also concordant (92·8% [91·4–94·1] *An funestus* SS; 4·2% [3·1–5·2] *Anopheles rivulorum*; 3·1% did not amplify).

In the PY-LLIN group, there were significant increases in α-cypermethrin-resistance intensity and permethrin-resistance intensity in *An funestus* SL during the first 2 years (table 1; table 2). A significant increase in permethrin-resistance intensity was also observed in year 3, but there was no increase in α-cypermethrin-resistance intensity. Increasing pyrethroid-resistance led to a significant decrease in piperonyl-butoxide synergy (ie, increasing mosquito mortality) in *An funestus* SL across all 3 years; greater permethrin concentrations were required after exposure to piperonyl butoxide to induce complete lethality over time.

In the chlorfenapyr PY-LLIN group, initial pyrethroid resistance in *An funestus* SL was lower than in the PY-LLIN group (table 1; table 2). No longitudinal increases in α-cypermethrin-resistance intensity or permethrin-resistance intensity were observed during 3 years in the chlorfenapyr PY-LLIN group (table 1; table 2). A significant decline in piperonyl-butoxide synergy was evident by the third year.

In the pyriproxyfen PY-LLIN group, pyrethroid resistance in *An funestus* SL was low, similar to the chlorfenapyr PY-LLIN group in the first year (table 1; table 2). However, over time, *An funestus* SL from the pyriproxyfen PY-LLIN group showed increasing α-cypermethrin-resistance intensity and permethrin-

| | Pyriproxyfen PY-LLIN (Royal Guard) | | | | Piperonyl butoxide PY-LLIN (Olyset Plus) | | | |
|---|---|---|---|---|---|---|---|---|
| | Year 1 | Year 2 | Year 3 | Relative median potency by year* | Year 1 | Year 2 | Year 3 | Relative median potency by year* |
| α-cypermethrin | 0·71 (0·02–3·02); 461 mosquitoes tested | 81·56 (48·91–205·84); 1193 mosquitoes tested | 203·75 (88·58–1143·21); 574 mosquitoes tested | Year 1 *vs* year 2† 0·01 (0·00–0·06, p<0·0001); year 1 *vs* year 3† 0·00 (0·00–0·03, p<0·0001); year 2 *vs* year 3† 0·40 (0·09–0·99, p=0·046) | 33·26 (23·54–47·41); 551 mosquitoes tested | 35·81 (27·18–47·20); 706 mosquitoes tested | 70·22 (50·15–102·64); 673 mosquitoes tested | Year 1 *vs* year 2 0·93 (0·59–1·44, p=0·76); year 1 *vs* year 3† 0·47 (0·26–0·78, p=0·0071); year 2 *vs* year 3† 0·51 (0·30–0·79, p=0·0064) |
| Permethrin | 5·68 (1·85–11·65); 492 mosquitoes tested | 50·14 (34·14–69·57); 1334 mosquitoes tested | 344·81 (198·64–796·18); 683 mosquitoes tested | Year 1 *vs* year 2† 0·11 (0·03–0·28, p< 0·0001); year 1 *vs* year 3† 0·02 (0·00–0·07, p<0·0001); year 2 *vs* year 3† 0·15 (0·04–0·31, p=0·0003) | 47·09 (13·10–174·93); 164 mosquitoes tested | 306·02 (130·17–8246·03); 624 mosquitoes tested | 2635·29 (264·98–3·98e+06); 731 mosquitoes tested | Year 1 *vs* year 2† 0·15 (0·00–0·58, p=0·0066); year 1 *vs* year 3† 0·02 (0·00–0·28, p<0·0001); year 2 *vs* year 3 0·12 (0·00–1·18, p=0·081) |
| Piperonyl butoxide and permethrin | 1·82 (0·09–5·82); 355 mosquitoes tested | 8·76 (1·25–18·61); 655 mosquitoes tested | 7·00 (0·78–16·72); 540 mosquitoes tested | Year 1 *vs* year 2† 0·21 (0·02–0·64, p=0·020); year 1 *vs* year 3† 0·26 (0·02–0·81, p=0·030); year 2 *vs* year 3 1·25 (0·49–3·76, p=0·68) | Insufficient mosquito numbers collected for testing | 7·26 (2·87–12·95); 813 mosquitoes tested | 61·73 (45·08–83·19); 1178 mosquitoes tested | Year 2 *vs* year 3† 0·12 (0·04–0·25, p<0·0001) |

Data are LD50 (95% CI) or RMP (95% CI, p value). RMP 95% CIs provide estimates to compare LD50 among years. LD50=lethal dose 50. NE=not estimable. PY-LLIN=pyrethroid long-lasting insecticidal nets. RMP=relative median potency. *RMP ratio 95% CIs not containing 1 are statistically different (p<0·05). †Significant pairwise difference.

***Table 2:*** **Summary statistics of LD50 values for *Anopheles funestus* sensu lato collected per trial group during 3 years**

resistance intensity and declining piperonyl-butoxide synergy, similar to the PY-LLIN group. The significant increases in pyrethroid-resistance intensity were greater in the pyriproxyfen PY-LLIN group than in the PY-LLIN group.

*An funestus* SL collected in two piperonyl butoxide PY-LLIN clusters were grouped together for analysis (individual cluster-level analysis; appendix p 13), showing the largest significant increases in pyrethroid resistance across trial years and concomitant declines in piperonyl-butoxide synergy. Differences in the rate of pyrethroid-resistance evolution were also apparent, with no significant increase in α-cypermethrin resistance in the first 2 years but a significant increase in permethrin-resistance intensity during the same time period.

Insecticide-resistance intensity varied between geographically proximate study clusters. The PY-LLIN cluster and pyriproxyfen PY-LLIN cluster were adjacent villages, presenting different initial pyrethroid-resistance intensity profiles in *An funestus* SL (table 1; table 2). Significant differences in pyrethroid-resistance intensity were observed between more geographically separate clusters (appendix p 14).

GLMM logistic results were consistent with the probit models, but also accounted for covariate effects (appendix p 23). For all insecticides, significant differences were found among years. For α-cypermethrin, pairwise trial-group differences were significant except for pyriproxyfen PY-LLINs versus PY-LLINs and pyriproxyfen PY-LLINs versus piperonyl butoxide PY-LLINs. Permethrin results were consistent, with significant pairwise comparisons for all trial groups except PY-LLINs versus piperonyl butoxide PY-LLINs and piperonyl butoxide PY-LLINs versus pyriproxyfen PY-LLINs. For piperonyl butoxide plus permethrin, all pairwise trial groups differed significantly (appendix p 23).

The effects of dual-active-ingredient LLINs on the evolution of pyrethroid-resistance intensity was more challenging to ascertain in *An gambiae* SL because of relative species complex availability throughout the trial. Longitudinal data were available for *An gambiae* SL collected from the piperonyl butoxide PY-LLIN group, which showed no significant increase in α-cypermethrin-resistance intensity during the trial years (appendix p 21), and collected from the pyriproxyfen PY-LLIN group, which showed a significant increase in α-cypermethrin-resistance intensity between year 1 and year 2 and year 1 and year 3 (appendix p 22). However, the magnitude of change was considerably less than observed for *An funestus* SL collected in the same study cluster.

All vectors exposed to pyrethroids were held for 72 h to assess any effects of insecticide on delayed-mortality rate. There was a significant reduction in survival during this holding period for *An funestus* SL exposed to α-cypermethrin, permethrin, and piperonyl butoxide across all trial groups (appendix pp 15–17, 26–31). An inverse relationship was apparent between pyrethroid-resistance intensity and delayed mortality; as resistance intensity increased over time, a decline in delayed mortality was observed (ie, exposure to higher concentrations of insecticide were required to elicit this effect; appendix pp 18–20, 26–31).

Regarding resistance to new active ingredients, populations of *An funestus* SL were susceptible to the diagnostic dose of chlorfenapyr in all study groups throughout the trial (appendix p 24). Populations of *An gambiae* SL had lower rates of mortality after chlorfenapyr exposure (appendix p 22). Populations of

| | Cluster number | Vector species | Pyriproxyfen dose tested, µg/mL* | Number of mosquitoes tested | Fertility reduction, % | Odds ratio (95% CI)† | p value |
|---|---|---|---|---|---|---|---|
| **Year 1** | | | | | | | |
| PY-LLINs | 72 | *Anopheles funestus* SL | 100 | 349 | 46·03% | 6·93 (3·53–13·95) | <0·0001 |
| Chlorfenapyr PY-LLINs | 63 | *An funestus* SL | 100 | 134 | 77·93% | 121·6 (35·67–382·9) | <0·0001 |
| Pyriproxyfen PY-LLINs | 73 | *An funestus* SL | 100 | 154 | 63·69% | 15·58 (7·38–32·21) | <0·0001 |
| Kisumu | Colony | *Anopheles gambiae* SS | 100 | 72 | 100·00% | .. | .. |
| **Year 2** | | | | | | | |
| PY-LLINs | 72 | *An funestus* SL | 100 | 309 | –4·68% | 0·84 (0·44–1·59) | 0·74 |
| Chlorfenapyr PY-LLINs | 63 | *An funestus* SL | 1000 | 116 | 11·33% | 5·26 (1·49–17·59) | 0·0093 |
| Pyriproxyfen PY-LLINs | 73; 73 | *An funestus* SL; *An gambiae* SL | 100; 100 | 128; 99 | 28·44%; 1·06% | 3·58 (1·78–7·28); 0·68 (0·36–1·27) | 0·0003; 0·26 |
| Piperonyl butoxide PY-LLINs | 78; 68 | *An funestus* SL; *An gambiae* SL | 100; 100 | 370; 118 | 2·90%; 37·28% | 0·90 (0·41–1·91); 10·67 (6·52–65·33) | 0·85; <0·0001 |
| Kisumu | Colony | *An gambiae* SS | 100 | 105 | 100·00% | .. | .. |
| **Year 3** | | | | | | | |
| PY-LLINs | 72 | *An funestus* SL | 1000 | 165 | 17·03% | 1·76 (0·92–3·31) | 0·10 |
| Pyriproxyfen PY-LLINs | 73 | *An funestus* SL | 1000 | 102 | 25·25% | 9·33 (3·31–25·43) | <0·0001 |
| Piperonyl butoxide PY-LLINs | 78; 68 | *An funestus* SL; *An gambiae* SL | 1000; 1000 | 206; 129 | 10·23%; 28·85% | 2·56 (1·01–6·51); 4·95 (2·33–10·22) | 0·049; <0·0001 |
| Kisumu‡ | Colony | *An gambiae* SS | 100 | 159 | 94·91% | 142·5 (48·22–358·0) | <0·0001 |

PY-LLIN=pyrethroid long-lasting insecticidal nets. SL=sensu lato. SS=sensu stricto. *Ten times the putative diagnostic dose of pyriproxyfen was tested in year 2 and year 3 due to low fertility reduction in year 1. †Odds ratio of reduction in fertility between mosquitoes exposed to pyriproxyfen and mosquitoes that were not exposed to pyriproxyfen. ‡Reduced sterility was attributed to an incomplete first blood meal that was required to create an entirely fertile egg batch among a subset of colony control mosquitoes.

***Table 3:*** **Effects of pyriproxyfen exposure on *Anopheles* fertility per trial group and study year**

both *An funestus* SL and *An gambiae* SL across all trial groups had some reduction in fertility after exposure to one or ten times the diagnostic dose of pyriproxyfen (table 3; appendix p 22). High or complete sterility was observed in the susceptible control colony (*An gambiae* SS Kisumu) after exposure to one or ten times the diagnostic dose of pyriproxyfen in each trial year.

Changes in expression of nine metabolic genes was monitored in PCR-confirmed *An funestus* SS in each group for 3 years after the intervention. Across all four groups, *CYP6M7*, *GSTE2*, and *CYP6M1* showed consistent minimal over-expression relative to the susceptible colony (data not shown).

In the PY-LLIN group, *CYP9K1* (fold change 9·43), *CYP4H17* (42·49), and *CYP6N1* (549·37) were all highly over-expressed in year 1, with expression levels significantly decreasing across trial years (year 3 *CYP9K1* 0·63; year 3 *CYP4H17* 7·03; year 3 *CYP6N1* 39·06; figure A). A similar decrease in expression level of *CYP9K1* (year 1 173·59 *vs* year 3 0·63), *CYP4H17* (year 1 29·18 *vs* year 3 7·03), and *CYP6N1* (year 1 233·68 *vs* year 3 39·06) was also observed in the pyriproxyfen PY-LLIN groups (figure C). There was evidence for ongoing selection of *CYP6P9b* (year 1 2·73 *vs* year 3 8·81 in the PY-LLIN group; year 1 2·61 *vs* year 3 8·81 in the pyriproxyfen PY-LLIN group; figure A, C).

In the chlorfenapyr PY-LLIN group, over-expression of several metabolic enzymes fluctuated dynamically across the 3 trial years (eg, year 1 *CYP9K1* fold change 7·27, year 2 99·91, year 3 9·90; year 1 *CYP6N1* 67·81, year 2 6·86, year 3 67·75; figure B). This group was the only group in which *CYP6P9a* was under selection (year 1 0·09 *vs* year 3 16·08; figure B).

In the piperonyl butoxide PY-LLIN group, there was evidence of selection for *CYP9K1* (year 1 FC=2·31 *vs* year 2 10·64), *CYP4H17* (year 1 6·89 *vs* year 2 31·19), and *CYP6N1* (year 1 195·20 *vs* year 2 520·06), which diminished by year three (figure 1D). Conversely, over-expression of both *CYP6Z1* (year 1 0·33 *vs* year 3 3·82) and *CYP6P9b* (year 1 4·18 *vs* year 3 11·93) increased significantly during the trial years (figure D).

## Discussion

The widespread distribution of three dual-active-ingredient LLINs and PY-LLINs had significant effects on insecticide-resistance selection in the primary malaria vector *An funestus* SL during 3 trial years in northwest Tanzania. Findings showed an increase in both α-cypermethrin-resistance intensity and permethrin-resistance intensity and a concomitant decline in piperonyl-butoxide synergy in most trial years for the PY-LLIN, pyriproxyfen PY-LLIN, and piperonyl butoxide PY-LLIN groups. These observations might be unsurprising in the PY-LLIN group but lead to substantial concerns about the long-term implications of PY-LLIN campaigns, which continue across many malaria-endemic areas. To date, there have been few studies, but

they have shown robust insecticide-resistance monitoring activities that effectively show the extent to which PY-LLINs intensify phenotypic resistance in established, resistant vector populations. This analysis presents novel evidence of phenotypic and genotypic resistance selection in vector populations during a short time span, suggesting that ongoing widespread use of PY-LLINs is unsustainable and advising against the reliance on current LLIN tools, including piperonyl butoxide PY-LLINs, for malaria-vector control and resistance management.

Arguments have been made for the ongoing use of PY-LLINs to provide personal protection as a physical barrier or by eliciting delayed mortality of vectors after exposure.[23,24] In most trial clusters, significant delayed mortality was observed, which might have also diminished overall malaria transmission. However, persistent deployment of PY-LLINs, as well as cross-resistance between type I and type II pyrethroids,[25] can contribute to the selection of alternate, generalist resistance mechanisms, including cuticular thickening or salivary-gland detoxification, which impart other fitness benefits to resistant vectors but have enduring consequences for the design and evaluation of candidate insecticides for malaria-vector control.[26]

In the pyriproxyfen PY-LLIN group, despite comparatively low initial pyrethroid resistance, populations of *An funestus* SL rapidly developed highly intense pyrethroid resistance, which might have been driven by cross-resistance mechanisms that have been reported between pyriproxyfen and pyrethroid insecticides in both *An gambiae* SL and *An funestus* SL.[27] The largest increase in pyrethroid-resistance intensity was shown in the piperonyl butoxide PY-LLIN group; by year 3, the concentration to kill 95% of *An funestus* SL was more than 50 times the diagnostic dose of permethrin despite pre-exposure to piperonyl butoxide. These results probably reflect selection for over-expressed metabolic-detoxification enzymes or alternate resistance mechanisms, providing a cautionary rationale for the distribution of piperonyl butoxide PY-LLINs to avoid further exacerbating the pyrethroid insecticide-resistance crisis.

Of the three dual-active-ingredient LLINs assessed, the chlorfenapyr PY-LLIN group showed no significant selection for pyrethroid resistance, supported by minimal reductions in chlorfenapyr susceptibility throughout the trial. Currently, chlorfenapyr PY-LLINs are a promising dual-active-ingredient LLIN to control malaria transmitted by pyrethroid-resistant vector populations. However, reliance on a single intervention for reactive resistance management is inadvisable because of the propensity of *Anopheles* to rapidly evolve resistance to new active ingredients and reports of incipient reduced chlorfenapyr susceptibility from west and central sub-Saharan Africa.[28] Our findings strengthen trial epidemiological outcomes, with chlorfenapyr PY-LLINs providing superior protection from malaria across multiple transmission seasons; piperonyl butoxide PY-LLINs remained effective for the first year and pyriproxyfen PY-LLINs had no significant effect on disease incidence.[9] The rapid loss of the protective efficacy of piperonyl butoxide PY-LLINs and overall lack of pyriproxyfen PY-LLIN intervention effect can be explained by the significant increase in permethrin-resistance intensity in the piperonyl butoxide PY-LLIN group and pre-existing tolerance to pyriproxyfen in the trial area in the pyriproxyfen PY-LLIN group. Furthermore, relative differences in LLIN textile and chemical durability, surface bioavailability, and household use also influenced the evolution of insecticide resistance in the Misungwi district. By the end of the trial, less than 20% of community members reported using their pyriproxyfen PY-LLINs or piperonyl butoxide PY-LLINs (unpublished). These nets were also in poor physical condition, with severe depletion of both pyrethroid (α-cypermethrin reduced by 40% and permethrin reduced by 59%) and partner active ingredients (pyriproxyfen reduced by 73% and piperonyl butoxide reduced by 93%; unpublished). Although the effective longevities of dual-active-ingredients are still being investigated, leaving pyriproxyfen PY-LLINs or piperonyl butoxide PY-LLINs in the field after their optimum operational lifespans might have exposed vector populations to sublethal insecticide doses, expediting resistance selection and potentially worsening the pyrethroid-resistance intensification observed in this analysis.[29,30]

Subanalyses and molecular monitoring of key detoxification enzymes that had been previously implicated in pyrethroid resistance in *An funestus* SL across central and southern Africa, and in other parts of the Lake Zone in Tanzania,[19,20] have revealed several insights. After LLIN distribution, *CYP4H17*, *CYP6N1*, and *CYP9K1*, which were already over-expressed in populations of *An funestus* SL, began to decrease, which is indicative of ongoing selection for other resistance mechanisms that were not under active surveillance in the cRCT.[9] In comparison, levels of *CYP6P9a* and *CYP6P9b* expression increased during the trial. In both *CYP6P9a* and *CYP6P9b*, cisregulatory polymorphisms, which drive transcriptional upregulation, have been significantly associated with reduced efficacy of PY-LLINs and piperonyl butoxide PY-LLINs in experimental hut trials.[31–33] Currently, *CYP6P9a* is the only enzyme incriminated in pyriproxyfen metabolism in *An funestus* in vitro.[27] However, selection for this gene was only observed in the chlorfenapyr PY-LLIN group, suggesting the presence of additional, undescribed cross-resistance mechanisms in the Misungwi district that require further elucidation. Study clusters that were fewer than 2–4 kilometres apart showed distinct genotypic and phenotypic profiles (table 1; table 2; figure), emphasising how focal and heterogeneous insecticide resistance can be on a microgeographical scale. Several of the metabolic enzymes assessed in this analysis

(ie, *CYP6M7* and *CYP6M1*) were previously over-expressed in *An funestus* populations in the first cRCT evaluating piperonyl butoxide PY-LLINs in a different part of the Lake Zone of Tanzania but did not appear to be under selection in the cRCT.[9,20] This variability advocates for widespread, in-depth, genetic characterisation of vector populations during trial baselines to better inform the effects of insecticidal interventions on the selection of novel-resistance mechanisms.

Our results should be interpreted in the context of several limitations. *An funestus* SL mosquitoes were collected as indoor resting adults due to difficulties in identifying reliable, productive breeding sites and rearing this vector species under controlled insectary conditions. Individual mosquitoes were held for 3 days to minimise the effect of blood-meal digestion on metabolic-gene expression; however, because adults were of unknown age and because phenotypic resistance declines over time,[34] pyrethroid resistance was possibly underestimated. During the first year, mosquitoes were challenging to collect from the piperonyl butoxide PY-LLIN group, probably due to an intervention effect after LLIN scale up as vectors that were available for resistance monitoring might have inherently been those that were more resistant to chemical exposure.[9] Furthermore, because collections were done in multiple months, population age, parity, life history, and species composition were variable. However, a logistic GLMM approach was used to account for potential differences in survival probability by treatment groups, study years, test replicates, and testing dates. Throughout the trial, the proportion of *An funestus* SL steadily declined compared with *An gambiae* SL.[22] Phenotypic results indicated different selection pressures acting on *An gambiae* SL (mostly *An arabiensis*), with little evidence for an escalation in pyrethroid-resistance intensity in this species complex. This finding might be explained by the propensity for *An arabiensis* to avoid insecticidal exposure by feeding and resting outdoors in areas with high intervention coverage.[35] Finally, contamination of larval breeding sites with agricultural pesticides probably also contributed to increased insecticide resistance via cross-resistance mechanisms, but was not directly assessed.[36]

This secondary analysis of a 3-year cRCT showed the differential effects of dual-active-ingredient LLINs containing either chlorfenapyr, piperonyl butoxide, or pyriproxyfen on phenotypic and genotypic insecticide-resistance selection in *An funestus* SL. Although chlorfenapyr PY-LLINs had no significant selection for phenotypic pyrethroid resistance and vector populations remained susceptible to the partner active ingredient throughout the trial, piperonyl butoxide PY-LLINs, pyriproxyfen PY-LLINs, and PY-LLINs all intensified insecticide resistance. Increased pyrethroid resistance by PY-LLINs was not surprising but continued

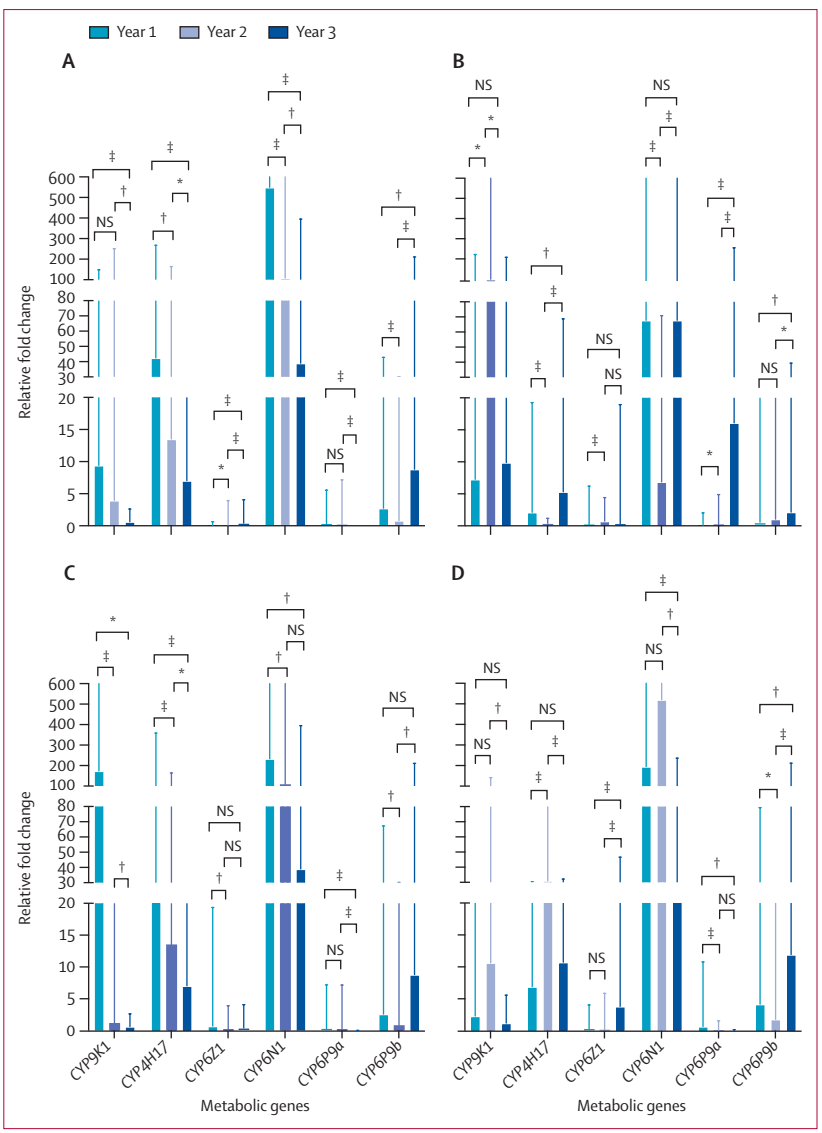


***Figure:*** **3-year changes in PCR-confirmed *Anopheles funestus* sensu stricto metabolic gene expression relative to a susceptible colony population**
(A) PY-LLIN (Interceptor). (B) Chlorfenapyr PY-LLIN (Interceptor G2). (C) Pyriproxyfen PY-LLIN (Royal Guard). (D) Piperonyl butoxide PY-LLIN (Olyset Plus). Error bars represent 95% CIs. NS=not significant. PY-LLIN=pyrethroid long-lasting insecticidal nets. *p<0·05. †p<0·01. ‡p≤0·001.

widespread distribution of these nets will have potentially severe consequences for the selection of cross-resistance mechanisms, as shown in this analysis between different types of pyrethroids and between pyrethroids and pyriproxyfen. Ongoing deployment of PY-LLINs might further drive the evolution of novel insecticide-resistance mechanisms, which will further complicate the design of new insecticidal interventions for malaria-vector control. Piperonyl butoxide PY-LLINs have now replaced PY-LLINs as the standard of care in most sub-Saharan African countries. However, our findings indicated that these LLINs could be worsening the pyrethroid insecticide-resistance crisis among

*Anopheles* species with anthropophilic and endophagic behaviours. Furthermore, pyriproxyfen PY-LLINs were largely inadequate for resistance management due to shared cross-resistance mechanisms with pyrethroids. At the molecular level, this analysis showed the focal nature of specific insecticide-resistance mechanisms among vectors with distinct life histories and supports improved genetic characterisation of diverse mosquito populations across different ecologies, transmission intensities, and malaria-endemic areas to better inform pre-emptive resistance monitoring and intervention development. Our findings also challenge the viability of tactical deployment of an already small number of insecticidal interventions and argue for rapid development and evaluation of novel insecticidal tools and alternative strategies for global malaria control.

#### Contributors

LAM, NSM, NP, AM, FWM, MR, and MAK designed the study. NSM led the fieldwork and bioassay testing, with support from LAM, MJ, JM, and JFM. LAM, NMP, RK, OM, BP, and KF did the molecular analysis. TW provided molecular laboratory resources. EL and EM curated the data. LAM, NSM, CLC, MAK, and NP accessed, verified, analysed, and interpreted the data. LAM drafted the manuscript, which was revised by all other authors. All authors had full access to all the data in the study, read and approved the final manuscript, and had final responsibility for the decision to submit for publication.

#### Declaration of interests

We declare no competing interests.

#### Data sharing

The datasets that were generated for or analysed in this analysis are provided in the Article and the appendix (pp 15–17). All other relevant data are available from the corresponding author upon request from the date of publication.

#### Acknowledgments

This analysis was funded by the UK Foreign, Commonwealth and Development Office; the UK Medical Research Council; the Wellcome Trust; the UK Department of Health and Social Care (MR/R006040/1); and The Bill and Melinda Gates Foundation via the Innovative Vector Control Consortium. TW is supported by the Wellcome Trust–Royal Society Fellowship (101285/Z/13/Z). We thank the entomology technicians for their support in the field work and the residents of the Misungwi district for their acceptance and cooperation. We thank Amy Guy for providing susceptible individuals from the FANG colony and Charles Thickstun for drawing the study map. Electronic data solutions were provided by London School of Hygiene & Tropical Medicine Global Health Analytics (odk.lshtm.ac.uk).

#### References

1 WHO. World Malaria Report 2021. 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021 (accessed Jan 13, 2023).
2 Bhatt S, Weiss DJ, Cameron E, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* 2015; **526:** 207–11.
3 Killeen GF, Sougoufara S. Getting ahead of insecticide-resistant malaria vector mosquitoes. *Lancet* 2023; **401:** 410–11.
4 Killeen GF. Control of malaria vectors and management of insecticide resistance through universal coverage with next-generation insecticide-treated nets. *Lancet* 2020; **395:** 1394–400.
5 WHO. World malaria report 2022. 2022. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022 (accessed Jan 13, 2023).
6 WHO. Norms, standards and processes underpinning development of WHO recommendations on vector control. 2020. https://www.who.int/publications/i/item/9789240017382 (accessed Jan 13, 2023).
7 Protopopoff N, Mosha JF, Lukole E, et al. Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two factorial design trial. *Lancet* 2018; **391:** 1577–88.
8 Staedke SG, Gonahasa S, Dorsey G, et al. Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *Lancet* 2020; **395:** 1292–303.
9 Mosha JF, Kulkarni MA, Lukole E, et al. Effectiveness and cost-effectiveness against malaria of three types of dual-active-ingredient long-lasting insecticidal nets (LLINs) compared with pyrethroid-only LLINs in Tanzania: a four-arm, cluster-randomised trial. *Lancet* 2022; **399:** 1227–41.
10 Accrombessi M, Cook J, Dangbenon E, et al. Efficacy of pyriproxyfen-pyrethroid long-lasting insecticidal nets (LLINs) and chlorfenapyr-pyrethroid LLINs compared with pyrethroid-only LLINs for malaria control in Benin: a cluster-randomised, superiority trial. *Lancet* 2023; **401:** 435–46.
11 Anopheles gambiae 1000 Genomes Consortium. Genetic diversity of the African malaria vector *Anopheles gambiae*. *Nature* 2017; **552:** 96–100.
12 Matowo NS, Martin J, Kulkarni MA, et al. An increasing role of pyrethroid-resistant *Anopheles funestus* in malaria transmission in the Lake Zone, Tanzania. *Sci Rep* 2021; **11:** 13457.
13 Mosha JF, Kulkarni MA, Messenger LA, et al. Protocol for a four parallel-arm, single-blind, cluster-randomised trial to assess the effectiveness of three types of dual active ingredient treated nets compared to pyrethroid-only long-lasting insecticidal nets to prevent malaria transmitted by pyrethroid insecticide-resistant vector mosquitoes in Tanzania. *BMJ Open* 2021; **11:** e046664.
14 Gillies MT, De Meillon B. The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region). Johannesburg, South Africa: South African Institute for Medical Research, 1968.
15 Brogdon WG, Chan A. Guideline for evaluating insecticide resistance in vectors using the CDC Bottle Bioassay. 2012. https://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf (accessed Jan 13, 2023).
16 WHO. Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. 2022. https://www.who.int/publications/i/item/9789240051089 (accessed Jan 13, 2023).
17 Soto A, Rowland M, Messenger LA, et al. Ovary dissection is a sensitive measure of sterility in *Anopheles gambiae* exposed to the insect growth regulator pyriproxyfen. *Insects* 2023; **14:** 552.
18 Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *Am J Trop Med Hyg* 2002; **66:** 804–11.
19 Sandeu MM, Mulamba C, Weedall GD, Wondji CS. A differential expression of pyrethroid resistance genes in the malaria vector *Anopheles funestus* across Uganda is associated with patterns of gene flow. *PLoS One* 2020; **15:** e0240743.
20 Matowo J, Weetman D, Pignatelli P, et al. Expression of pyrethroid metabolizing P450 enzymes characterizes highly resistant *Anopheles* vector species targeted by successful deployment of PBO-treated bednets in Tanzania. *PLoS One* 2022; **17:** e0249440.
21 Lei C, Sun X. Comparing lethal dose ratios using probit regression with arbitrary slopes. *BMC Pharmacol Toxicol* 2018; **19:** 61.
22 Matowo NS, Kulkarni MA, Messenger LA, et al. Differential impact of dual-active ingredient long-lasting insecticidal nets (LLINs) on primary malaria vectors: results of a three-year cluster-randomized controlled trial in rural Tanzania. *Lancet Planet Health* 2023; **7:** e370–80.
23 Kleinschmidt I, Bradley J, Knox TB, et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. *Lancet Infect Dis* 2018; **18:** 640–49.
24 Viana M, Hughes A, Matthiopoulos J, Ranson H, Ferguson HM. Delayed mortality effects cut the malaria transmission potential of insecticide-resistant mosquitoes. *Proc Natl Acad Sci USA* 2016; **113:** 8975–80.

25 Lissenden N, Kont MD, Essandoh J, et al. Review and meta-analysis of the evidence for choosing between specific pyrethroids for programmatic purposes. *Insects* 2021; **12:** 826.

26 Messenger LA, Impoinvil LM, Derilus D, Yewhalaw D, Irish S, Lenhart A. A whole transcriptomic approach provides novel insights into the molecular basis of organophosphate and pyrethroid resistance in *Anopheles arabiensis* from Ethiopia. *Insect Biochem Mol Biol* 2021; **139:** 103655.

27 Yunta C, Hemmings K, Stevenson B, et al. Cross-resistance profiles of malaria mosquito P450s associated with pyrethroid resistance against WHO insecticides. *Pestic Biochem Physiol* 2019; **161:** 61–67.

28 Tchouakui M, Assatse T, Tazokong HR, et al. Detection of chlorfenapyr resistance in the malaria vector *Anopheles gambiae* contrasts with full susceptibility in *Anopheles funestus* across Africa. *Sci Rep* 2023; **13:** 2363.

29 Andreazza F, Oliveira EE, Martins GF. Implications of sublethal insecticide exposure and the development of resistance on mosquito physiology, behavior, and pathogen transmission. *Insects* 2021; **12:** 917.

30 Zoh MGTJ, Tutagata J, Fodjo BK, et al. Exposure of *Anopheles gambiae* larvae to a sub-lethal dose of an agrochemical mixture induces tolerance to adulticides used in vector control management. *Aquat Toxicol* 2022; **248:** 106181.

31 Mugenzi LMJ, Menze BD, Tchouakui M, et al. Cis-regulatory *CYP6P9b* P450 variants associated with loss of insecticide-treated bed net efficacy against *Anopheles funestus*. *Nat Commun* 2019; **10:** 4652.

32 Weedall GD, Mugenzi LMJ, Menze BD, et al. A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. *Sci Transl Med* 2019; **11:** eaat7386.

33 Menze BDML, Mugenzi LMJ, Tchouakui M, Wondji MJ, Tchoupo M, Wondji CS. Experimental hut trials reveal that *CYP6P9a/b* P450 alleles are reducing the efficacy of pyrethroid-only Olyset net against the malaria vector *Anopheles funestus* but PBO-based Olyset Plus net remains effective. *Pathogens* 2022; **11:** 638.

34 Jones CMSA, Sanou A, Guelbeogo WM, Sagnon N, Johnson PC, Ranson H. Aging partially restores the efficacy of malaria vector control in insecticide-resistant populations of *Anopheles gambiae* s.l. from Burkina Faso. *Malar J* 2012; **11:** 24.

35 Killeen GFGN, Govella NJ, Lwetoijera DW, Okumu FO. Most outdoor malaria transmission by behaviourally-resistant *Anopheles arabiensis* is mediated by mosquitoes that have previously been inside houses. *Malar J* 2016; **15:** 225.

36 Urio NHPP, Pinda PG, Ngonzi AJ, et al. Effects of agricultural pesticides on the susceptibility and fitness of malaria vectors in rural south-eastern Tanzania. *Parasit Vectors* 2022; **15:** 213.