LLIN Evaluation in Uganda Project (LLINEUP) – Effect of long-lasting insecticidal nets (LLINs) with, and without, piperonyl butoxide on malaria indicators in Uganda: a pragmatic cluster-randomised trial embedded in a national LLIN distribution campaign

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Abstract

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 to LLINs containing piperonyl butoxide (PBO), a synergist that can partially restore pyrethroid susceptibility in mosquito vectors.

Methods. Overall, 104 clusters (health sub-districts) were included, covering 40% of Uganda. Proportionate randomisation was used to assign clusters to one of four arms, including LLINs with PBO (32 PermaNet 3·0, 20 Olyset Plus), and conventional LLINs (37 PermaNet 2·0, 15 Olyset Net). At baseline, 6, 12, and 18 months after LLIN distribution, cross-sectional surveys were conducted in 50 randomly selected households per cluster (5,200 per survey); a sub-set of 10 households per cluster (1,040 per survey) were randomly selected for entomology surveys. The primary outcome was parasite prevalence by microscopy in children aged 2-10 years.

Findings. LLINs were delivered from March 2017 to March 2018. 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 (31 PermaNet 3·0, 18 Olyset Plus) and 52 non-PBO LLIN clusters (39 PermaNet 2·0, 13 Olyset Net). At six months, parasite prevalence was 11% in the PBO arm vs 15% in the non-PBO arm (prevalence ratio [PR] adjusted for baseline values 0·74 [95% CI: 0·62–0·87], p<0·001). Results were similar at 12 months (11% vs 13%, PR 0·73 [95% CI: 0·63–0·85], p<0·001) and at 18 months (12% vs 14%, PR 0·84 [95% CI: 0·72–0·98], p=0·03).

Interpretation. In Uganda, where pyrethroid resistance is high, PBO LLINs reduced parasite prevalence more effectively than conventional LLINs for up to 18 months.

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Trial registration

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Keywords

malaria, long-lasting insecticidal nets (LLINs), piperonyl butoxide, cluster-randomised trial, Uganda

Research in context

Evidence before this study

We searched titles and abstracts in 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. We found one trial that evaluated the impact of long-lasting insecticidal nets (LLINs) with piperonyl butoxide (PBO) on epidemiological outcomes in Tanzania. This cluster-randomised, controlled trial compared Olyset® Plus (LLINs with PBO) to Olyset® Net (LLINs without PBO) in a single district of Tanzania, where pyrethroid resistance is high. At 9, 16, and 21 months 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, the World Health Organization (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 epidemiologic and entomologic outcomes. Fifteen 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 epidemiologic 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 limited evidence base on 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 may still have a role in settings where insecticide resistance and malaria transmission are lower. 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.

Introduction

Long-lasting insecticidal nets (LLINs) are the foundation of malaria control in sub-Saharan Africa.[1,2] Over the past twenty years, substantial efforts have been made to expand LLIN coverage in malaria endemic countries. From 2000-2015, the incidence of *Plasmodium falciparum* fell 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] Recent reports suggest that malaria control has stalled in Africa, particularly in high burden areas.[5] The World Health Organization (WHO) has called for aggressive action to preserve malaria control gains and to ensure that the ambitious 2030 targets, including eliminating malaria from at least 35 countries, are met.[6] To achieve this, effectiveness of LLINs must be maintained.

Currently, all LLINs are impregnated with pyrethroid insecticides, due to 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; 'knock down resistance' (*kdr*) caused by mutations in the voltage-gated sodium channel where pyrethroids bind, and metabolic resistance resulting from alterations in 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, as compared to non-PBO LLINs.[12] A cluster-randomised, clinical trial of the effectiveness of a PBO LLINs (Olyset Plus), conducted in Tanzania, found that PBO LLINs were associated with lower parasite prevalence, than conventional LLINs, at 9, 16, and 21 months after distribution.[13] Subsequently, the 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 may have variable specificity;[15] insecticide resistance was assessed by *kdr* mutations, 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 (IRS), 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] Recently, malaria cases in Uganda have increased, underscoring the need to intensify malaria control efforts.[5] The Ministry of Health has committed to distributing free LLINs in Uganda through mass campaigns every 3-4 years. In 2017-18, two brands of LLINs (PermaNet and Olyset) including LLINs with, and without, PBO 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, allowing us to rigorously evaluate the impact of the LLINs at an unprecedented scale.

Methods

The trial protocol has been published previously.[18]

Study design

Our primary objective was to evaluate the impact of combination LLINs (with PBO), as compared to conventional LLINs (without PBO), on parasite prevalence in eastern and western Uganda. For this trial, clusters were defined as one health sub-district (serving approximately 100,000 people); 104 (47%) of 221 health sub-districts nationwide were included, covering 48 districts (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). Following sensitisation of authorities and local communities, baseline cross-sectional community and entomology surveys were conducted.[17,19,20] 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.

Study setting

Health sub-districts in eastern and western Uganda, which were not scheduled to receive IRS with pirimiphos-methyl (Actellic),[14] were included in the trial. Districts in the north and east where IRS was ongoing or planned, were excluded. 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).[20] Overall, parasite prevalence in children aged 2-10 years was 26%, ranging from 8% in the South West region to 53% in East Central.[17] Very high levels of pyrethroid resistance due to target-site (primarily *Vgsc*-L1014S) and metabolic mechanisms (markers *Cyp4j5*-L43F and *Coeae1d*) were observed.[19]

Assignment of interventions

Initial randomisation

LLINs were procured in advance of the randomisation. We utilised the entire production capacity for the PBO LLINs due to the scale of the trial; thus, the total number of the four LLIN types varied. Proportionate randomisation was carried out by a co-investigator based outside of Uganda using STATA Version 14.2 (StataCorp, Texas, USA), as described previously.[18] 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 based on the targeted number of each individual type of net / 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 arm were observed at baseline. LLIN allocation was not blinded.

LLIN distribution

The following LLINs were distributed: PermaNet® 3·0 and PermaNet® 2·0 (Vestergaard Frandsen SA, Denmark), and Olyset® Plus and Olyset® Net (Sumitomo Chemical, Japan). The Ministry of Health, local government, and partners distributed the assigned LLINs from March 2017 to March 2018, as part of the national LLIN distribution campaign, according to the net allocation list generated from the randomisation. Using household registration data collected prior to 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).

Revised randomisation and net allocation

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 re-randomised, using the same process of proportionate randomisation, resulting in 52 clusters being assigned to each of the two main study arms (PBO vs non-PBO).[18]

Community surveys

Cross-sectional surveys were conducted at 6-, 12-, and 18-months following 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.[18] 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, 5,200 per survey). Households were included if: (1) at least one resident was 2-10 years of age, (2) at least one adult ($\geq$ 18 years) was present, (3) the adult was usually resident and slept in the sampled household on the night before the survey, and (4) the adult agreed to provide written, informed-consent to participate in the survey. Households were excluded if: (1) the dwelling was destroyed or could not be found, (2) the house was vacant, (3) 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.[18,20] Children residing in the household had blood drawn by finger-prick, if they met the following selection criteria: (1) aged 2-10 years, (2) usually resident and slept in the sampled household on the night before the survey, (3) provision of written informed consent by parent/guardian, (4) provision of assent by child $\geq$ 8 years of age. Children who could not be located were excluded. Blood samples were taken for a thick blood smear from all children enrolled; haemoglobin was measured in children aged 2-4 years, as anaemia is predominant in children under-five in Uganda.[21] Participants who had a temperature of $\geq$ 38.0^{o}C, or who reported fever in the past 48 hours, had a rapid diagnostic test performed and were managed as previously reported.[18]

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 (1,040 per survey). Households were included if: (1) at least one adult (aged $\geq$ 18 years) was present; (2) the adult was a usual

resident who slept in the sampled household on the night before the survey; (3) 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., USA). A standardised collection duration of ten minutes 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 (LSTM) for molecular analysis.

LLIN assessment

Twelve 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.[22] LLINs were fitted over a frame and visually examined. The number and size (length and width) of observed holes was classified into standardised categories based on hole size. A sub-set of 138 LLINs (35 PermaNet 3·0, 31 Olyset Plus, 38 PermaNet 2·0, 34 Olyset Net) were selected at random for chemical analysis of insecticide and synergist content using high-performance liquid chromatography (HPLC). LLINs that were withheld from the national distribution were used as baseline controls (five for each net type).

Laboratory procedures

Microscopy and haemoglobin testing

Thick blood smears were dried and transported to the Infectious Diseases Research Collaboration Molecular Research Laboratory in Kampala within seven days for processing and reading. Slides were stained with 2% Giemsa for 30 minutes and read by experienced laboratory technologists. Parasite densities were calculated by counting the number of asexual parasites, per 200 leukocytes (or per 500, if the count was less than 10 parasites per 200 leukocytes), assuming a leukocyte count of

8,000/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 (1) positive versus a negative thick blood smear, (2) parasite density differing by $\geq$ 25%. Haemoglobin measurements were made using a portable HemoCue analyzer (HemoCue, Anglom, Sweden).

High-performance liquid chromatography

HPLC analysis was performed using standard procedures (supplemental file 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 (PESTANAL®, analytical standard, Sigma-Aldrich, UK) and corrected against internal standard dicyclohexyl phthalate readings.

Bioassays

Standard WHO cone bioassays were conducted using a standard lab strain of *A. gambiae* from Kisumu, western Kenya, which is fully susceptible to both permethrin and deltamethrin.[23] In brief, five unfed 3- to 5-day-old mosquitoes were exposed for three minutes 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 minutes post-exposure and mortality after 24 hours. Two cone tests were conducted per net together with appropriate controls.

Sample size calculations

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 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 arm,[24] and coefficient of variation between clusters of 0.3 (derived from the Tanzanian trial),[13] we had 80% power (two-sided significance level of 0·05) to detect a relative reduction in parasite prevalence of at least 17% (prevalence ratio of 0·83).

Analytical issues

All data were collected by survey teams using hand-held tablet computers, were transferred daily to our core data facilities, and were stored on a secure server, as previously described.[18] All analyses were conducted using both an intention-to-treat and 'as treated' approach. Before conducting 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 study arm according to the 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 the 4 study LLIN types 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 > 75%; clusters in which the dominant net type received was $\leq$ 75% were excluded. Analyses for outcomes measured at multiple time points following LLIN distribution were conducted 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 arm. For all analyses, a p-value < 0.05 was considered statistically significant.

The primary outcome was parasite prevalence, defined as the proportion of children 2-10 years of age with asexual parasites detected by microscopy. For comparison of the primary outcome between study arms, a log-binomial regression model was used with generalized 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/prevalence in the control arm).

Secondary outcomes included prevalence of any anaemia (haemoglobin < 11 g/dL), moderate/severe anaemia (haemoglobin < 10 g/dL), vector density (the number of female Anopheles collected per household), and 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). HPLC 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 arms, a negative binomial regression model was used with generalized estimating equations to allow for within-cluster correlations and adjustment for baseline cluster-level vector density. The effect of the intervention was expressed as the density ratio (density in the intervention arm/density in the control arm). Analyses of anaemia and vector density included adjustment for baseline cluster-level values.

Research ethics approval

The trial was approved by the Ugandan National Council for Science and Technology (UNCST Ref HS 2176), Makerere University School of Medicine Research & Ethics Committee (SOMREC 2016-133),

London School of Hygiene & Tropical Medicine Ethics Committee (LSHTM Ref 12019), and the Liverpool School of Tropical Medicine (LSTM Ref 16-072), which sponsored the study.

Role of the funder

The content of the manuscript is solely the responsibility of the authors. The funders played no role in data collection, analysis, interpretation, writing of the manuscript or the decision to submit.

Results

LLIN delivery

Of the 104 clusters (figure 2), 52 were randomised to each study arm (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 were reassigned to a different study arm (figure 2); thus, 52 non-PBO LLIN clusters (39 PermaNet 2·0, 13 Olyset Net) and 49 PBO LLIN clusters (31 PermaNet 3·0, 18 Olyset Plus) were included.

Baseline survey

Baseline cross-sectional community and entomology surveys were carried out from March to June 2017 (table 1), and were published previously.[17,19,20] Characteristics of households (n=5,196, median 50 per cluster, range 48–50) were similar across study arms. Most households were constructed of traditional materials (73%) and owned at least one LLIN (65%), but few were adequately covered with LLINs, defined as one LLIN for every two residents (18%). In children aged 2-10 years tested for parasitaemia (n=8,836, median 83 per cluster, range 56–121), median cluster-level parasite prevalence (19%) was similar between the study arms, but ranged widely (0%–77%), resulting in a coefficient of variation of 0.86, which was not anticipated. When the study population was stratified by manufacturer, median parasitaemia was higher in the PermaNet arms than in the Olyset arms

(25% vs. 10%, p=0·03). In children aged 2-4 years tested for anaemia (n=3,762, median 36 per cluster, range 11—57), median anaemia prevalence (31%) was similar between the study arms. In households included in the entomology survey (n=1,028, median 10 per cluster, range 8—10), median household vector density (0·4 female anopheles/house) was also similar between the study arms.

LLIN ownership, coverage and use

Cross-sectional surveys were carried out at 6, 12, and 18 months after LLIN distribution, from September 2017 to September 2019 (figure 2). Trends in LLIN ownership, coverage and use were similar in both study arms (supplemental file 3). Nearly all households reported owning at least one LLIN at 6 months (97%). LLIN ownership remained high at 12 months (95%), and at 18 months (91%). In contrast, at 6 months, adequate coverage of LLINs (one LLIN for every two residents) had increased markedly from baseline (18% to 71%), but decreased at 12 months (63%) and fell even further at 18 months (51%), indicating that LLIN attrition post-distribution is an issue. In the 6-month community surveys, most household residents reported sleeping under a LLIN the previous night (85·4%); reported use of LLINs remained high at 12 months (78·6%) and at 18 months (73·1%).

LLIN assessment

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; 73% of LLINs were found to have at least one hole, with an average of ten holes per net. Over two-thirds of LLINs were in good condition as per WHO classification (total area of holes/net <80cm^2): PermaNet 2·0: 79%, PermaNet 3·0: 71%, Olyset Net: 68%, Olyset Plus: 67%.

HPLC analysis revealed 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=0·04), Olyset Net (p<0·001), and Olyset Plus (p=0·001). The proportion of used nets found to have less than the manufacturers' declared minimum insecticide concentration for a new net was as follows: PermaNet 2·0: 45%; PermaNet 3·0: 3%; Olyset Net: 44%; Olyset Plus: 55%. In PBO LLINs withdrawn at 12 months, PBO concentrations were significantly lower than in unused nets with 80·0% of PermaNet 3·0 (p<0·001) and 90% of Olyset Plus (p<0·001) 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 *Anopheles gambiae* s.s., all LLINs met the WHO criteria for efficacy (>80% mortality 24 hours post-exposure). The control-corrected mortality estimates for each LLIN type included: PermaNet 2·0: 98% (95% CI: 97·3–99·3%); PermaNet 3·0: 100% (95% CI: 99·4–100%); Olyset Net: 94% (95% CI: 91·9–95·4%) and Olyset Plus: 98% (95% CI: 96·6–99·2%).

Impact on primary outcome: parasite prevalence

In the 'as treated' analysis, parasite prevalence was lower in the PBO arm than the non-PBO arm at 6, 12, and 18 months after LLIN distribution (table 2). Parasite prevalence at 6 months was 11% in the PBO arm vs 15% in the non-PBO arm (prevalence ratio adjusted for baseline values [PR] 0·74 [95% CI: 0·62–0·87], p<0·001). Similar results were observed at the 12- and 18-month timepoints. In both the PBO and non-PBO arms, parasite prevalence decreased from baseline (supplemental file 4); the changes from baseline were greater for both arms than the differences between the study arms.

In the sub-group analysis stratified by brand, parasite prevalence was lower in the PermaNet 3·0 (with PBO) arm than the PermaNet 2·0 (non-PBO) arm at 6 and 12 months, but not at 18 months (table 2). The greatest impact was observed at the 6-month time-point (12%vs 16%, PR 0·67 [95% CI: 0·56–0·81], p<0·001). For Olyset, parasite prevalence was lower in the Olyset Plus (with PBO) arm than the Olyset Net (non-PBO) arm only at 12 months (4% vs 7%, PR 0·62 [95% CI: 0·46–0·85], p=0·003) and 18 months (6% vs 13%, PR 0·66 [95% CI: 0·47–0·93], p=0·02).

In the sub-group analysis stratified by region (supplemental file 2), in the East, parasite prevalence was lower in the PBO arm than the non-PBO arm at 6 months (16% vs 22%, PR 0·66 [95% CI: 0·53–0·82], p<0·001) and 12 months (18% vs 21%, PR 0·69 [95% CI: 0·58–0·82], p<0·001), but not at 18 months. In contrast, in the West, parasite prevalence was lower in the PBO arm than the non-PBO arm only at 18 months (4% vs 10%, PR 0·56 [95% CI: 0·42–0·74), p<0·001.

Impact on secondary outcomes: anaemia

In the 'as treated' analysis, the prevalence of any anaemia (haemoglobin < 11 g/dL) at 6 months was lower in the PBO arm than the non-PBO arm (19% vs 26%, PR 0·72 [95% CI: 0·54─0·95], p=0·02). Although a similar pattern was observed at 12 and 18 months, differences were not statistically significant (table 4). In the sub-group analysis stratified by brand, no statistically significant differences in prevalence of anaemia were seen between the PermaNet arms at any timepoint. However, anaemia prevalence was significantly lower in the Olyset Plus (with PBO) arm than the Olyset Net (non-PBO) arm at 6 and 18 months. No differences in the prevalence of moderate/severe anaemia (haemoglobin < 10 g/dL) were observed between the study arms (supplemental file 5).

Impact on secondary outcomes: vector density

Vector density of all female anopheles was lower in the PBO arm than the non-PBO arm at all three timepoints (table 5). In the 'as treated' analysis, 82 mosquitoes were identified at 6 months in 490 household collections in the PBO arm, compared to 363 mosquitoes in 517 collections in the non-PBO arm (density ratio [DR] 0·14 [95% CI: 0·09–0·22], p<0·001). At 12 and 18 months, vector density remained lower in the PBO arm, although the number of mosquitoes collected increased in both arms. In the sub-group analysis, vector density was lower in the PBO than the non-PBO arm at all timepoints for both PermaNet and Olyset nets.

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 superior 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, compared to 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, the WHO recommends that countries aim for universal LLIN coverage by distributing nets free-of-charge through mass campaigns conducted every three years, supplemented by continuous distribution through different channels.[25] Results from this trial,[20] and evidence from elsewhere,[26,27] raise concerns about the three-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 impact of pyrethroid resistance on the effectiveness of LLINs is less clear.[28] 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, 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.[28] Given this, 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, following 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 following experimental hut trials, the Tanzanian cluster randomised control trial, and a series of modelling studies.[12-14,29] Modelling predicted that the greatest impact 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.[29] In our study area, WHO-assay based estimates of mortality are at or below these thresholds, consistent with higher-level insecticide resistance.[30,31] Moreover, studies suggest that cytochrome P450-mediated insecticide resistance is near ubiquitous in both *An. gambiae* and *An. funestus*.[32,33] These results, demonstrating superiority of PBO LLINs in the setting of high-level pyrethroid resistance, suggest that the range of endemicities and levels of resistance levels for which PBO LLINs are recommended may need to be expanded.

Despite the many strengths of this study, it also had some limitations. First, the 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 sub-group analyses, stratified by manufacturer and region, suggest some differences in LLIN performance. However, these analyses are limited by small sample size, 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 lacked 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, 93 (92%) had 85-100% of the dominant LLIN brand, while 8 (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 may have been different than the originally allocated LLIN type. However, this low-level contamination would have likely biased towards the null. Fourth, we relied on self-report to measure LLIN use. At 18 months, although adequate LLIN coverage fell markedly in both study arms, 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, or that three or more residents slept under the same LLIN, but reporting bias may also be a factor; residents may report using LLINs because they believe this to be the correct answer. Fifth, we decided before conducting 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, given the exceptional nature of this trial (large-scale, effectiveness trial of a national LLIN distribution campaign), we opted to report the 'as treated' results as the main outcome as 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 may be considered a limitation, we view this as a strength, as 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 policy makers.

Conclusions

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 limited evidence-base on the use of PBO LLINs. Our results highlight that conventional LLINs provide protection and may still have a role in vector control programmes in settings where insecticide resistance and malaria transmission are lower. Future studies should investigate the cost-effectiveness of PBO LLINs, the effectiveness of new generation LLINs including those with two active ingredients, and approaches for integrating LLINs with IRS and other new malaria control tools, as these become available.

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AUTHORS' CONTRIBUTIONS

SGS, GD, MRK and MJD conceived of the study with input from JO and JH. SGS, GD, and MJD developed the procedures and drafted the protocol with MRK and JH. CMS, SG, and AL developed the standard operating procedures. SG, AK, MK and AL led the data collection in the field, with oversight from SGS, CMS, JO, MRK and MJD. PM and SPK managed the data, with support from SGS, SG, AL and GD. GD led the data analysis, with support from SGS, MJD, MRK and JH. All authors reviewed the manuscript and gave permission for publication. GD and SGS, the corresponding author, had full access to all the data in the study and SS had final responsibility for the decision to submit for publication.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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DATA SHARING STATEMENT

De-identified participant data and a data dictionary defining each field in the set will be made publicly available at the time of publication on the ClinEpiDB website (URL). The study protocol has been published (https://trialsjournal.biomedcentral.com/articles/10.1186/s13063-019-3382-8).

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620 **FIGURE LEGEND**

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622 **Figure 1: Map of study area.** The study included 104 clusters, defined as one health sub-district.

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624 **Figure 2: Trial profile.** LLIN=long-lasting insecticidal net. PBO=piperonyl butoxide. Hb=haemoglobin.

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Table 1. Baseline characteristics

Characteristic	Combined study arms		Individual study arms			
	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, mean (SD) [a]	33% (17%)	34% (20%)	30% (16%)	35% (20%)	39% (20%)	33% (21%)
Households with modern house construction, mean (SD) [a]	26% (21%)	28% (22%)	29% (21%)	28% (22%)	19% (21%)	28% (24%)
Households with at least 1 LLIN, mean (SD) [a]	64% (14%)	66% (14%)	64% (13%)	65% (13%)	65% (18%)	69% (15%)
Households with adequate LLINs, mean (SD) [a]	17% (11%)	19% (12%)	16% (11%)	17% (11%)	19% (12%)	21% (13%)
Number of children 2-10 years tested for parasitaemia	4328	4506	3134	2845	1194	1661
Parasite prevalence children 2-10 years, median (IQR) [a]	19% (10-42%)	19% (4-40%)	24% (13-42%)	25% (7-42%)	16% (2-38%)	8% (3-34%)
Number of children 2-4 years tested for anaemia	1865	1897	1332	1219	533	678
Anaemia prevalence children 2-4 years, median (IQR) [a]	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, median (IQR) [b]	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)

[a] Proportions at the level of each cluster

[b] Mean values at the level of each cluster

Table 2. Efficacy analysis of parasite prevalence

Duration following the intervention	Study arm	Intention-to-treat analysis			As treated analysis		
		n/N (%)	PR (95% CI)	p-value	n/N (%)	PR (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)	0.004	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)	0.009	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)	0.03
6 months	PermaNet 2·0 [a]	440/2713 (16%)	reference	-	451/2836 (16%)	reference	-
	PermaNet 3·0 [b]	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 [a]	412/2760 (15%)	reference	-	427/2906 (15%)	reference	-
	PermaNet 3·0 [b]	346/2532 (14%)	0·83 (0·69-0·99)	0·04	343/2456 (14%)	0·76 (0·65-0·90)	0·001
18 months	PermaNet 2·0 [a]	410/2825 (15%)	reference	-	429/2966 (15%)	reference	-
	PermaNet 3·0 [b]	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 [a]	112/1154 (10%)	reference	-	105/1008 (10%)	reference	-
	Olyset Plus [b]	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 [a]	74/1031 (7%)	reference	-	66/896 (7%)	reference	-
	Olyset Plus [b]	81/1386 (6%)	0·82 (0·56-1·20)	0·30	49/1246 (4%)	0·62 (0·46-0·85)	0·003
18 months	Olyset Net [a]	134/1155 (12%)	reference	-	129/1010 (13%)	reference	-
	Olyset Plus [b]	113/1389 (8%)	0·78 (0·58-1·04)	0·09	81/1268 (6%)	0·66 (0·47-0·93)	0·02

[a] *Non-PBO LLIN*

[b] *PBO LLIN*

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

Duration following the intervention	Study arm	Intention-to-treat analysis			As treated analysis		
		n/N (%)	PR (95% CI)	p-value	n/N (%)	PR (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)	0·06	283/1509 (19%)	0·72 (0·54-0·95)	0·02
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 [a]	297/1193 (25%)	reference	-	327/1259 (26%)	reference	-
	PermaNet 3·0 [b]	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 [a]	335/1223 (27%)	reference	-	357/1282 (28%)	reference	-
	PermaNet 3·0 [b]	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 [a]	283/1211 (23%)	reference	-	299/1281 (23%)	reference	-
	PermaNet 3·0 [b]	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 [a]	119/446 (27%)	reference	-	106/388 (27%)	reference	-
	Olyset Plus [b]	73/537 (14%)	0·48 (0·28-0·84)	0·009	63/474 (13%)	0·46 (0·26-0·80)	0·006
12 months	Olyset Net [a]	100/468 (21%)	reference	-	93/410 (23%)	reference	-
	Olyset Plus [b]	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 [a]	131/446 (29%)	reference	-	117/390 (30%)	reference	-
	Olyset Plus [b]	80/569 (14%)	0·48 (0·24-1·00)	0·05	76/515 (15%)	0·46 (0·23-0·96)	0·04

[a] *Non-PBO LLIN*

[b] *PBO LLIN*

Table 4. Efficacy analysis of vector density

Duration following the intervention	Study arm	Intention-to-treat analysis				As treated analysis			
		# of female mosquitoes	# of collections	DR (95% CI)	p-value	# of mosquitoes	# of collections	DR (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 [a]	229	368	reference	-	259	388	reference	-
	PermaNet 3·0 [b]	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 [a]	418	370	reference	-	526	390	reference	-
	PermaNet 3·0 [b]	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 [a]	734	370	reference	-	643	390	reference	-
	PermaNet 3·0 [b]	488	320	0·21 (0·15-0·30)	<0.0001	488	310	0·25 (0·17-0·36)	<0.0001
6 months	Olyset Net [a]	104	149	reference	-	104	129	reference	-
	Olyset Plus [b]	60	200	0·65 (0·32-1·30)	0·23	29	180	0·19 (0·10-0·36)	<0.0001
12 months	Olyset Net [a]	145	150	reference	-	143	130	reference	-
	Olyset Plus [b]	180	200	1·30 (0·78-2·18)	0·32	41	180	0·16 (0·09-0·28)	<0.0001
18 months	Olyset Net [a]	87	150	reference	-	86	130	reference	-
	Olyset Plus [b]	48	200	0·43 (0·24-0·78)	0·006	35	180	0·27 (0·15-0·47)	<0.0001

[a] *Non-PBO LLIN*

[b] *PBO LLIN*

Water Bodies

Other Districts

Assignment

Olyset Net

Olyset Plus

PermaNet 2.0

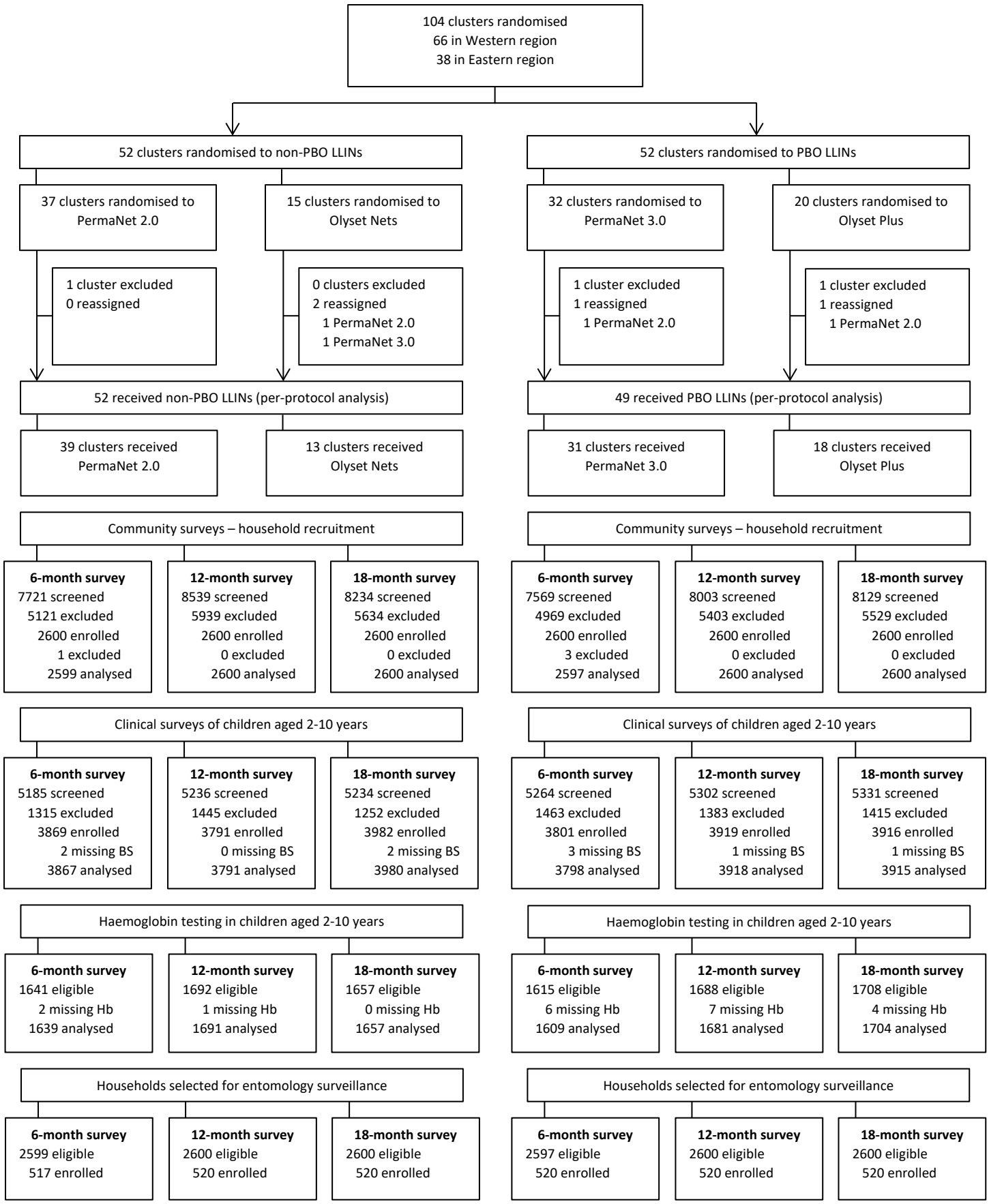
PermaNet 3.0

N

W E

S

0 75 150 Km


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 clusters randomised to PermaNet 2.0
15 clusters randomised to Olyset Nets
32 clusters randomised to PermaNet 3.0
20 clusters randomised to Olyset Plus
1 cluster excluded
0 reassigned
0 clusters excluded
2 reassigned
1 PermaNet 2.0
1 PermaNet 3.0
1 cluster excluded
1 reassigned
1 PermaNet 2.0
1 cluster excluded
1 reassigned
1 PermaNet 2.0
52 received non-PBO LLINs (per-protocol analysis)
49 received PBO LLINs (per-protocol analysis)
39 clusters received PermaNet 2.0
13 clusters received Olyset Nets
31 clusters received PermaNet 3.0
18 clusters received Olyset Plus
Community surveys – household recruitment
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
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
Haemoglobin testing in children aged 2-10 years
Haemoglobin testing in children aged 2-10 years
6-month survey
1641 eligible
2 missing Hb
1639 analysed
12-month survey
1692 eligible
1 missing Hb
1691 analysed
18-month survey
1657 eligible
0 missing Hb
1657 analysed
6-month survey
1615 eligible
6 missing Hb
1609 analysed
12-month survey
1688 eligible
7 missing Hb
1681 analysed
18-month survey
1708 eligible
4 missing Hb
1704 analysed
Households selected for entomology surveillance
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