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

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Summary

Background Long-lasting insecticidal nets (LLINs) are the primary malaria prevention tool, but their effectiveness is threatened by pyrethroid resistance. We embedded a pragmatic cluster-randomised trial into Uganda's national LLIN campaign to compare conventional LLINs with those containing piperonyl butoxide (PBO), a synergist that can partially restore pyrethroid susceptibility in mosquito vectors.

Methods 104 health sub-districts, from 48 districts in Uganda, were randomly assigned to LLINs with PBO (PermaNet 3.0 and Olyset Plus) and conventional LLINs (PermaNet 2.0 and Olyset Net) by proportionate randomisation using an iterative process. At baseline 6, 12, and 18 months after LLIN distribution, cross-sectional surveys were done in 50 randomly selected households per cluster (5200 per survey); a subset of ten households per cluster (1040 per survey) were randomly selected for entomological surveys. The primary outcome was parasite prevalence by microscopy in children aged 2–10 years, assessed in the as-treated population at 6, 12, and 18 months. This trial is registered with ISRCTN, ISRCTN17516395.

Findings LLINs were delivered to households from March 25, 2017, to March 18, 2018, 32 clusters were randomly assigned to PermaNet 3.0, 20 to Olyset Plus, 37 to PermaNet 2.0, and 15 to Olyset Net. In the as-treated analysis, three clusters were excluded because no dominant LLIN was received, and four clusters were reassigned, resulting in 49 PBO LLIN clusters (31 received PermaNet 3.0 and 18 received Olyset Plus) and 52 non-PBO LLIN clusters (39 received PermaNet 2.0 and 13 received Olyset Net). At 6 months, parasite prevalence was 11% (386/3614) in the PBO group compared with 15% (556/3844) in the non-PBO group (prevalence ratio [PR] adjusted for baseline values 0·74, 95% CI 0·62–0·87; p=0·0003). Parasite prevalence was similar at month 12 (11% *vs* 13%; PR 0·73, 95% CI 0·63–0·85; p=0·0001) and month 18 (12% *vs* 14%; PR 0·84, 95% CI 0·72–0·98; p=0·029).

Interpretation In Uganda, where pyrethroid resistance is high, PBO LLINs reduced parasite prevalence more effectively than did conventional LLINs for up to 18 months. This study provides evidence needed to support WHO's final recommendation on use of PBO LLINs.

Funding The Against Malaria Foundation, UK Department for International Development, Innovative Vector Control Consortium, and Bill and Melinda Gates Foundation.

Introduction

Long-lasting insecticidal nets (LLINs) are the foundation of malaria control in sub-Saharan Africa.[1,2] Over the past 20 years, substantial efforts have been made to expand LLIN coverage in malaria-endemic countries. From 2000 to 2015, the incidence of *Plasmodium falciparum* decreased by 40% in Africa, largely attributable to widespread use of LLINs.[3] However, the effectiveness of LLINs is threatened by pyrethroid resistance, which could severely compromise malaria control efforts.[4] Reports suggest that malaria control has stalled in Africa, particularly in high burden areas.[5] WHO has called for aggressive action to preserve gains made in malaria

control and ensure that the ambitious 2030 targets, including eliminating malaria from at least 35 countries, are met.[6] To achieve these goals, effectiveness of LLINs must be maintained.

Currently, all LLINs are impregnated with pyrethroid insecticides, because of their favourable safety profile, low cost, and rapid insecticidal activity.[7] However, resistance to pyrethroids is now widespread in Africa.[8] In African *Anopheles* mosquitoes, pyrethroid resistance is primarily mediated through two mechanisms: knockdown resistance (*kdr*) caused by mutations in the voltage-gated sodium channel where pyrethroids bind, and metabolic resistance resulting from alterations in

Research in context

Evidence before this study

We searched PubMed with the terms "piperonyl butoxide, PBO, Olyset* or PermaNet*", AND "insecticide-treated bednets, nets, bednet*, ITN*, LLIN*, insecticide-treated bednet*, OR insecticidal net*" for studies published in English with no date restrictions. We found one trial that evaluated the effect of long-lasting insecticidal nets (LLINs) with piperonyl butoxide (PBO) on epidemiological outcomes in Tanzania. The cluster-randomised, controlled trial compared Olyset Plus (LLINs with PBO) with Olyset Net (LLINs without PBO) in a single district of Tanzania, where pyrethroid resistance is high. At months 9, 16, and 21 after LLIN distribution, prevalence of malaria parasites was lower in community residents who received PBO LLINs than in those who received conventional (non-PBO) LLINs. Supported largely by these results, WHO provided an interim endorsement of PBO LLINs in 2017, recommending their use in areas where pyrethroid resistance is mediated by metabolic mechanisms and is classified by moderate intensity using standard procedures. A Cochrane systematic review published in 2018 assessed the effectiveness of PBO LLINs on epidemiological and entomological outcomes. 15 studies were included: two laboratory trials, eight experimental hut trials, and five cluster-randomised controlled village trials, including the Tanzanian trial, which was the only study to report epidemiological outcomes. This review suggested that PBO LLINs would be most effective in areas of high-level

pyrethroid resistance; the effectiveness of PBO LLINs in areas of moderate or low-level pyrethroid resistance was less clear. Further epidemiological evidence of the effectiveness of PBO LLINs is urgently needed to guide WHO recommendations and malaria control policy throughout Africa, where pyrethroid resistance in malaria vectors is widespread.

Added value of this study

This study makes an important contribution to the small evidence base on the use of PBO LLINs. In this large, cluster-randomised, controlled trial we found that PBO LLINs provided superior protection against malaria in the setting of high-level insecticide resistance in Uganda. Both PBO and non-PBO LLINs effectively reduced parasite prevalence from baseline, suggesting that conventional LLINs provide protection and can still have a role in settings where insecticide resistance and malaria transmission are low. This innovative trial, embedded within a national LLIN distribution campaign, serves as a paradigm for future assessment of malaria control interventions.

Implications of all the available evidence

This study provides the evidence needed to support WHO's final recommendation on use of PBO LLINs. LLINs are the cornerstone of malaria control in Africa. Deploying PBO LLINs in areas of high-level pyrethroid resistance will help to extend the useful lifespan of this important vector control tool.

enzymes that detoxify pyrethroids, notably cytochrome P450s.[9,10] To address P450-based resistance, newer LLINs combine pyrethroids with a synergist, piperonyl butoxide (PBO), which inhibits P450s enzymes, blocking the mosquito's defence against pyrethroids and at least partially restoring pyrethroid susceptibility.[11]

A systematic review of PBO LLINs found that they were associated with higher mosquito mortality and lower blood-feeding rates in areas of high-level insecticide resistance than were non-PBO LLINs.[12] A cluster-randomised, clinical trial of the effectiveness of PBO LLINs (Olyset Plus), done in Tanzania, found that PBO LLINs were associated with lower parasite prevalence than were conventional LLINs at 9, 16, and 21 months after distribution.[13] Subsequently, WHO issued an interim endorsement of PBO LLINs, recommending them for areas of intermediate-level pyrethroid resistance, due at least partly to metabolic mechanisms.[14] However, the Tanzanian study had several limitations: the study was restricted to one district; parasite prevalence was measured using rapid diagnostic tests, which could have variable specificity;[15] insecticide resistance was assessed by *kdr* mutations, which are not markers of metabolic resistance; and 21-month data were potentially compromised by routine distribution of new LLINs within the study area. Thus, additional epidemiological evidence of PBO LLINs in different contexts is urgently needed to support robust guidance to countries.

In Uganda, despite mass distribution of LLINs, targeted indoor residual spraying, and treatment of symptomatic malaria cases with artemisinin-based combination therapies, progress on malaria control has been slow and control gains have been difficult to sustain.[16,17] In 2015–18, malaria incidence in Uganda increased, underscoring the need to intensify malaria control efforts.[5,18] The Ministry of Health has committed to distributing free LLINs in Uganda through mass campaigns every 3–4 years. In 2017–18, LLINs with PBO (PermaNet 3.0 and Olyset Plus) and conventional nets without PBO (PermaNet 2.0 and Olyset Net) were distributed across Uganda. With support from the Ministry of Health, donors, and partners, a large cluster-randomised trial was embedded within the national LLIN distribution campaign, which allowed us to rigorously evaluate the effect of LLINs at an unprecedented scale.

Methods

Study design and setting

The trial protocol has been published previously.[19] Our primary objective was to evaluate the effect of combination LLINs (with PBO) compared with conventional LLINs (without PBO), on parasite prevalence in eastern and western Uganda.

Briefly, we did a pragmatic, cluster-randomised trial in 104 health sub-districts in eastern and western Uganda, which were not scheduled to receive indoor residual

spraying with pirimiphos-methyl (Actellic).[14] Districts in the north and east where indoor residual spraying was ongoing or planned, were excluded. Following sensitisation of authorities and local communities, baseline cross-sectional community and entomology surveys were conducted.[17,20,21] At baseline, 65% of households owned at least one LLIN, but only 18% met the WHO definition of adequate coverage (at least one LLIN per two residents).[21] Overall, parasite prevalence in children aged 2–10 years was 26%, ranging from 8% in the southwest region to 53% in the east central region.[17] Very high levels of pyrethroid resistance due to target-site (primarily *Vgsc*-L1014S) and metabolic mechanisms (markers *Cyp4j5*-L43F and *Coeae1d*) were observed.[21] For this trial, clusters were defined as one health sub-district (serving approximately 100 000 people); 104 health sub-districts nationwide were included, covering 48 districts, approximately 40% of the country (figure 1). Clusters were randomly assigned to one of four study arms: (1) PermaNet 3.0 [n=32], (2) Olyset Plus [n=20] (both PBO LLINs); and (3) PermaNet 2.0 [n=37], (4) Olyset Net [n=15] (both conventional LLINs without PBO). From 2017–2018, LLINs were delivered through a mass-distribution campaign. Cross-sectional community and entomology surveys were carried out at 6, 12, and 18 months after net distribution; net durability and bio-efficacy were assessed at 12 months. The primary outcome was parasite prevalence measured by microscopy in children aged 2–10 years. The trial was approved by the Ugandan National Council for Science and Technology (reference HS 2176), Makerere University School of Medicine Research & Ethics Committee (SOMREC 2016–133), London School of Hygiene & Tropical Medicine Ethics Committee (LSHTM reference 12019), and the Liverpool School of Tropical Medicine (LSTM reference 16–072). This trial is registered with ISRCTN, ISRCTN17516395.

Randomisation and net distribution

LLINs were procured in advance of the randomisation. We used the entire production capacity for the PBO LLINs because of the scale of the trial; thus, the total number of the four LLIN types varied. Proportionate randomisation was done by a co-investigator based outside of Uganda using STATA (version 14.2), as described previously.[19] Briefly, an iterative process was used to assign net types to each cluster using cumulative probability ranges generated for each of the four types of nets on the basis of the targeted number of each individual type of net divided by targeted number of total nets and random numbers between 0 and 1 generated for each cluster. The randomisation was stratified by region, with 66 clusters in the west and 38 clusters in the east, in case regional differences in insecticide resistance were found. However, no significant differences in resistance marker frequency by region or study group were observed at baseline (data not shown). LLIN allocation was not masked.

PermaNet 3.0 and PermaNet 2.0 (Vestergaard SA, Lausanne, Switzerland) and Olyset Plus and Olyset Net (Sumitomo Chemical, Tokyo, Japan) nets were distributed. The Ministry of Health, local government, and partners distributed the assigned LLINs from March 25, 2017, to March 18, 2018, as part of the national LLIN distribution campaign, according to the net allocation list generated from the randomisation. Using household registration data collected before the campaign, an allocation formula was applied by the Ministry of Health to determine the number of LLINs each household should receive (total number of people in the household divided by two, and rounded up if the number of household members was uneven).
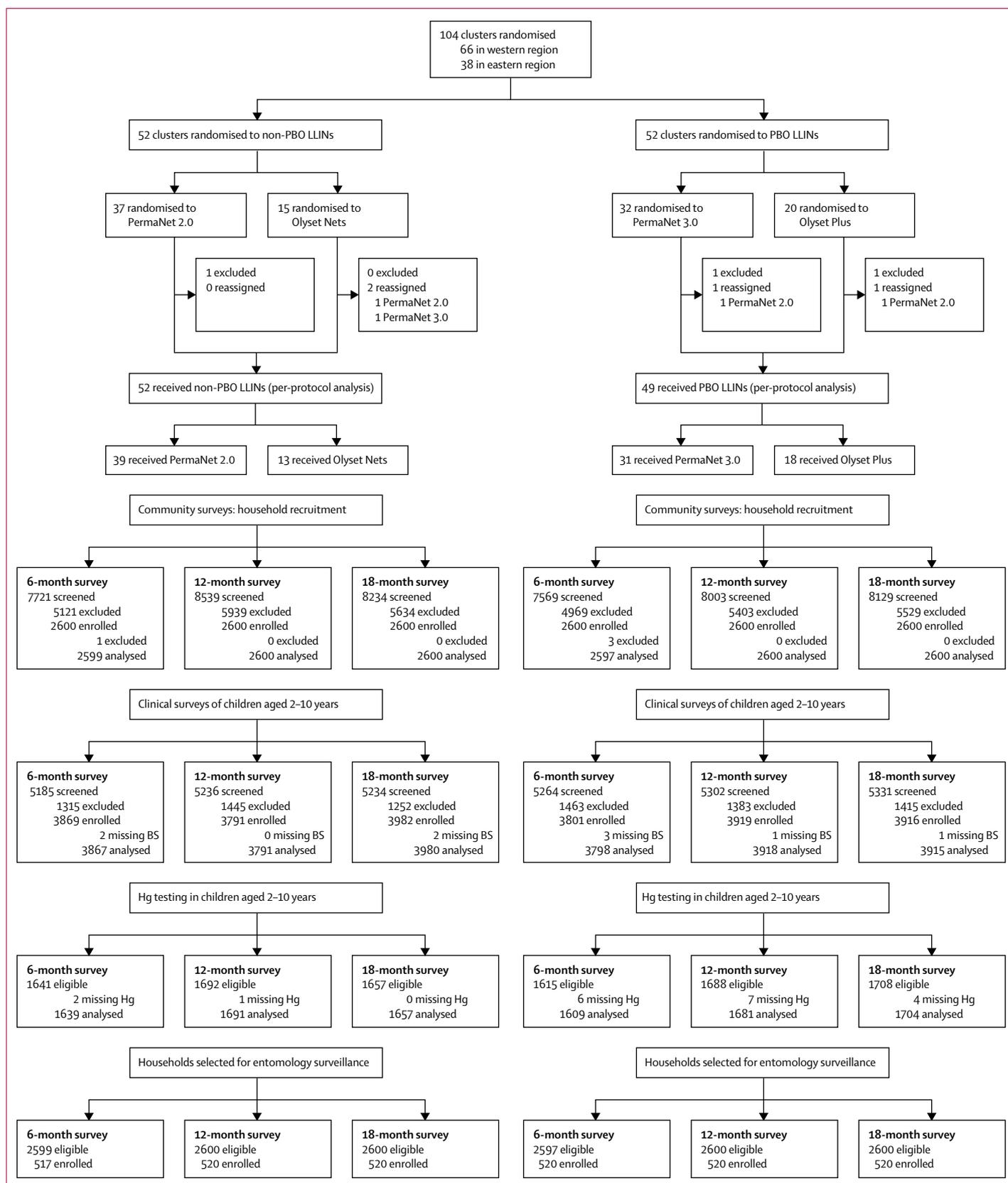
LLINs were delivered in the first 44 clusters (24 PBO and 20 non-PBO) as scheduled. However, the total number of nets needed was underestimated and additional LLINs had to be procured. Subsequently, the 60 remaining clusters were randomly assigned again, using the same process of proportionate randomisation, resulting in 52 clusters being assigned to each of the two main study groups.[19]

Community surveys

Cross-sectional surveys were done at 6, 12, and 18 months after LLIN distribution by study staff. Two-stage cluster sampling was applied using enumeration areas as the primary sampling unit; ten enumeration areas (defined as a natural village or urban city block) within each cluster were randomly selected.[19] All households in the selected areas were mapped and assigned an identification number. A list of randomly selected households was generated for each area. Households were approached sequentially until five were enrolled from each area (50 households per cluster, 5200 per survey). Households were included if at least one resident was aged 2–10 years, at least one adult (aged ≥18 years) was present, the adult was usually resident and slept in the sampled household on the night before the survey, and the adult agreed to provide written informed consent to participate in the survey. Households were excluded if the dwelling was destroyed or could not be found, the house was vacant, or there was no adult resident home on more than three occasions.

Heads of household, or their designate, were asked to complete a household survey questionnaire, to gather information on households, residents, and LLIN ownership and use.[19,21] Children residing in the household had blood drawn by finger-prick if they were aged 2–10 years, usually resident and slept in the sampled household on the night before the survey, had provision of written informed consent by parent or guardian, and assented if they were aged 8 years or older. Children who could not

Figure 1: Trial profile
Hg=Haemoglobin. BS=blood slide. LLIN=long-lasting insecticidal net. PBO=piperonyl butoxide.

104 clusters randomised
66 in western region
38 in eastern region

52 clusters randomised to non-PBO LLINs

52 clusters randomised to PBO LLINs

37 randomised to PermaNet 2.0

15 randomised to Olyset Nets

32 randomised to PermaNet 3.0

20 randomised to Olyset Plus

1 excluded
0 reassigned

0 excluded
2 reassigned
 1 PermaNet 2.0
 1 PermaNet 3.0

1 excluded
1 reassigned
 1 PermaNet 2.0

1 excluded
1 reassigned
 1 PermaNet 2.0

52 received non-PBO LLINs (per-protocol analysis)

49 received PBO LLINs (per-protocol analysis)

39 received PermaNet 2.0

13 received Olyset Nets

31 received PermaNet 3.0

18 received Olyset Plus

Community surveys: household recruitment

6-month survey
7721 screened
 5121 excluded
 2600 enrolled
 1 excluded
 2599 analysed

12-month survey
8539 screened
 5939 excluded
 2600 enrolled
 0 excluded
 2600 analysed

18-month survey
8234 screened
 5634 excluded
 2600 enrolled
 0 excluded
 2600 analysed

6-month survey
7569 screened
 4969 excluded
 2600 enrolled
 3 excluded
 2597 analysed

12-month survey
8003 screened
 5403 excluded
 2600 enrolled
 0 excluded
 2600 analysed

18-month survey
8129 screened
 5529 excluded
 2600 enrolled
 0 excluded
 2600 analysed

Clinical surveys of children aged 2–10 years

6-month survey
5185 screened
 1315 excluded
 3869 enrolled
 2 missing BS
 3867 analysed

12-month survey
5236 screened
 1445 excluded
 3791 enrolled
 0 missing BS
 3791 analysed

18-month survey
5234 screened
 1252 excluded
 3982 enrolled
 2 missing BS
 3980 analysed

6-month survey
5264 screened
 1463 excluded
 3801 enrolled
 3 missing BS
 3798 analysed

12-month survey
5302 screened
 1383 excluded
 3919 enrolled
 1 missing BS
 3918 analysed

18-month survey
5331 screened
 1415 excluded
 3916 enrolled
 1 missing BS
 3915 analysed

Hg testing in children aged 2–10 years

6-month survey
1641 eligible
 2 missing Hg
 1639 analysed

12-month survey
1692 eligible
 1 missing Hg
 1691 analysed

18-month survey
1657 eligible
 0 missing Hg
 1657 analysed

6-month survey
1615 eligible
 6 missing Hg
 1609 analysed

12-month survey
1688 eligible
 7 missing Hg
 1681 analysed

18-month survey
1708 eligible
 4 missing Hg
 1704 analysed

Households selected for entomology surveillance

6-month survey
2599 eligible
 517 enrolled

12-month survey
2600 eligible
 520 enrolled

18-month survey
2600 eligible
 520 enrolled

6-month survey
2597 eligible
 520 enrolled

12-month survey
2600 eligible
 520 enrolled

18-month survey
2600 eligible
 520 enrolled

be located were excluded. Blood samples were taken for a thick blood smear from all children enrolled and haemoglobin was measured in children aged 2–4 years because anaemia is predominant in children younger than 5 years in Uganda.[22] Participants who had a temperature of 38·0°C or higher or who reported fever in the past 48 h had a rapid diagnostic test done and were managed as previously reported.[19]

Entomology surveys

In each cluster, ten households were randomly selected for inclusion into the entomology survey from the list of 50 households enrolled into the community surveys (1040 per survey). Households were included if at least one adult (aged ≥18 years) was present, the adult was a usual resident who slept in the sampled household on the night before the survey, and the adult resident agreed to provide written informed consent. The household was excluded if no adult resident was home on more than three occasions. Mosquitoes resting on interior surfaces were collected by entomology technicians using Prokopack aspirators (John W Hock Co, Gainesville, FL, USA). A standardised collection duration of 10 min per house was used, which was sufficient to mechanically aspirate mosquitoes from all resting surfaces in a typical house, while minimising disruptions. Female *Anopheles* mosquitoes were identified phenotypically and stored on silica gel in the field, before being shipped to the Liverpool School of Tropical Medicine (Liverpool, UK) for molecular analysis.

LLIN assessment

12 months after LLINs were distributed, 400 LLINs (100 of each of LLIN type) were withdrawn (and replaced) from selected households enrolled in the community surveys. Net integrity was assessed using WHO guidelines.[23] LLINs were fitted over a frame and visually examined. The number and size (length and width) of observed holes was classified into standardised categories on the basis of hole size. A subset of 138 LLINs (35 PermaNet 3.0, 31 Olyset Plus, 38 PermaNet 2.0, and 34 Olyset Net) were selected at random for chemical analysis of insecticide and synergist content using high-performance liquid chromatography. Five LLINs of each type that were withheld from the national distribution were used as baseline controls.

Procedures

Thick blood smears were dried and transported to the Infectious Diseases Research Collaboration Molecular Research Laboratory in Kampala within 7 days for processing and reading. Slides were stained with 2% Giemsa for 30 min and read by experienced laboratory technologists. Parasite densities were calculated by counting the number of asexual parasites per 200 leucocytes (or per 500 if the count was less than ten parasites per 200 leucocytes), assuming a leucocyte count of 8000 per µL. A thick blood smear was considered negative when the

examination of 100-high power fields did not reveal asexual parasites. For quality control, all slides were read by a second microscopist and a third reviewer settled discrepant readings, defined as positive versus a negative thick blood smear and parasite density differing by 25% or more. Haemoglobin measurements were made using a portable HemoCue analyzer (HemoCue, Anglom, Sweden).

High-performance liquid chromatography analysis was done using standard procedures (appendix p 1). The quantities of pyrethroid and PBO were calculated in grams per kilogram of net material from standard curves established with known concentrations of authenticated standards for PBO, permethrin, and deltamethrin (individual compound PESTANAL-analytical standards, Sigma-Aldrich, Gillingham, UK), and corrected against internal standard dicyclohexyl phthalate readings.

WHO cone bioassays were done using a standard lab strain of *Anopheles gambiae* from Kisumu, western Kenya, which is fully susceptible to both permethrin and deltamethrin.[24] In brief, five unfed mosquitoes aged 3–5 days were exposed for 3 min to a sample of each LLIN. Testing was restricted to fabric from the top of each LLIN to ensure comparability between products. Knockdown was recorded 60 min after exposure and mortality after 24 h. Two cone tests were done per net together with appropriate controls.

Outcomes

The primary outcome was parasite prevalence, defined as the proportion of children aged 2–10 years with asexual parasites detected by microscopy. For comparison of the primary outcome between study groups, a log-binomial regression model was used with generalised estimating equations to allow for within-cluster correlations and adjustment for baseline cluster-level parasite prevalence. The effect of the intervention was expressed as the prevalence ratio (prevalence in the intervention arm divided by prevalence in the control arm).

Secondary outcomes were prevalence of any anaemia (haemoglobin <11 g/dL), moderate or severe anaemia (haemoglobin <10 g/dL), vector density (the number of female *Anopheles* collected per household), measures of LLIN ownership (the proportion of households that owned at least one LLIN), adequate LLIN coverage (the proportion of households that owned at least one LLIN for every two occupants), LLIN use (the proportion of household residents who slept under an LLIN the previous night), LLIN integrity (number and estimated area of holes in the net fabric), and bio-efficacy (proportion of susceptible female *Anopheles* mosquitoes surviving a standard WHO cone test exposure). High-performance liquid chromatography results for withdrawn LLINs and their unused controls were compared using the Wilcoxon rank-sum test.

For secondary outcomes measured as proportions, the same analytical approach was used as for the primary outcome. For comparison of vector density and LLIN

integrity between treatment groups, a negative-binomial regression model was used with generalised estimating equations to allow for within-cluster correlations and adjustment for cluster-level vector density at baseline. The effect of the intervention was expressed as the density ratio (density in the intervention arm divided by density in the control arm). Analyses of anaemia and vector density included adjustment for baseline cluster-level values.

Statistical analysis

The study sample size (number of clusters and allocation of interventions) was determined by the number of LLINs available and the estimated number of LLINs required per cluster. We aimed to sample all eligible children aged 2–10 years from 50 households in the 104 clusters in each round of surveys, estimating that up to 10 400 children would be sampled per survey, assuming an average of two children aged 2–10 years per household. Assuming a parasite prevalence of 40% in the control group,[25] and coefficient of variation between clusters of 0·3 (derived from the Tanzanian trial),[13] we had 80% power (two-sided α of 0·05) to detect a relative reduction in parasite prevalence of at least 17% (prevalence ratio of 0·83).

All data were collected by survey teams using hand-held tablet computers, transferred daily to our core data facilities, and stored on a secure server as previously described.[19] All analyses were done using both an intention-to-treat and as-treated approach. Before doing the final analyses, we decided to present the as-treated analyses as the main study findings, because this approach most accurately reflects the type of LLINs actually received in each cluster, and was deemed appropriate for this unique, large-scale, effectiveness study. For the as-treated analysis, clusters were grouped by type of LLINs received according to household data from the 6-month survey. For clusters with mixed LLIN distribution, the number of nets from the dominant type received (numerator) was divided by the total number of LLINs received in that cluster (denominator); non-study nets were excluded. To be included in the as-treated analyses, the proportion of the dominant net had to be more than 75%; clusters in which the dominant net type received was 75% or less were excluded. Analyses for outcomes measured at multiple timepoints after LLIN distribution were done independently. No allowance was made for multiplicity of testing in the analyses. An individual-level approach to the analysis was used due to the large number of clusters per group. For all analyses, a p value of less than 0·05 was considered significant.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.
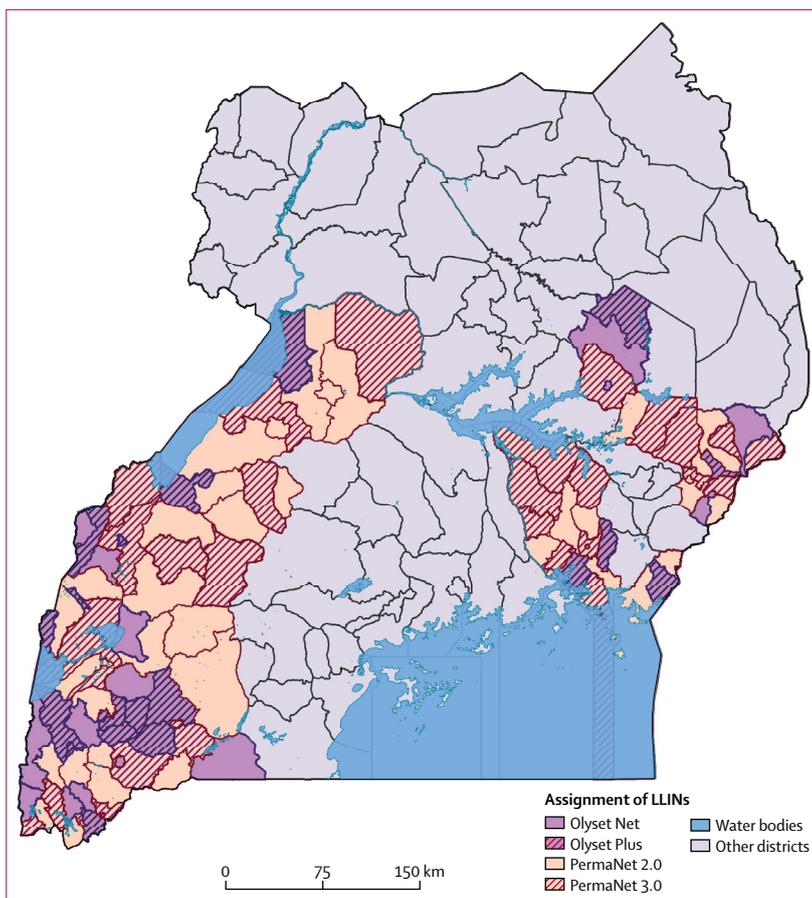
Figure 2: **Map of study area**
The study included 104 clusters, defined as one health sub-district. LLIN=long-lasting insecticidal net.

Results

Of the 104 clusters (figure 1, figure 2), 52 were randomly assigned to each study group (non-PBO LLINs *vs* PBO LLINs) and were included in the intention-to-treat analysis. For the as-treated analysis, three clusters were excluded because no dominant LLIN type was received, and four clusters were reassigned to a different study group (figure 1); thus, 52 non-PBO LLIN clusters (39 PermaNet 2.0 and 13 Olyset Net) and 49 PBO LLIN clusters (31 PermaNet 3.0 and 18 Olyset Plus) were included.

Baseline cross-sectional community and entomology surveys were done from March 16, to June 12, 2017 (table 1), and were published previously.[17,20,21] Characteristics of households (n=5196; median 50 households per cluster, range 48–50) were similar across study groups. Most households were constructed of traditional materials and owned at least one LLIN, but few were adequately covered with LLINs, defined as one LLIN for every two residents (table 1). In children aged 2–10 years tested for parasitaemia (n=8836; 83 per cluster, 56–121), median cluster-level parasite prevalence was 19%, but ranged widely (0–77%), resulting in a coefficient of variation of

	Combined study group		Individual study groups			
	Non-PBO LLIN	PBO LLIN	PermaNet 2.0 Non-PBO LLIN	PermaNet 3.0 PBO LLIN	Olyset Net Non-PBO LLIN	Olyset Plus PBO LLIN
Number of clusters	52	52	37	32	15	20
Eastern region	17	21	13	16	4	5
Western region	35	31	24	16	11	15
Number of households surveyed	2598	2598	1849	1598	749	1000
Household in the lowest tertile of wealth*	33% (17)	34% (20)	30% (16)	35% (20)	39% (20)	33% (21)
Households with modern house construction*	26% (21)	28% (22)	29% (21)	28% (22)	19% (21)	28% (24)
Households with at least one LLIN*	64% (14)	66% (14)	64% (13)	65% (13)	65% (18)	69% (15)
Households with adequate LLINs*	17% (11)	19% (12)	16% (11)	17% (11)	19% (12)	21% (13)
Number of children aged 2–10 years tested for parasitaemia	4328	4506	3134	2845	1194	1661
Parasite prevalence in children aged 2–10 years*	19% (10–42)	19% (4–40)	24% (13–42)	25% (7–42%)	16% (2–38)	8% (3–34)
Number of children aged 2–4 years tested for anaemia	1865	1897	1332	1219	533	678
Anaemia prevalence in children aged 2–4 years*	35% (23–45)	28% (20–44)	36% (25–45)	27% (20–42%)	28% (17–36)	29% (20–48)
Number of households selected for entomological collections	514	514	365	316	149	198
Household vector density†	0·3 (0–2·5)	0·4 (0–6·9)	0·3 (0–2·6)	0·8 (0·2–10·7)	0·3 (0–1·8)	0·1 (0–3·1)

Data are n, mean (SD), or median (IQR). PBO=piperonyl butoxide. LLIN=long-lasting insecticidal nets. *Proportions at the level of each cluster. †Mean values at the level of each cluster.

Table 1: Baseline characteristics

0·86, which was not anticipated (table 1). When the study population was stratified by manufacturer, median parasitaemia was higher in the PermaNet group than in the Olyset group (25% [IQR 11–42] *vs* 10% [IQR 3–35], p=0·033). In children aged 2–4 years tested for anaemia (n=3762; median 36 per cluster, range 11–57), median anaemia prevalence (31%) was similar between the study arms (table 1). In households included in the entomology survey (n=1028; median 10 per cluster, range 8–10), median household vector density (0·4 female anopheles per house) was also similar between the study arms (table 1).

Cross-sectional surveys were done at 6, 12, and 18 months after LLIN distribution, from Sept 11, 2017, to Sept 26, 2019 (figure 2). Trends in LLIN ownership, coverage, and use were similar in both study arms (appendix p 3). Nearly all households reported owning at least one LLIN at 6 months 97% (4872/5046 households). LLIN ownership remained high at months 12 (95%; 4783/5050) and 18 (91%; 4595/5050). By contrast, at month 6, adequate coverage of LLINs (one LLIN for every two residents) had increased markedly from baseline (18% [928/5196] to 71% [3585/5046]), but decreased at month 12 (63%; 3182/5050) and decreased even further at month 18 (51%; 2581/5050), indicating that LLIN attrition after distribution is an issue. In the community surveys done at month 6, 23 745 of 27 817 (85%) household residents reported sleeping under a LLIN the previous night; reported use of LLINs remained high at months 12 (79%) and 18 (73%).

Nets withdrawn from households participating in the community surveys were analysed for net durability.

After 12 months, fabric integrity of all LLIN types had degraded markedly; 287 (73%) of 395 LLINs were found to have at least one hole, with an average of ten holes per net. Over two-thirds of LLINs, 281 (71%) of 395, were in good. Over two-thirds of LLINs, 281 (71%) of 395, were in good condition as per WHO classification (total area of holes per net <80 cm²): 77 (79%) of 98 PermaNet 2.0, 71 (71%) of 100 PermaNet 3.0, 68 (68%) of 100 Olyset Net, and 65 (67%) of 97 Olyset Plus.

High-performance liquid chromatography analysis showed that all unused nets had insecticide and PBO concentrations within the manufacturers' specifications. After 12 months, withdrawn LLINs had less insecticide than unused nets for PermaNet 3.0 (p=0·038), Olyset Net (p=0·00021), and Olyset Plus (p=0·0010). The proportion of used nets found to have less than the manufacturers' declared minimum insecticide concentration for a new net was 45% (17/38 nets) of PermaNet 2.0, 3% (1/35) of PermaNet 3.0, 44% (15/34) of Olyset Net, and 55% (17/31) of Olyset Plus. In PBO LLINs withdrawn at 12 months, PBO concentrations were significantly lower than in unused nets with 80% of PermaNet 3.0 (used mean 15·28 [SD 5·15] *vs* unused mean 26·81 [3·26; p=0·00013]) and 90% of Olyset Plus (used mean 5·04 [1·68] *vs* unused mean 8·17 [0·23; p<0·0001]) having less than the manufacturers minimum target dose of PBO. No data were collected on frequency of LLIN washing, which might contribute to insecticide loss.

In a bio-efficacy study of the withdrawn LLINs using WHO cone assays and a pyrethroid-susceptible strain of *A gambiae*, all LLINs met the WHO criteria for efficacy (>80% mortality 24 h after exposure). The

	Intention-to-treat analysis			As-treated analysis		
	n/N (%)	Prevalence rate (95% CI)	p value	n/N (%)	Prevalence rate (95% CI)	p value
6 months						
Non-PBO LLIN	552/3867 (14%)	Reference	..	556/3844 (15%)	Reference	..
PBO LLIN	418/3798 (11%)	0·78 (0·66–0·92)	0·0038	386/3614 (11%)	0·74 (0·62–0·87)	0·0003
12 months						
Non-PBO LLIN	486/3791 (13%)	Reference	..	493/3802 (13%)	Reference	..
PBO LLIN	427/3918 (11%)	0·80 (0·68–0·95)	0·0086	392/3702 (11%)	0·73 (0·63–0·85)	0·0001
18 months						
Non-PBO LLIN	544/3980 (14%)	Reference	..	558/3976 (14%)	Reference	..
PBO LLIN	474/3915 (12%)	0·94 (0·80–1·10)	0·46	437/3708 (12%)	0·84 (0·72–0·98)	0·029
6 months						
PermaNet 2.0*	440/2713 (16%)	Reference	..	451/2836 (16%)	Reference	..
PermaNet 3.0†	275/2385 (12%)	0·70 (0·57–0·85)	0·0002	271/2334 (12%)	0·67 (0·56–0·81)	<0·0001
12 months						
PermaNet 2.0*	412/2760 (15%)	Reference	..	427/2906 (15%)	Reference	..
PermaNet 3.0†	346/2532 (14%)	0·83 (0·69–0·99)	0·038	343/2456 (14%)	0·76 (0·65–0·90)	0·0013
18 months						
PermaNet 2.0*	410/2825 (15%)	Reference	..	429/2966 (15%)	Reference	..
PermaNet 3.0†	361/2526 (14%)	0·98 (0·82–1·18)	0·85	356/2440 (15%)	0·90 (0·76–1·07)	0·22
6 months						
Olyset Net*	112/1154 (10%)	Reference	..	105/1008 (10%)	Reference	..
Olyset Plus†	143/1413 (10%)	1·08 (0·77–1·51)	0·67	115/1280 (9%)	0·99 (0·69–1·42)	0·96
12 months						
Olyset Net*	74/1031 (7%)	Reference	..	66/896 (7%)	Reference	..
Olyset Plus†	81/1386 (6%)	0·82 (0·56–1·20)	0·30	49/1246 (4%)	0·62 (0·46–0·85)	0·0027
18 months						
Olyset Net*	134/1155 (12%)	Reference	..	129/1010 (13%)	Reference	..
Olyset Plus†	113/1389 (8%)	0·78 (0·58–1·04)	0·087	81/1268 (6%)	0·66 (0·47–0·93)	0·017

PBO=piperonyl butoxide. LLIN=long-lasting insecticidal nets. *Non-PBO LLIN. †PBO LLIN.

Table 2: Efficacy analysis of parasite prevalence

control-corrected mortality estimates were 98% (95% CI 97·3–99·3) with PermaNet 2.0, 100% (99·4–100) with PermaNet 3.0, 94% (91·9–95·4) with Olyset Net, and 98% (96·6–99·2) with Olyset Plus.

In the as-treated analysis, parasite prevalence was lower in the PBO group than in the non-PBO group at months 6, 12, and 18 after LLIN distribution (table 2). Parasite prevalence at month 6 was 11% in the PBO group compared with 15% in the non-PBO arm (prevalence ratio adjusted for baseline values [PR] 0·74, 95% CI 0·62–0·87; p=0·0003; table 3). In the PBO group and non-PBO group, parasite prevalence decreased from baseline (appendix p 3); the changes from baseline were greater for both groups than the differences between the study groups.

In the sub-group analysis stratified by brand, parasite prevalence was lower in the PermaNet 3.0 (with PBO) group than the PermaNet 2.0 (non-PBO) group at months 6 and 12, but not at month 18 (table 2). The greatest effect was observed at the 6-month timepoint (table 2). For Olyset, parasite prevalence was lower in the Olyset Plus (with PBO) group than the Olyset Net (non-PBO) group only at 12 and 18 months (table 2).

In the subgroup analysis stratified by region (appendix p 4), parasite prevalence in eastern Uganda was lower in the PBO group than in the non-PBO group at month 6 (16% [247/1506] vs 22% [317/1427]; PR 0·66, 95% CI 0·53–0·82; p<0·0001) and month 12 (18% [297/1633] vs 21% [311/1455]; 0·69, 0·58–0·82; p<0·0001), but not at month 18. By contrast, in western Uganda, parasite prevalence was lower in the PBO group than in the non-PBO group only at month 18 (4% [87/2161] vs [10% 257/2510]; 0·56, 0·42–0·74; p<0·0001).

In the as-treated analysis, the prevalence of any anaemia (haemoglobin <11 g/dL) at 6 months was lower in the PBO group than in the non-PBO group (table 3). Differences were not statistically significant at 12 and 18 months. In the subgroup analysis stratified by net brand, no statistically significant differences in prevalence of anaemia were seen between the PermaNet groups at any timepoint. However, anaemia prevalence was significantly lower in the Olyset Plus (with PBO) group than the Olyset Net (non-PBO) group at 6 and 18 months (table 3). No differences in the prevalence of moderate or severe anaemia (haemoglobin <10 g/dL)

	Intention-to-treat analysis			As-treated analysis		
	n/N (%)	Prevalence rate (95% CI)	p value	n/N (%)	Prevalence rate (95% CI)	p value
6 months						
Non-PBO LLIN	416/1639 (25%)	Reference	..	433/1647 (26%)	Reference	..
PBO LLIN	307/1609 (19%)	0·76 (0·57–1·01)	0·055	283/1509 (19%)	0·72 (0·54–0·95)	0·019
12 months						
Non-PBO LLIN	435/1691 (26%)	Reference	..	450/1692 (27%)	Reference	..
PBO LLIN	345/1681 (21%)	0·83 (0·60–1·14)	0·25	325/1584 (21%)	0·79 (0·57–1·08)	0·14
18 months						
Non-PBO LLIN	414/1657 (25%)	Reference	..	416/1671 (25%)	Reference	..
PBO LLIN	308/1704 (18%)	0·76 (0·53–1·09)	0·13	292/1607 (18%)	0·75 (0·52–1·07)	0·11
6 months						
PermaNet 2.0*	297/1193 (25%)	Reference	..	327/1259 (26%)	Reference	..
PermaNet 3.0†	234/1072 (22%)	0·94 (0·68–1·29)	0·70	220/1035 (21%)	0·85 (0·62–1·16)	0·31
12 months						
PermaNet 2.0*	335/1223 (27%)	Reference	..	357/1282 (28%)	Reference	..
PermaNet 3.0†	252/1101 (23%)	0·89 (0·60–1·31)	0·56	238/1060 (23%)	0·84 (0·57–1·23)	0·36
18 months						
PermaNet 2.0*	283/1211 (23%)	Reference	..	299/1281 (23%)	Reference	..
PermaNet 3.0†	228/1135 (20%)	0·93 (0·62–1·38)	0·71	216/1092 (20%)	0·86 (0·58–1·29)	0·48
6 months						
Olyset Net*	119/446 (27%)	Reference	..	106/388 (27%)	Reference	..
Olyset Plus†	73/537 (14%)	0·48 (0·28–0·84)	0·0094	63/474 (13%)	0·46 (0·26–0·80)	0·0062
12 months						
Olyset Net*	100/468 (21%)	Reference	..	93/410 (23%)	Reference	..
Olyset Plus†	93/580 (16%)	0·76 (0·44–1·31)	0·32	87/524 (17%)	0·74 (0·42–1·29)	0·29
18 months						
Olyset Net*	131/446 (29%)	Reference	..	117/390 (30%)	Reference	..
Olyset Plus†	80/569 (14%)	0·48 (0·24–1·00)	0·049	76/515 (15%)	0·46 (0·23–0·96)	0·038

PBO=piperonyl butoxide. LLIN=long-lasting insecticidal nets. *Non-PBO LLIN. †PBO LLIN.

Table 3: Efficacy analysis of anaemia (haemoglobin <11 gm/dL) prevalence

were observed between the study groups (appendix p 5).

Vector density of all female Anopheles was lower in the PBO group than in the non-PBO group at all three timepoints (table 4). In the as-treated analysis, 82 mosquitoes were identified at 6 months in 490 household collections in the PBO group compared with 363 mosquitoes in 517 collections in the non-PBO group (table 4). At 12 and 18 months, vector density remained lower in the PBO group, although the number of mosquitoes collected increased in both groups (table 4). In the subgroup analysis, vector density was lower in the PBO group than in the non-PBO group at all timepoints for both PermaNet and Olyset nets (table 4).

Discussion

The results of this innovative, large-scale, cluster-randomised trial suggest that although both PBO and non-PBO LLINs effectively reduced parasite prevalence from baseline, PBO LLINs provided better protection against malaria in the setting of high-level pyrethroid resistance. In the as-treated analysis, PBO LLINs were associated with lower parasite prevalence in children aged 2–10 years than were conventional LLINs, up to 18 months after distribution. These findings are supported by the secondary outcomes, particularly vector density. To ensure community-level benefits of LLINs, WHO recommends that countries aim for universal LLIN coverage by distributing nets free of charge through mass campaigns done every 3 years, supplemented by continuous distribution through different channels.[26] Results from this trial,[21] and evidence from elsewhere,[27,28] raise concerns about the 3-year lifespan of LLINs. Strategies for achieving and maintaining high LLIN coverage, including more frequent mass campaigns, and expanding routine distribution channels, must be considered.

Although insecticide resistance poses a major threat to vector control,[4] the effect of pyrethroid resistance on the effectiveness of LLINs is less clear.[29] We found that PBO LLINs were more effective, but that even non-PBO LLINs were associated with lower parasite prevalence than at baseline at all timepoints. The findings are consistent with prospective cohort studies conducted in Benin,

	Intention-to-treat analysis				As-treated analysis			
	Number of female mosquitoes	Number of collections	Density ratio (95% CI)	p value	Number of mosquitoes	Number of collections	Density ratio (95% CI)	p value
6 months								
Non-PBO LLIN	333	517	Reference	..	363	517	Reference	..
PBO LLIN	113	520	0·27 (0·17–0·41)	<0·0001	82	490	0·14 (0·09–0·22)	<0·0001
12 months								
Non-PBO LLIN	563	520	Reference	..	669	520	Reference	..
PBO LLIN	345	520	0·57 (0·41–0·79)	0·0007	202	490	0·17 (0·12–0·25)	<0·0001
18 months								
Non-PBO LLIN	821	520	Reference	..	729	520	Reference	..
PBO LLIN	536	520	0·25 (0·18–0·34)	<0·0001	523	490	0·25 (0·18–0·35)	<0·0001
6 months								
PermaNet 2.0*	229	368	Reference	..	259	388	Reference	..
PermaNet 3.0†	53	320	0·14 (0·08–0·24)	<0·0001	53	310	0·13 (0·08–0·23)	<0·0001
12 months								
PermaNet 2.0*	418	370	Reference	..	526	390	Reference	..
PermaNet 3.0†	165	320	0·23 (0·16–0·33)	<0·0001	161	310	0·18 (0·12–0·27)	<0·0001
18 months								
PermaNet 2.0*	734	370	Reference	..	643	390	Reference	..
PermaNet 3.0†	488	320	0·21 (0·15–0·30)	<0·0001	488	310	0·25 (0·17–0·36)	<0·0001
6 months								
Olyset Net*	104	149	Reference	..	104	129	Reference	..
Olyset Plus†	60	200	0·65 (0·32–1·30)	0·23	29	180	0·19 (0·10–0·36)	<0·0001
12 months								
Olyset Net*	145	150	Reference	..	143	130	Reference	..
Olyset Plus†	180	200	1·30 (0·78–2·18)	0·32	41	180	0·16 (0·09–0·28)	<0·0001
18 months								
Olyset Net*	87	150	Reference	..	86	130	Reference	..
Olyset Plus†	48	200	0·43 (0·24–0·78)	0·0055	35	180	0·27 (0·15–0·47)	<0·0001

PBO=piperonyl butoxide. LLIN=long-lasting insecticidal nets. *Non-PBO LLIN. †PBO LLIN.

***Table 4:* Efficacy analysis of vector density**

Cameroon, India, Kenya, and Sudan, which found no evidence of an association between pyrethroid resistance (as measured by WHO bioassays) and parasite prevalence or malaria incidence in children.[29] Given these findings, when and where should PBO nets be used? PBO LLINs received a conditional endorsement as a new class of vector control products by WHO in 2017, after review of the data from the Tanzanian trial.[14] Full endorsement is contingent upon the Vector Control Advisory Group reviewing data from a second epidemiological trial; we hope that the data presented here will support a full endorsement. Interim guidelines for the deployment of PBO LLINs were drawn up after experimental hut trials, the Tanzanian cluster-randomised control trial, and a series of modelling studies.[12–14,30] Modelling predicted that the greatest effect of PBO LLINs would be in areas where pyrethroid resistance was deemed to be at an intermediate level—defined as mosquito mortality of 10–80% after exposure to a pyrethroid insecticide in a standard assay and mediated at least in part by cytochrome P450s.[30] In our study area, WHO-assay based estimates of mortality

are at or below these thresholds, consistent with higher-level insecticide resistance.[31,32] Moreover, studies suggest that cytochrome P450-mediated insecticide resistance is near ubiquitous in both *A gambiae* and *Anopheles funestus*.[33,34] These results, showing that PBO LLINs in the setting of high pyrethroid resistance are more effective, suggest that the range of endemicities and levels of resistance for which PBO LLINs are recommended might need to be expanded.

Our study had some limitations. First, our trial was not powered to directly compare the different LLIN brands (PermaNet *vs* Olyset), which prevents us from drawing any conclusions about the superiority of either brand. Our subgroup analyses, stratified by manufacturer and region, suggest some differences in LLIN performance. However, these analyses are limited by a small sample size, an uneven distribution of the LLIN brands, and imbalances in the distribution of LLINs between regions. Second, we used parasite prevalence rather than malaria incidence as the primary outcome measure. Although incidence is considered the gold standard for measuring

malaria burden, we did not have the financial resources to measure incidence in this study. Third, the distribution of LLINs in this study was imperfect. LLINs were distributed over 12 months. Although malaria transmission in Uganda is seasonal, we think that prolonged distribution is unlikely to have resulted in bias, because the trial was randomised. Considering the 101 clusters included in the as-treated analyses, 92% had 85–100% of the dominant LLIN brand, while 8% had 75–84%, possibly due to errors in net distribution, movement of nets between clusters, or reporting errors. Errors in net distribution occurred when the number of allocated LLINs shipped to the districts was insufficient to achieve universal coverage, requiring additional LLINs to be sourced from neighbouring districts to cover shortages, which could have resulted in people receiving different LLIN than the originally allocated type. However, this low-level contamination would have likely biased towards the null. Fourth, we relied on self-report assessment to measure LLIN use. At 18 months, although adequate LLIN coverage decreased markedly in both study groups, LLIN use remained fairly high. It is possible that households that retained their nets may have been more likely to value and use the LLINs. The discrepancy between reports of fewer bed nets but high use might be explained by three or more residents sleeping under the same bed net; we will investigate this further in future analyses. Reporting bias could also be a factor, and residents might report using LLINs because they believe this to be the correct answer. Fifth, we decided before doing the final analyses, as outlined in our statistical analysis plan, to use the results of the as-treated analysis as our primary results. Although we recognise that employing an intention-to-treat approach for the primary analysis is standard practice, because of the exceptional nature of this trial, we opted to report the as-treated results as the main outcome because these results reflect the LLINs that were actually distributed in each cluster. Sixth, we used prokopack aspirators to collect mosquitoes, not the more commonly used Centers for Disease Control light traps or human landing catches. Although this method could be considered a limitation, we view this as a strength because using aspirators allowed us to sample mosquitoes in all clusters, increasing the granularity of our evaluation and mirroring the epidemiological outcomes. Collecting mosquitoes using prokopack aspirators proved to be a pragmatic, cost-effective, scalable, and sensitive approach and should be considered for future intervention assessments. Finally, we did not assess the cost-effectiveness of PBO LLINs, which is an important question for policymakers.

In this pragmatic, cluster-randomised trial, embedded within a national LLIN distribution campaign, we found that PBO LLINs were more effective than conventional LLINs in Uganda, where resistance to pyrethroid insecticides is high. This study makes an important contribution to the evidence base on the use of PBO LLINs. Our results highlight that conventional LLINs provide protection and can still have a role in vector control programmes in settings where insecticide resistance and malaria transmission are low. Future studies should investigate the cost-effectiveness of PBO LLINs, the effectiveness of new generation LLINs, including those with two active components, and approaches for integrating LLINs with indoor residual spraying and other new malaria control tools, as these become available.

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