Statistical Analysis Plan

TRIAL FULL TITLE	**Characterizing the humoral immune response against salivary antigens of Southeast Asian mosquito vectors of malaria and dengue with a human challenge model**
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SAP VERSION	1.2
SAP VERSION DATE	23-MAR-2023

SAP Revision History

Version	Key Change	Reason(s) for change	Date	Page
1.1	No change	Version prior to sample collection generation	14-JUN-2020	
1.2	Antibody levels were $\log_2$ transformed to represent a two-fold change in antibody levels	To account for positively skewed antibody data and aid interpretation,	23-MAR-2023	16
1.2	The overall effect of time will be estimated (independent of any exposure and thus representing the baseline period), with the exposure and post-exposure periods fitted as a categorical time-varying variable	Due to differences (in terms of time) between individuals transitioning from baseline, to exposure and post-exposure periods, linear splines representing these periods of fixed time were unable to be estimated.	23-MAR-2023	16
1.2	The change in antibody levels over time, and in response to *Anopheles* biting exposure, will be analysed using generalised estimating equations (GEEs).	High-levels of serial correlation in antibody levels within individuals over time	23-MAR-2023	16
1.2	Additional models will be fitted to determine the effect of concordant and discordant exposure species and antibody responses.	Concordance of the exposure species is likely to have biological relevance to the antibody outcome.	23-MAR-2023	17
1.2	Biostatisticians were un-blinded to participant exposure species and dose	In order to perform and interpret concordant exposure species and antibody analyses.	23-MAR-2023	11

SAP Signatures

I give my approval for the attached SAP entitled SG6-SHC dated 23-MAR-2023:

On behalf of the Principal Investigators

Name	Signature	Date/Time
Prof François Nosten (Principal investigator)		07-MAR-2024/10:00

SAP Authors

Name	Signature	Date/Time
Dr Ellen Kearney (Trial statistician)		23-MAR-2023/10:00
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A/Prof Paul Agius (Senior statistician)		23-03-2023/14:00

Abbreviations and Definitions

ABBREVIATION	DEFINITION
Ab	Antibody
aegNterm34kDa	*Aedes aegpyti* Nterm34kDa
albNterm34kDa	*Aedes albopictus* Nterm34kDa
CI	Confidence Interval
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
dirSG6-P1	*Anopheles dirus* Salivary Gland 6 Peptide 1
ELISA	Enzyme-Linked Immunosorbent Assay
gSG6-P1	*Anopheles gambiae* Salivary Gland 6 Peptide 1
Ig	Immunoglobulin
macSG6-P1	*Anopheles maculatus* Salivary Gland 6 Peptide 1
minSG6-P1	*Anopheles minimus* Salivary Gland 6 Peptide 1
OD	Optical Density
SAP	Statistical Analysis Plan
SMRU	Shoklo Malaria Research Unit

Contents

1 Introduction

1.1 Preface

Mosquito-borne diseases cause significant burden in populations exposed to bites of vector species across Southeast Asia (1). Malaria and dengue are endemic and pose the greatest challenges. Malaria is transmitted in rural areas and multi-drug resistant falciparum malaria has been identified as a major threat to public health in these areas (2). Consequently, considerable investment has been made to eliminate *Plasmodium falciparum* in the Greater Mekong Subregion, the epicenter of antimalarial drug resistance (3). In this area, the main vectors are *Anopheles dirus*, *An. maculatus* and *An. minimus*; several other species also contribute to the transmission (4). The efficacy of conventional vector-control measures is low (5-7) because of the ecology and biology of relevant vector species (8-10), and is particularly difficult to evaluate due to the complex transmission dynamics and low rates of disease incidence (8). Dengue viruses are transmitted by aedine mosquitoes; the main vectors are *Aedes aegypti* and *Ae. albopictus* (11). Infection can cause death and overall disease burden has drastically increased over the past decades (12). As there is no specific treatment for dengue, prevention of infection is critical to reduce morbidity and mortality. Integrated management strategies and active involvement of homeowners is necessary to control *Aedes* mosquitoes in urban and semi-urban environments. In rural areas where vector breeding sites also include a variety of natural water bodies (rock pools, tree holes and bamboo stumps), control is particularly challenging and personal protection with long-sleeve clothes and skin repellents is often the only option available (13).

Exposure to mosquito bites is a key parameter of the vectorial capacity equation (14) and its assessment is extremely informative for disease surveillance and trials of vector-control interventions. Exposure results from a combination of parameters including mosquito population density, aggressivity to humans, people movements and sleeping habits, and personal protection conferred by vector-control interventions (15). It is currently not possible to measure exposure accurately; mosquito biting-rate estimates are sometimes combined with data on human behaviors and vector-control to produce elusive estimates, but the cost and challenges associated with data collection are often prohibitive (15).

When blood feeding, mosquitoes inject saliva into the skin of vertebrates (16). Mosquito saliva is composed of hundreds of biologically active molecules that play essential roles in the physiology of blood feeding (17). Many saliva components have immunogenic properties and some of these antigens elicit detectable levels of antibody responses in the blood following biting exposure (18). Assessment of antibody responses directed against mosquito salivary antigens as a surrogate measure of human exposure to mosquito bites has been proposed (19). Individuals repeatedly bitten by mosquitoes develop a long-lasting broad and variable sero-reactivity to mosquito salivary antigens of the biting species (20). Serum can cross-react with salivary antigens of other mosquito species to which an individual has never been exposed to (21). As a result, it can be difficult to identify antigenic peptides that elicit transient antibody responses with adequate sensitivity and specificity. Only two *Anopheles gambiae* (gSG6-P1 and cE5) and two *Aedes* sp. (Nterm-34kDa and D7) peptides have been used for assessing exposure in large-scale epidemiological surveys and trials of vector-control (22, 23). However, critical parameters including sensitivity, specificity and half-life of the antibody responses have not

been assessed precisely: only one prospective study strived to characterize the immune responses of humans (n = 1 subject exposed to *Culex quinquefasciatus* bites) and rabbits (n = 1 subject exposed to *Ae. aegypti* bites) to mosquito salivary antigens in a challenge model of controlled exposure (18). We therefore propose to conduct a world-first clinical trial of controlled exposure to bites of uninfected laboratory-reared *Anopheles* and *Aedes* mosquitoes to identify and validate biomarkers of exposure to dengue and malaria vector bites.

The primary objective of this study is to identify and validate biomarkers of exposure to bites of *An. dirus*, *An. maculatus*, *An. minimus*, *Ae. aegypti* and *Ae. albopictus*. The secondary objectives are to characterize the dose-response relationship between the number of mosquito bites and antibody titers and to compare the performance of capillary blood spotted on filter paper and serum from venous blood for measuring antibody responses to mosquito salivary antigens.

This study is an exploratory, factorial randomized control trial of controlled exposure to mosquito bites with 10 arms corresponding to different species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and numbers of bites (35 or 305 bites in total over 6 weeks). Participants will be assigned randomly to one of the 10 study arms with a 1:1 ratio using a block randomization schedule. Those with incomplete follow-up will be replaced until complete follow-up of 15 participants per arm is achieved. Serum from venous blood and capillary blood spotted on filter paper will be collected weekly from each study participant before, during and after the challenges. In addition to those described in the published literature, candidate peptides will be identified with antigen prediction algorithms using mosquito DNA sequences data (either deposited in open access databases (24) or generated using wild-caught mosquito specimens) and immunoproteomic assays carried out using protein extracts of dissected mosquito salivary glands and participants samples (20). Antibody titers will be determined with high-throughput ELISA (25) and mesoscale screening assays (26).

The Statistical Analysis Plan (SAP) for the analysis of “Characterizing antibody responses to mosquito salivary antigens of the Southeast Asian vectors of malaria and dengue with a human challenge model of controlled exposure” (ClinicalTrials.gov Identifier: NCT04478370) describes and expands upon the statistical information presented in the protocol.

1.2 Purpose of the analyses

The purpose of this SAP is to outline the pre-planned analyses to be completed to support the main publication of the analysis of the trial and is based on version 2.0 of the protocol. Versions of the SAP will be tracked. This document contains a review of the study design, general statistical considerations, and statistical analysis methods for antibody outcomes. The reader of this SAP is encouraged to also review the study protocol (ClinicalTrials.gov Identifier: NCT04478370) for details on conduct of the study and the operational aspects of clinical assessments.

2 Study Objectives and Endpoints

2.1 Study Objectives

Primary objectives:

The primary objective of this study is to identify and validate biomarkers of exposure to bites of *An. dirus*, *An. maculatus*, *An. minimus*, *Ae. aegypti* and *Ae. albopictus*.

Secondary objectives:

1. To characterize the relationship between levels of mosquito exposure and humoral response.
2. To assess the feasibility of using dry blood spots made from capillary blood for the monitoring of exposure to bites of mosquito vectors of malaria and dengue.

2.2 Endpoints

Primary endpoints:

1. The levels (continuous OD (405nm)) and seroprevalence (binary) of total IgG antibodies against *Anopheles* and *Aedes* species-specific salivary peptides mesured in human serum; including gSG6-P1, minSG6-P1, macSG6-P1, dirSG6-P1, aegN-term34kDa, albN-term34kda.

Secondary endpoints:

1. The levels (continuous OD (405nm)) and seroprevalence (binary) of antibodies (including different isotypes and subclasses) against *Anopheles* and *Aedes* species-specific salivary peptides; including gSG6-P1, minSG6-P1, macSG6-P1, dirSG6-P1, aegN-term34kDa, albN-term34kda.
2. The levels (continuous OD (405nm)) and seroprevalence (binary) of antibodies (any isotype or subclasses) against novel *Anopheles* and *Aedes* species-specific salivary peptides mesured in human serum.
3. The levels (continuous OD (405nm)) and seroprevalence (binary) of antibodies against *Anopheles* and *Aedes* species-specific salivary peptides mesured in human dried blood spots.

3 Study Methods

3.1 General Study Design and Plan

Study setting

The study will be conducted at the research center of the Shoklo Malaria Research Unit (SMRU) in Mae Sot, Thailand. Critical to the trial design, Mae sot is a small town in Thailand where *Anopheles* mosquitoes have disappeared after decades of development and urbanization (27). Therefore, people who live in Mae Sot are not exposed to *Anopheles* bites if they do not travel in rural areas located outside the city.

Eligibility criteria

Volunteer eligibility to the study will be assessed using the criteria presented in Table 1.

Intervention

Participants will be exposed to bites of laboratory-adapted colonies of *An. minimus* (*s.s.*), *An. maculatus* (*s.s.*), *An. dirus* (*s.s.*), *Ae. aegypti* or *Ae. albopictus* weekly for six weeks (seven challenges per participant in total). Participants in the low-exposure arms will be challenged on each occasion with five mosquito bites (35 bites in total), those in the high-exposure arms will be challenged once with five bites and then six times with 50 bites (305 bites in total). In order to assess participant skin reactions to mosquito bites, three bites will be administered on one participant arm using single mosquitoes in 50 mL tubes topped with netting material. The remaining mosquitoes will be put into plastic cups of 10 cm in diameter topped with netting material and offered to feed on participant counter arm, calf, thigh or back skin. The mosquitoes will be left undisturbed and allowed to feed for 30 minutes. The number of bites actually received by the participant will be assessed by counting the number of engorged mosquitoes at the end of the exposure time. If the mosquitoes are not all successfully engorged, the participant will be exposed to additional mosquitoes using the same procedure in order to reach the target number of bites. All challenges will be performed with 5- to 7-day-old starved nulliparous female imagoes (*i.e.*, that have never blood fed before) of laboratory-adapted mosquito colonies reared in insectaries. Participants with hypersensitivity to mosquito bites will be withdrawn from the study. In order to increase adherence to the study protocol, the study coordinator will call participants on the day before a scheduled visit and remined them about the appointment. Participants who miss a visit will be given the opportunity to come for a retake visit until the day of the next scheduled visit. All participants will be provided antipruritic medication (chlorphenoxamine cream and cetirizine pills) to relieve itching from mosquito bites. Participants will be informed during the screening visit about the importance of avoiding being bitten by *Anopheles* mosquitoes during the study and how to do so. They will be provided with an insecticide-impregnated mosquito bed net (PermaNet 2.0®, Vestergaard) and skin repellent (N,N-diethyl-meta-toluamide, D.E.E.T., 20%). They will be asked not to travel to rural areas and the travel history will be recorded at every visit.

Immunological assays

Previous wok by Poinsignon, *et al.* (28) generated synthetic peptides of the *An. gambiae* salivary protein gSG6, and identified the peptide of the P1 region (gSG6-P1) as an immunogenic biomarker of exposure to *Anopheles* bites. As the homologues from the primary malaria vectors of Southeast Asia share varied sequence identity to gSG6-P1 (Table 1), we used the published sequences of the SG6 proteins (24) to synthesise species-specific *Anopheles* salivary antigens corresponding to the SG6-P1 region of *An. gambiae* (gSG6-P1), *An. minimus* (minSG6-P1), *An. maculatus* (macSG6-P1) and *An. dirus* (dirSG6-P1)(GenScript, New Jersey, USA). Total IgG and IgM antibodies against *Anopheles* species-specific SG6-P1 antigens were measure using a high-throughput ELISA protocol. Detection of total IgG and IgM against salivary antigens was performed by adapting previously published ELISA protocols (29-31), and optimising them into a high-throughput protocol outlined below. Spectraplates (Perkin Elmer) were coated with 0.5µg/mL of *Anopheles* salivary peptides (Genscript) resuspended in autoclaved MilliQ water and diluted in PBS, and incubated for 3 hours at 37°C. Plates were washed and blocked for one hour at 37°C with Blocking Buffer (Pierce, Thermo Scientific USA). After a subsequent wash step, sera were added at a desired concentrations (diluted in 10% Blocking Buffer with PBS) and incubated overnight at 4°C. Following sera incubation, plates were washed and secondary antibody added. To detect human IgG and IgM, HRP-conjugated goat anti-human IgG (Millipore) and HRP-conjugated goat anti-human IgM (Millipore) was used at a 1:500 dilution. Plates were incubated at 37°C for 1.5 hours and then washed. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-

sulfonic acid)) substrate was added to each well, covered and left to develop at room temperature, then stopped with 1% sodium dodecyl sulphate (SDS), and the OD was read in a spectrophotometer at 405nm.

A minimum of 6 wells per plate were incubated with positive control sera consisting of pooled samples from naturally exposed participants found to have high levels of antibodies to *Anopheles* salivary proteins from a mass drug administration trial in Thailand (Positive Pool 1) and from our biting trial cohort (Positive Pool 2). A panel of negative control sera collected from individuals living in Melbourne with no history of malaria or travel to a malaria endemic region were also incubated in a minimum of 6 wells per plate. Additionally, each plate included 8 wells incubated without participant sera used to measure background reactivity.
In order to account for any variation between plates, plate-specific conversion factors were calculated and used to normalise OD values across multiple plates as follows. First, the mean OD of non-sera containing wells from each plate were subtracted from the OD of each sample to remove any background reactivity. Next, the mean of each positive control was calculated per plate and was then divided by the mean of that positive control calculated across all plates. Subsequently, the mean of these values on each plate was calculated to generate a plate-specific conversion factor. Finally, a normalised OD value for each sample was calculated by dividing it by the conversion factor for that plate. The OD of negative Melbourne controls was used to calculate the seropositivity thresholds. Seropositivity was defined as having a normalised OD greater than the mean OD of the negative controls plus three standard deviations.

Table 1. *Anopheles* salivary SG6-P1 peptide antigen amino acid sequences and their similarity to gSG6-P1.

Vector species	Antigen	Peptide Sequence	% gSG6-P1 Homology
An. gambiae	gSG6-P1	E K V W V D R D N V Y C G H L D C T R V A T F	-
An. dirus	dirSG6-P1	G Q T W I D R D K T Y C E H I D C T K L A K Y	48%
An. maculatus	macSG6-P1	A K V W V D R D K V Y C E H I D C T R L A T F	78%
An. minimus	minSG6-P1	E K V W V D R D R V Y C G H I D C T R V A T Y	87%

Note. Green shading references differences in amino acid sequence from gSG6-P1.

3.2 Inclusion-Exclusion Criteria and General Study Population

Participants will be randomised only if they fulfilled all the inclusion criteria and none of the exclusion criteria. These criteria were checked at the screening visit (Day -14 to Day -1).

Inclusion Criteria:

To be included in this study, an individual must satisfy all the following criteria:

1. Generally healthy male or female aged ≥ 18 to <60 years old as assessed by a medical doctor
2. Thai, Burmese or Karen ethnicity
3. Living in Mae Sot city for the last 12 months
4. Able to tolerate direct mosquito exposure

Exclusion Criteria:

If an individual meets any of the following criteria, they are ineligible for this study:

1 History of travel in a rural area (i.e., where participant may be exposed Anopheles bites) in the last 12 months, or plan to do so during the study
2 Medication or condition deemed to interfere with the outcome measure or increase the risk of an adverse reaction to the study procedures (hypersensitivity to mosquito bites, atopy, systemic mastocytosis, immunodeficiencies, Epstein-Barr virus-associated lymphoproliferative disease, and longue-course oral treatment with a steroidal anti-inflammatory drug)
3 Moderate and severe anaemia (haemoglobin concentration less than 110 g/L of blood)
4 Pregnancy
5 Breastfeeding

5.1 Randomisation and Blinding

A block randomization schedule will be generated using the block.random function of the R package psych version 1.8.12 (28) with variables species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and dose (35 or 305 bites in total), yielding an ordered list of 15 blocks with 10 participants per block randomly assigned to one of the 10 study arms.

An allocation sequence will be implemented using individual, sealed and sequentially numbered envelopes. Following screening and eligibility assessment, participants will be assigned to a study arm during visit 2 using the randomization schedule.

An allocation sequence will be generated by a study investigator. At the beginning of the study, the study coordinator will prepare a set of case report forms (CRFs) with preprinted subject identification codes and attach the sealed envelope containing intervention allocation to the CRF. Study nurses will then assign a subject identification code to participants by chronological order of enrollment in the study and the envelope will be opened during visit 2.

Allocation of intervention will be masked to outcome assessors (laboratory personnel who will process the serum samples) and data analysts. In order to do so, allocation of intervention in study datasets that contain this information will be masked until the results of the final analysis are made available to study investigators.

Amendment to SAP 23/03/2023
In order to fit additional models assessing the effects of concordant vs discordant species of exposure on antibody analyses (detailed in Section 9.2), after consultation with Principal and Study Investigators, the antibody data were locked and the biostatisticians were un-blinded.

5.2 Study Visit Schedule

Study variables are collected according to the study visit schedule in Table 2.

Table 2 Study Visit Schedule

Visit	Study period	Day [a]	Informed consent obtained	Medical History	PE and VS	Randomization	Mosquito exposure [b]	DBS Collection [c]	VP collection [c,d]
1	screening	30 days or less before enrolment	Yes	Yes	Yes	No	No	No	Yes
2	baseline	0	No	No	No	Yes	No	Yes	Yes
3		7	No	No	No	No	No	Yes	Yes
4	exposure	14	No	No	Yes	No	Yes	Yes [c]	Yes [c]
5		15	No	No	Yes	No	No	No	No
6		21	No	No	Yes	No	Yes	Yes	Yes
7		22	No	No	Yes	No	No	No	No
8		28	No	No	Yes	No	Yes	Yes	Yes
9		35	No	No	Yes	No	Yes	Yes	Yes
10		42	No	No	Yes	No	Yes	Yes	Yes
11		49	No	No	Yes	No	Yes	Yes	Yes
12		56	No	No	Yes	No	Yes	Yes	Yes
13	post-exposure	63	No	No	No	No	No	Yes	Yes
14		70	No	No	No	No	No	Yes	Yes
15		77	No	No	No	No	No	Yes	Yes
16		84	No	No	No	No	No	Yes	Yes
17		91	No	No	No	No	No	Yes	Yes
18		98	No	No	No	No	No	Yes	Yes
19		105	No	No	No	No	No	Yes	Yes
20		112	No	No	No	No	No	Yes	Yes

[d] 2 mL during the screening visit, 8.6 mL during visits 2, 3, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19 and 18.6 mL during visits 4,

[a] In case participant fail to come on the exact day a visit is scheduled, he/she will be allowed to come for a retake any other working day of the study team until the next scheduled visit;

[b] 5 bites for the screening of hypersensitivity to mosquito bites during visit 4, then 5 or 50 bites/visit according to the randomisation schedule (total of 35 and 305 bites/participant in low- and high-exposure groups respectively, split into 7 direct feeding assays);

[c] before exposure to mosquito bites.
13 and 20.

6 Sample Size

There is no data to calculate the sample size *a priori* because the characteristics of immune responses to candidate peptides is not known at this stage. A sample size of 15 participants with complete follow-up per study arm was deemed appropriate for this study given the number of repeated assessments and expected variation in the continuous individual antibody responses. Participants with incomplete follow-up will be replaced to ensure there are 15 participants per study arm with complete follow-up.

7 General Considerations

7.1 Timing of Analyses

Study data entered and stored into the Excel files by the SMRU research team and assay data entered and stored in Excel files managed by the responsible laboratories will be transferred to researchers at the Burnet Institute and the Centre for Epidemiology and Biostatistics at the University of Melbourne (Melbourne, Australia). After the last subject has concluded study participation to Day 112 follow-up and all study data were available, the following topics were reviewed:

1. Study participants who have withdrawn consent, in relation to the use of the study participant's data (or part of it) in any of the analyses (Protocol Section 7.9). No subjects withdrew consent as part of the 112-day reporting period.

2. Subjects with (minor or major) protocol violations as defined in Section 8.3, in relation to the use of the subject's data (or part of it) in the per protocol analysis set (Section 8.3).

After the SAP is signed, the randomized exposure species and dose allocation will be obtained from the SMRU study team. No database may be locked, random code unblinded, or analyses completed until the SAP has been approved. The planned analyses in this SAP will be conducted after unblinding of the database and any changes to this SAP after unblinding will be documented in an amendment of this SAP and considered post-hoc analyses.

7.2 Analysis Populations

Study participants will be reported and analysed according to their randomisation order ("as-randomised"), except when mentioned otherwise. The following analysis populations are planned.

7.2.1 Intention To Treat Set

The Intention To Treat (ITT) Set consists of all enrolled participants who were randomised and provided at least one valid measurement of antibody response and will be analysed according to randomisation group.

7.2.2 Per Protocol Set

The Per Protocol Set consists of all participants included in the Intention To Treat Set who do not have any major protocol deviations (see Section 8.3).

7.3 Covariates and Subgroups

The protocol does not define any formal subgroup analyses.

7.4 Missing Data

Missing data will not be imputed, instead an available case analysis will be performed of the longitudinal outcomes, antibody levels and sero-prevalence.

7.5 Multiple Testing

There are no adjustments planned for multiple comparisons.

8 Summary of Study Data

Antibody data listings will be sorted by participant identifier and timepoint, as well as mosquito exposure species and biting dose, and enrolment month.

Data will be summarised as described in the sections below. Summaries will consist of descriptive statistics whereby continuous variables will be summarized using n (non-missing sample size), mean, standard deviation, median, minimum, and maximum and categorical variables using counts and percentages (based on the non-missing sample size) of observed category levels.

All confidence intervals (CIs) will be two-sided 95% CIs. Comparative analyses will consist of qualitatively describing the difference.

8.1 Subject Disposition

The flow of participants will be presented in accordance with the Consolidated Standards of Reporting Trials (CONSORT) criteria.

We will report the following:

- Number of participants assessed for eligibility
- Number of participants not meeting the inclusion criteria and declined to participate
- Number of participants allocated to each mosquito species exposure and dose group
- Number of participants attending day 112 visit and number of participants who withdrew from the study and lost to follow-up
- Number of participants included in the Intention to Treat Set and Per Protocol Set

8.2 Derived Variables

The following measures will be derived:

- Linear splines will be derived using the visit variable to represent the baseline (visits 2-3), exposure to *Anopheles* spp. bites (visits 4-12) and post exposure (visits 13-20) periods to model the boosting and decay of antibody responses.
- Exposure and post-exposure periods will be derived as a categorical time-varying variable.
- Concordant and discordant exposure species and antibody responses variables will be derived by collapsing across categories of the mosquito species participants were exposed to, according to the following groups: (i) exposure to *Anopheles* spp. compared to *Aedes* spp. (explored for all anti-SG6-P1 antibodies), (ii) exposure to *An*.

dirus bites, compared to *An. maculatus* and *An. minimus* bites, compared to *Aedes* spp. bites (explored for anti-dirSG6-P1 antibodies), (iii) exposure to *An. maculatus* bites, compared to *An. dirus* and *An. minimus* bites, compared to *Aedes* spp. bites (explored for anti-macSG6-P1 antibodies), (iv) exposure to *An. min* bites, compared to *An. maculatus* and *An. dirus* bites, compared to *Aedes* spp. bites (explored for anti-minSG6-P1 antibodies).

8.3 Protocol Deviations

The following will be considered as protocol deviations:

- Variation of Inclusion/Exclusion criteria
- Significant protocol deviations, which may have an effect on the integrity of study data

Protocol deviations were reviewed prior to un-blinding to determine which protocol deviations may affect the analysis. The Per Protocol Set will then be defined for the antibody endpoints with the following exclusions:

- Data from all available visits for subjects found to violate inclusion/exclusion criteria.
- Data from all visits subsequent for the protocol deviations that are considered to affect the integrity of the antibody data.
- Data from any visit that occurs substantially out of window.

Protocol deviations will not be excluded from the Intention to Treat Set, but may result in subjects being excluded from the Per Protocol Set. After review of the data prior to un-blinding, no subjects were excluded from the Per Protocol Set.

8.4 Demographic and Baseline Variables

Demographic and baseline variables will be summarized, including age and sex, and presented by exposure species and dosing group using frequencies and percentages for categorical variables, mean and standard deviation and median (minimum – maximum) for continuous variables. If variables have missing values, we will report the non-missing sample either in the table or in the footnote of the table.

9 Statistical Analyses

9.1 Effect of mosquito biting exposure on antibody level and seropositivity

Measurement of mosquito species-specific antibodies will be using high-throughput ELISA are described in Table 3:

Table 3 Overview of assays

Assay	Visits	Outcomes
High-throughput ELISA	Visits 2-20	Total Anti-IgG levels and seropositivity against: gSG6-P1 minSG6-P1 macSG6-P1 dirSG6-P1 aegNterm34kDa

Assay	Visits	Outcomes
		albNterm34kDa
High-throughput ELISA	Visits 2-20	Total Anti-IgM levels and seropositivity against: gSG6-P1 minSG6-P1 macSG6-P1 dirSG6-P1 aegNterm34kDa albNterm34kDa
High-throughput ELISA	Visits 2-20	Total antibody (including any isotype or subclass) levels and seropositivity against novel candidate *Anopheles* and *Aedes* species-specific antigens

Antibody markers will be summarised at each scheduled time point, including changes from baseline. Values will be presented visually with dotplots at each time point and spaghetti plots to visualise within-participant changes from baseline.

The change in antibody levels (as a continuous outcome and binary response) over time will be analysed using generalised linear mixed-effects models. Optical density measurements (*i.e.* antibody levels) will be analysed using a Gaussian distribution family and identity link function (*i.e.* a linear mixed-effects model) and binary responder status will be analysed using a Binomial distribution family and a logit link function (*i.e.* a logistic mixed-effects model). Linear splines will be fitted to represent the baseline (visits 2-3), exposure to mosquito bites (visits 4-12) and post exposure (visits 13-20) periods to model the boosting and decay of antibody responses. Mixed-effects models will include a random effect (*i.e.* intercept) for participant to allow random variations of immune responses in individuals. Estimates from the linear mixed-effects models will be used to calculate the half-lives of each antibody measure.

Amendment to SAP 23/03/2023

To account for positively skewed antibody data and aid interpretation, antibody levels were $\log_2$ transformed to represent a two-fold change in antibody levels.

Due to differences (in terms of time) between individuals transitioning from baseline, to exposure and post-exposure periods, linear splines representing these periods of fixed time were unable to be estimated. Instead, the overall effect of time will be estimated (independent of any exposure and thus representing the baseline period), with the exposure and post-exposure periods fitted as a categorical time-varying variable. To estimate the extent to which antibody levels associated with biting are dependent on time, interaction terms for the intervention periods by time will be estimated.

The change in antibody levels (as a continuous outcome and binary response) over time, and in response to *Anopheles* biting exposure, will be analysed using generalised estimating equations (GEEs). The specification of an autoregressive correlation structure will better account for high-levels of serial correlation in antibody levels within individuals over time. Furthermore, a marginal model will allow for robust inference of the estimated effects of the exposure and post-exposure periods on antibody levels.

9.2 Moderating effect of mosquito biting species or dose of exposure on antibody level and seropositivity

To investigate if changes in antibody levels over time vary according to mosquito species or biting dose, interaction terms will be fitted between time, exposure species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and biting dose (35 or 305 bites), respectively.

Amendment to SAP 23/03/2023
Additional models will be fitted to determine the effect of concordant and discordant exposure species and antibody responses, as the exposure species is likely to have biological relevance to the antibody outcome. This will be done by collapsing across categories of the mosquito species participants were exposed to, according to the following groups: (i) exposure to *Anopheles* spp. compared to *Aedes* spp. (explored for all anti-SG6-P1 antibodies), (ii) exposure to *An. dirus* bites, compared to *An. maculatus* and *An. minimus* bites, compared to *Aedes* spp. bites (explored for anti-dirSG6-P1 antibodies), (iii) exposure to *An. maculatus* bites, compared to *An. dirus* and *An. minimus* bites, compared to *Aedes* spp. bites (explored for anti-minSG6-P1 antibodies), (iv) exposure to *An.* bites, compared to *An. maculatus* and *An. dirus* bites, compared to *Aedes* spp. bites (explored for anti-minSG6-P1 antibodies). Any effect modification of the association between the intervention (exposure and post-exposure periods) and antibody levels will be estimated by fitting interaction terms between the intervention and these variables representing concordant and discordant mosquito exposure.
In order to collapse across these categories and perform concordant exposure species and antibody analyses, the locked dataset must be un-blinded.

10 Other Analyses
No further analyses will be performed.

11 Reporting Conventions
The mean, standard deviation, and any other statistics other than quantiles, will be reported to one decimal place greater than the original data. Quantiles, such as median, or minimum and maximum will use the same number of decimal places as the original data.

12 Technical Details
The statistical analyses will be performed by the trial statistician under supervision of independent senior biostatisticians who are un-blinded at the individual level. The individual level randomisation code will not be accessible to other biostatisticians involved in the study until the database is locked for the final analysis.

Analyses will be conducted using Stata and R. We will report the software and version used at the time of reporting.

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