Protocol

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Official Title of Study: An Ancillary Study Protocol to GlaxoSmithKline Phase IIb RTS,S/AS01E Malaria Vaccine Trial (Study Number 204889 [MALARIA-094]) entitled, "Efficacy, safety and immunogenicity study of GSK Biologicals' candidate malaria vaccine (SB257049) evaluating schedules with or without fractional doses, early Dose 4 and yearly doses, in children 5-17 months of age

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Molecular Detection and Genotyping of *Plasmodium falciparum* Parasites in Young African Children after Immunization with RTS,S/AS01$_E$ Malaria Vaccine

Protocol Number: 206213 (MALARIA-095)

"An Ancillary Study Protocol to GlaxoSmithKline Phase IIb RTS,S/AS01$_E$ Malaria Vaccine Trial (Study Number 204889 [MALARIA-094]) entitled, "Efficacy, safety and immunogenicity study of GSK Biologicals' candidate malaria vaccine (SB257049) evaluating schedules with or without fractional doses, early Dose 4 and yearly doses, in children 5-17 months of age."

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SYNOPSIS

The goal of the proposed amplicon sequencing and genotyping study (hereafter referred to as 'genotyping study') is to assess vaccine efficacy against clinical and asymptomatic malaria infection using ultra-sensitive molecular amplification and sequencing methodology to detect *Plasmodium falciparum* (*P. falciparum*) parasites from serial blood samples to be collected from children immunized with the primary and yearly booster immunizations of the RTS,S/AS01$_E$ vaccine as part of their participation in Protocol MALARIA-094. Genomic analysis will be performed on parasites from blood spot samples collected from children aged 5-17 months immunized with RTS,S/AS01$_E$ on different dosage and schedule regimens under the parent clinical study protocol.

Specifically, the aims of this protocol are:

Primary Objective: To estimate and compare vaccine efficacy (VE) against new malaria infection(s) across all genotypes among the vaccine arms of the trial, using amplicon sequencing and genotyping to detect new infections. Asymptomatic *P falciparum* infections will be detected in monthly cross-sectional samples and clinical infection will be detected by passive case detection.

Two independent measures of VE will be determined:

a. **Vaccine efficacy against first new infection**

   VE against first new *P. falciparum* infection (i.e., time-to-infection; clinical case or cross-sectional) in all vaccine groups for the first 12 months following the third immunization in each vaccine group.

b. **Vaccine efficacy against all new infections**

   VE against all new *P. falciparum* infections (clinical case or cross-sectional) in all vaccine groups for the first 12 months following the third immunization. The comparator group (rabies) window of follow-up will differ depending on the vaccine group to which it is compared.

Exploratory Objectives: The following exploratory objectives will be addressed using data generated for the primary objective:

a. **Exploratory Objective 1:** To assess whether VE against new malaria infection based on blood slides (passive case detection) and molecular detection (active case detection) depends on genotypic characteristics of the malaria parasites, based on cross-sectional and clinical case samples.

b. **Exploratory Objective 2:** To study whether prior infection or vaccination has a relationship to subsequent infection by measuring the molecular Force of Infection (mol[FOI]) determining the relationship between (mol)FOI and subsequent malaria infection risk.

c. **Exploratory Objective 3:** To determine whether prior infection by a particular parasite genotype, not necessarily the vaccine strain, reduces the likelihood of re-infection by a parasite with the same genotype.

d. **Exploratory Objective 4:** To understand how gaps in time as measured by consecutive monthly dried blood spots impact the evaluation of VE against new infections.

Study outcomes to be evaluated:

Time to first new malaria infection - Infection is defined as sequence positive status. As there is no guarantee of complete clearance of asymptomatic infections by drug treatment, a new infection may originate from a sample that goes from negative to positive, or from a positive genotype X to a positive genotype Y.

Sequence-based parasite positive/negative status - Positive status defined by the minimal threshold number of sequencing reads (defined in the Statistical and Analysis Plan) for at least one amplicon following filtration of polymerase chain reaction (PCR) and sequencing errors.

Genotype data for the *P. falciparum* circumsporozoite (CS) C-terminus and SERA2 loci - Defined as the haplotype(s) observed at each amplicon following filtration of PCR and sequencing errors.

New infections - New infection(s) for the primary objective is defined as a haplotypic genotype not previously detected in any sample that is different from baseline sample collected prior to immunization # 1.

This study is proposed to be an ancillary study of the GlaxoSmithKline (GSK) RTS,S/AS01$_E$ phase IIb randomized trial (Study Number 204889) and is to be performed across all study sites. The original randomized trial (MALARIA-094) will be vaccinating approximately 1,500 subjects 5-17 months of age in multi-centers according to the following groups:

i. **Control group**, i.e., rabies vaccine at Month 0, Month 1, Month 2; or

ii. **Group R012-20** - a course of RTS,S/AS01$_E$ full dose at Month 0, Month 1, Month 2 + a full dose at Month 20; or

iii. **Group R012-14-mD -** a course of RTS,S/AS01$_E$ full dose at Month 0, Month 1, Month 2 + yearly full doses at Month 14, Month 26, Month 38; or

iv. fractionated (Fx) **Group Fx012-14-mFxD -** a course of RTS,S/AS01$_E$ full dose at Month 0, Month 1 + RTS,S/AS01$_E$ 1/5th dose at Month 2, + RTS,S/AS01$_E$ 1/5th dose at Month 14, Month 26, and Month 38;

v. **Group Fx017-mFxD** - a course of RTS,S/AS01$_E$ full dose at Month 0, Month 1 + RTS,S/AS01$_E$ 1/5th dose at Month 7, + RTS,S/AS01$_E$ 1/5th dose at Month 20, and Month 32.

A total of 300 subjects will be allocated to each group.

This genotyping study of malaria parasites collected from serial blood samples following RTS,S/AS01$_E$ immunization will assess VE using molecular genetic data to identify first infections following vaccination, and to distinguish new from existing infections, using

an amplicon sequencing-based strategy: deep sequencing of small, highly variable regions of the parasite genome to allow for both:

i. highly sensitive detection of parasitemia (analogous to conventional PCR-based detection), and
ii. identification of genetically distinct parasite populations within or between affected individuals.

Given the similarities between this project and the ancillary genotyping study performed for the phase 3 MALARIA-055 study (MALARIA-066), there is confidence to perform the necessary data generation and analyses to accomplish the listed Specific Aims. The following sequential approach is proposed for sample evaluation to investigate the described Specific Aims, with Tier 1 sampling essential to addressing the Primary Objective, and Tier 2 sampling to be performed after primary immunization series and booster immunization series. Two analyses will be performed:

Tier 1: All cross-sectional dried blood spot samples collected monthly from all subjects during the first 20 months (approximately 31,500 samples) and clinical malaria cases (approximately 14,400) of the primary series from all arms of the study.

Tier 2: All cross-sectional dried blood spot samples collected for three consecutive months and then quarterly thereafter for one year following the first booster RTS,S dose (approximately 9,000 samples) and clinical malaria cases in all arms of the study up through Month 32 (approximately 8,640 samples).

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$AS01_E$	GlaxoSmithKline's proprietary Adjuvant System containing MPL, QS-21 and liposome (25 μg MPL and 25 μg QS-21)
ATP	According to protocol
BAM	Binary alignment/map
CDC	Center for Disease Control
CHMI	Controlled human malaria infection
CI	Confidence interval
COI	Complexity of infection
CRF	Case report form
CRO	Clinical research organization
CS	Circumsporozoite
CSP	Circumsporozoite protein of *Plasmodium falciparum*
csp	DNA seq for circumsporozoite gene
C-terminus	C-terminal domain from CSP protein
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
FOI	Force of infection
Fx	Fractionated / fractional
GP	Genomics platform
GSK	GlaxoSmithKline
HLA	Human leukocyte antigen
LIMS	Laboratory information management system
LL	Lower limit
mol(FOI)	Molecular force of infection

MVI	Malaria Vaccine Initiative
NANP	Amino acid repeat region of *P. falciparum* circumsporozoite protein
NCBI	National Center for Biotechnology Information
NVDP	Non-repetitive island
PCR	Polymerase chain reaction
P. falciparum	*Plasmodium falciparum*
PI	Principal investigator
POC	Proof of concept
pyr	Per year
rcdfs	Reverse cumulative distribution functions
RTS,S	Particulate antigen, containing both RTS and S (hepatitis B surface antigen) proteins
SAP	Statistical Analysis Plan
SERA2	Serine repeat antigen 2 from *P. falciparum*
SNP	Single nucleotide polymorphism
SQL	Structured query language
TVC	Total vaccinated cohort
VE	Vaccine efficacy

1. OBJECTIVES

GlaxoSmithKline (GSK) Biologicals and the PATH-Malaria Vaccine Initiative (MVI) are committed to develop a malaria vaccine for reduction of malaria disease burden in children and contribution to the malaria elimination goal. Characterization of an optimal dosing regimen and boosting schedules will be critical.

The results of the phase 3 RTS,S/AS01$_E$ malaria vaccine trial (MALARIA-055) previously reported, including the long term follow-up data and efficacy of the fourth dose, and the preliminary results of the controlled human malaria challenge study, MALARIA-071, were reviewed by the European Medicines Agency (EMA) during the assessment of the initial application of the RTS,S/AS01$_E$ candidate vaccine. The EMA asked GSK to address the following points in ongoing or future studies: 1) the timing of the fourth dose and evaluation of the safety and efficacy of an early fourth dose; 2) the efficacy and safety of multiple yearly doses and whether the vaccine predisposes to some degree of hyporesponsiveness to sequential doses; and 3) the plans to further investigate the potential utility of a delayed and fractionated third dose schedule in the target age range.

The primary protocol MALARIA-094 is designed to address these points in the pediatric population. It aims to:

- Establish proof of concept (POC) for a fractional (Fx) dose schedule under conditions of natural exposure,
- Establish the role of third dose spacing in a Fx dose schedule,
- Describe the effect of an earlier full fourth dose at Month 14, and
- Describe the effect of multiple Fx or full yearly doses.

1.1. Rationale for the MALARIA-094 Study Design

The study intends to establish POC for a Fx dose schedule under conditions of natural exposure. The study will be conducted in children 5-17 months of age at first vaccination living in areas of mid to high malaria transmission. In addition to assessing vaccine efficacy (VE) against clinical malaria disease, this study will for the first time determine VE against asymptomatic and sub-microscopic parasitemia in dried blood spots collected in monthly cross-sectional surveys from every child enrolled in this study using ultra-sensitive molecular genotyping methods. This ancillary protocol, addressing RTS,S VE against infection as defined by genetic methods, is in support of the MALARIA-094 clinical protocol entitled, “Efficacy, safety and immunogenicity study of GSK Biologicals’ candidate malaria vaccine (SB257049) evaluating schedules with or without fractional doses, early Dose 4 and yearly doses, in children 5-17 months of age”.

The study proposed herein will include the five study groups according to the MALARIA-094 study:

a. Control group will receive three doses of rabies vaccine at Month 0, Month 1 and Month 2.

b. Group R012-20 will receive the standard schedule consisting of RTS,S/AS01$_E$ full dose given at Month 0, Month 1, Month 2 and a fourth dose at Month 20. This corresponds to the schedule that received a positive scientific opinion from EMA. The four other groups will receive alternative schedules as follows:

c. Group R012-14-mD will receive RTS,S/AS01$_E$ full dose at Month 0, Month 1, Month 2, an early fourth dose at Month 14 and yearly full doses at Month 26 and Month 38.

d. Group Fx012-14-mFxD will receive RTS,S/AS01$_E$ full dose at Month 0 and Month 1, RTS,S/AS01$_E$ Fx dose (1/5th dose) at Month 2 and yearly Fx doses at Month 14, Month 26, Month 38.

e. Group Fx017-mFxD will receive RTS,S/AS01$_E$ full dose at Month 0 and Month 1, RTS,S/AS01$_E$ delayed Fx third dose (1/5th dose) at Month 7 and yearly Fx doses at Month 20 and Month 32.

Results from this study will be critical in forming future possibilities for the development of vaccine-based strategies which, in combination with other interventions, may contribute to the malaria elimination agenda.

1.2. MALARIA-095: Genetic Analysis of MALARIA-094 Infections

Genomic and statistical analysis approaches can be applied to the MALARIA-094 trial to help understand and improve the efficacy of RTS,S, forming the basis of the ancillary protocol MALARIA-095 described here. Whereas traditional polymerase chain reaction (PCR)-based infection detection methods yield a simple binary outcome of positive or negative infection status, amplicon sequencing data will provide information of the complexity of infection (COI), a measure of the number of genetically distinct parasite lineages in each participant sample in the trial, and indicate the rate with which new infections occur and parasite lineages turn over. The resulting data will allow assessment of VE of each vaccine study arm to prevent new genetically distinct malaria infection by yielding not only the infection status of each sample, but also information on the incidence rates of new and distinct malaria infection.

In addition to supporting the primary objective of evaluating differences in overall VE against new malaria infection among the arms of the MALARIA-094 trial, amplicon sequencing data from MALARIA-095 will enable investigations into the nature of immunity generated by vaccination and natural disease exposure. The dense cross-sectional sampling design will allow for exploration of the impact of prior infection history on susceptibility to new infections from the perspective of parasite genotype. There can also be evaluation of the degree to which allele-specific protection contributes to overall VE, and how VE depends on genetic distance of malaria infections from the vaccine strain, as were performed in the MALARIA-066 ancillary genotyping study. The

sampling schedule of this trial, in combination with the unique data generation capacity developed at the Broad Institute for previous RTS,S work, creates an opportunity to significantly increase the understanding of RTS,S efficacy, and to investigate the dynamics of naturally acquired immunity to malaria infection.

In summary, this proposed ancillary genotyping study is an evaluation of the RTS,S/AS01$_E$ VE when administered as different immunization schedules for the prevention of *P. falciparum* malaria infection upon molecular detection by amplicon sequencing and parasite genotyping. Sample collection methodology is described in the MALARIA-094 trial (Study Number 204889).

1.3. Statement of Specific Objectives

1.3.1. Primary Objective

Primary Objective: To estimate and compare vaccine efficacy (VE) against new malaria infection(s) across all genotypes among the vaccine arms of the trial, using amplicon sequencing and genotyping to detect new infections. Asymptomatic *P falciparum* infections will be detected in monthly cross-sectional samples and clinical infection detected by passive case detection.

Two independent measures of VE will be determined:

a. **Vaccine efficacy against first new infection**

   VE against first new *P. falciparum* infection (i.e., time-to-infection; clinical case or cross-sectional) in all vaccine groups. The comparator group (rabies) window of follow-up will differ depending on the vaccine group to which it is compared.

b. **Vaccine efficacy against all new infections**

   VE against all new *P. falciparum* infections (clinical case or cross-sectional) in all vaccine groups. The comparator group (rabies) window of follow-up will differ depending on the vaccine group to which it is compared.

1.3.2. Exploratory Objectives

The following exploratory objectives will be addressed using data generated for the primary aim:

a. **Exploratory Objective 1:** To assess whether VE against new malaria infection based on blood slides (passive case detection) and molecular detection (active case detection) depends on genotypic characteristics of the identified malaria parasites, based on cross-sectional and clinical case samples.

b. **Exploratory Objective 2:** To study whether prior infection or vaccination has a relationship to subsequent infection by measuring the molecular Force of Infection (mol[FOI]) determining the relationship between (mol)FOI and subsequent malaria infection risk.

c. **Exploratory Objective 3:** To determine whether prior infection by a particular parasite genotype, not necessarily the vaccine strain, reduces the likelihood of re-infection by a parasite with the same genotype.

d. **Exploratory Objective 4:** To understand how gaps in time as measured by consecutive monthly dried blood spots impact the evaluation of VE against new infections.

2. BACKGROUND AND SIGNIFICANCE

2.1. RTS,S Vaccine-Induced Immune Responses

Previous investigations have identified candidate mechanisms of both natural and vaccine-induced immune response to the CS protein (CSP) [Nardin and Nussenzweig, 1993]. Natural malaria infection induces a non-sterilizing protective immune response against blood stage merozoites (that do not express *csp*) targeted towards epitopes that bind antibodies as well as human leukocyte antigen (HLA) I and HLA II molecules. The mechanism of RTS,S/AS01$_E$ vaccine-induced protection remains unresolved as there is not a clear immunological surrogate of protection which is predictive of outcome. The efficacy of the vaccine has been most closely associated with anti-CSP antibody responses, more specifically, antibody response to the NANP amino acid repeat region. The NANP repeat region is interrupted by a non-repetitive island (NVDP), and variably exhibits 10-17 NANP repeats to the left of this island and 17-23 repeats to the right of this island. Early studies found that antibodies bind to CSP at two identical B cell epitopes [Zavala et al., 1983], suggesting that each NANP repeat region may independently bind antibodies. Antibodies elicited against epitopes within the amino acid NANP repeat region of the CS antigen correlate with protection (while not predictive) as demonstrated in the controlled human malaria infection (CHMI) challenge model [Stoute, et al., 1998; Kester, et al., 2001; Kester, et al., 2009, Ockenhouse, et al., 2015] and in field studies [Olotu, et al., 2011; White, et al., 2013]. Recently, Ockenhouse, et al. have identified antibodies in human immune sera detected after immunization with RTS,S/AS01 that react with conformational strain specific epitopes present in the C-terminal domain of the CSP (unpublished).

Studies in the Gambia [Bojang et al., 2001; Alloueche et al., 2003], Mozambique [Enosse et al., 2006] and Kenya [Waitumbi et al., 2009] comparing RTS,S and comparator vaccinated subjects could not identify any particular polymorphism or variants targeted by the vaccine in the *csp* gene and association with protection.

The RTS,S/AS02 vaccine is capable of eliciting CD4+ T-cell production, and IFN-γ secretory CD4+ T-cell responses, particularly of the Th1 type [Lalvani et al., 1999]. It has been hypothesized that polymorphism observed in the Th2R and Th3R CD4 epitopes [Weedall et al., 2007] may indicate that T-cell response to these regions of CSP may provide some degree of protective immunity against infection by providing T-cell help. The vaccine is also known to induce a strong CD4+ T-cell response to the non-variable CST3 epitope [Bojang et al., 2001].

2.2. Immunity and Genetic Diversity

The malaria parasite is one of the most widespread and deadly human pathogens, causing an estimated 214 million cases per year with 88% of cases occurring in sub-Saharan Africa where approximately 300,000 malaria deaths occur in children annually [World Malaria Report 2015]. The parasite has demonstrated great evolutionary plasticity, and has developed mechanisms to evade the host immune response and intervention strategies such as chemotherapeutics. Sequence analysis of *csp* demonstrates significant genetic diversity in the extant parasite populations consistent with immune-mediated selection at this locus. It is therefore anticipated that vaccination with a monovalent vaccine such as RTS,S/AS01$_E$ may provide differential protection across haplotypic variants for *csp* and possibly select those variants that can evade clearance by the vaccinated host.

Investigations of the immune responses to RTS,S in malaria-naïve subjects suggested that the protection conferred by RTS,S/AS02 could be allele-specific. For instance, Lalvani et al. [1999] have found that the CD4+ T-cell response to the Th2R and Th3R epitopes did not provide cross-reactive immunity to non-vaccine epitope haplotypes in malaria-naïve subjects. However, studies conducted with cells primed through natural infection or with subjects previously exposed to malaria could detect responses against non-vaccine parasite variants [Pinder et al., 2004].

Results of three studies comparing RTS,S and comparator vaccinated subjects in endemic areas also suggested that protection mediated by this vaccine is not allele specific. Investigators in the Gambia [Bojang et al., 2001; Alloueche et al., 2003], Mozambique [Alonso et al., 2004; Enosse et al., 2006] and Kenya [Waitumbi et al., 2009] could not find differences in parasite diversity in the Th2R and Th3R regions between isolates from RTS,S recipients and isolates from subjects receiving comparator vaccines, either in the occurrence of Th2R and Th3R vaccine haplotypes or in the occurrence of subject point mutations in those regions.

Next generation (Illumina MiSeq, PacBio) sequencing of PCR amplicons from infected participant samples from the phase 3 RTS,S clinical study (MALARIA-055) was used to conduct more sensitive genetic investigations of allele-specific protection, and to perform analyses at a potentially more immunologically relevant level: epitope haplotypes, rather than individual polymorphic residues. A sieve analysis methodology was used to analyze the CS data for differential VE at three levels: 1) the entire C-terminal domain from CSP protein (C-terminus) amplicon haplotype (95 residues), 2) defined haplotypic regions of the C-terminus (10-17 residues), and 3) individual polymorphic residues, for two defined trial endpoints in the two age cohorts [Neafsey et al., 2015]. PCR amplicon directed next-generation sequencing of deoxyribonucleic acid (DNA) extracted from 4,985 participant samples was used to survey polymorphisms in CS and evaluate the impact of polymorphic CS residues and several haplotypic regions on VE to prevent first or only episodes of clinical malaria within a year of vaccination. VE was significantly greater against clinical malaria with infections matching the vaccine strain in several haplotype regions and individual residues of the CS C-terminus in the 5-17 month old per-protocol cohort of 4,557 vaccinated and 2,328 control vaccinated participants. For matched versus mismatched malaria based on the entire CS C-terminus, VE cumulatively through 12

months was 50.3% (95% confidence interval [CI], 34.6 to 62.3) versus 33.4% (95% CI, 29.3 to 37.2), P = 0.04 for differential VE; and VE based on a hazard ratio was 62.7% (95% CI, 51.6 to 71.3) versus 54.2% (95% CI, 49.9 to 58.1), P = 0.06. VE did not vary with CS C-terminus haplotypes for the 6-12 week old per-protocol cohort. Given the low frequency of parasites matching the vaccine strain at many of the phase 3 trial study sites, these results partially explain the incomplete protective efficacy of RTS,S/AS01 vaccination.

2.3. Genotyping Technologies

A high-throughput pipeline was developed at the Broad Institute to generate PCR amplicon-based next generation sequencing data for the MALARIA-066 ancillary genotyping study from over 5,000 dried blood spot samples (see Appendix from Neafsey et al., 2015 for details on sequencing methodology). This capacity yielded highly useful information about parasite samples that could not be obtained via any other practical means. By generating deep sequencing coverage of hundreds to thousands of sequencing reads from a collection of short (~300 nucleotide) PCR amplicons spanning highly variable antigenic regions from the *P. falciparum* genome, complete phasing information (i.e., ordering of the variable positions within each amplicon for each patient sample), was recovered. From this information, the haplotypes of common to extremely rare (~1% abundance) parasite strains within complex infections were reconstructed. Because the sequenced regions exhibit a high diversity of haplotypes at the parasite population level, it could be expected that genetically distinct parasites would only very rarely exhibit the same haplotype at more than one amplicon locus by chance, so the data therefore allowed detection of all infections within patient samples with high sensitivity.

In the current study it is proposed to replace conventional 18S PCR-based detection of parasitemia with the amplicon sequencing approach. Parasite positivity will be defined as ≥200 sequence reads aligning to at least one amplicon. This is based on findings of the MALARIA-066 study, in which 200 reads was found to be the threshold below which true parasite detection could not be resolved from background signal, and at which parasitemia was detected in 90% of samples. In MALARIA-066, where samples did not yield a positive result there was no correlation with parasitemia; the lack of sequence reads was likely due to poor sample quality or other technical issues. For the filtration of PCR and sequencing errors within samples meeting the standard of parasite positivity, we will require that filtered haplotypes be supported by a minimum of 1% and with at least 5 reads associated with a given sample-amplicon. There will be a continuance to optimize this methodology and further benchmark it against standard PCR-based parasite detection.

There has been a reduction in the cost of the DNA extraction and amplicon sequencing, and tailoring it to new study designs and sequencing platforms. Simulations conducted based on data collected in the MALARIA-066 ancillary protocol indicate that with two sequenced amplicons per sample (specifically, the CS C-terminus and serine repeat antigen 2 from Pf [SERA2] amplicons), there will be greater than 99% power to detect new superinfections in samples containing a single initial strain, and approximately 95%

power to detect new superinfections in samples already containing five parasite strains (see Figure 1).

2.4. Study Objectives

2.4.1. Primary objective

- To estimate and compare vaccine efficacy (VE) against new malaria infection(s) across all genotypes among the vaccine arms of the trial, using amplicon sequencing and genotyping to detect new infections. Asymptomatic *P falciparum* infections will be detected in monthly cross-sectional samples and clinical infection detected by passive case detection.

To achieve this aim it is proposed to use data generation methodology very similar to what was employed in the MALARIA-066 ancillary genotyping study (see Supplementary appendix, Neafsey et al., 2015 for details). We will be using two of the same PCR amplicons analyzed for MALARIA-055, one spanning 288 base pairs in the C-terminus of the CS locus (coding sequence nucleotide coordinates 878–1165) and a second amplicon spanning 286 base pairs in the N-terminus of the SERA2 locus (coding sequencing nucleotide coordinates 72–357).

In the MALARIA-066 trial sequence data were successfully generated from approximately 90% of the samples received. No association between sequence read coverage and parasite density (Pearson correlation = 0.15) was observed, suggesting that PCR performs robustly on samples with a parasite density of at least 200/ul. The sensitivity of the CS and SERA2 amplicons against traditional PCR assays for *Plasmodium* detection, such as 18S amplicons, will be benchmarked.

Execution of the primary objective will require the capacity to distinguish new infections from existing infections in cross-sectional samples. Analysis of data gathered from the MALARIA-066 trial to estimate the power to detect new infections as a function of the initial COI and the number of PCR amplicon loci sequenced per sample (one or two) have been performed. Because power for this application is contingent on the local haplotypic diversity of the amplicon regions in the parasite population, simulation analyses have been conducted using MALARIA-066 data in both a West African site (Agogo) and an East African site (Siaya), given the differences in population haplotypic composition in those two regions. Infections were simulated of varying degrees of initial COI by randomly sampling haplotypes from the MALARIA-066 dataset according to their frequency in the parasite populations in Agogo and Siaya, and evaluated how often the addition of one more haplotype (also sampled according to population frequency) resulted in a distinct genotypic composition. One thousand simulations for each combination of parameters were performed.

As indicated by Figure 1, sequence data from two amplicons (CS and SERA2) yield much greater power to detect new infections than data from a single amplicon (CS), in both Agogo and Siaya. For infections that start with a COI of 5, data from two amplicons is sufficient to detect the addition of a sixth super-infecting strain at a frequency over 95%. There is confidence that, using data from two amplicons, new infections will be

resolved from existing infections in cross-sectional samples to address the key questions of the Primary and Exploratory Aims.

The Statistical Analysis Plan (SAP) (see Section 6.1) for the Primary Objective will assess VE against each of the two primary endpoints listed in Section 3.3.2 and for each of the ATP and TVC cohorts (see Section 3.3.3).

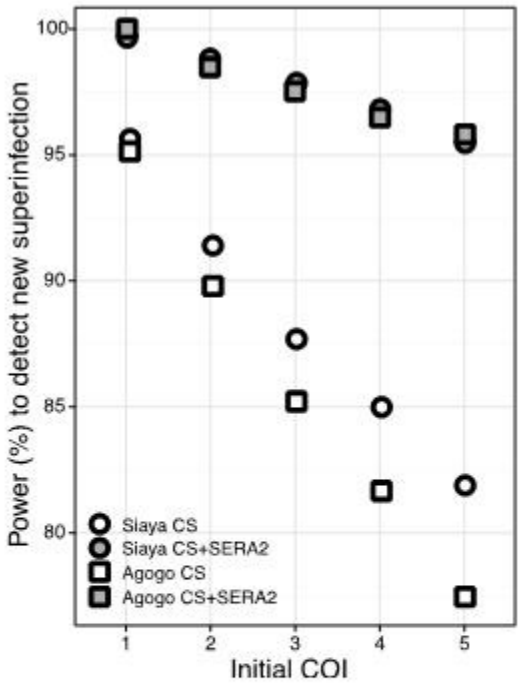

Figure 1. Power to detect new superinfections. Simulations indicate the power of one (CS) or two (CS and SERA2) amplicons to detect new superinfections on a background of 1-5 pre-existing infections in West Africa (Agogo) and East Africa (Siaya).

2.4.2. Exploratory objective 1

- To assess whether VE against new malaria infection based on blood slides (passive case detection) and molecular detection (active case detection) depends on genotypic characteristics of the identified malaria parasites, based on cross-sectional and clinical case samples.

2.4.3. Exploratory objective 2

- To study whether prior infection or vaccination has a relationship to subsequent infection by measuring the molecular Force of Infection (mol[FOI]) determining the relationship between (mol)FOI and subsequent malaria infection risk.

Molecular FOI, the number of parasite infections accrued over a given time period detected using molecular tools, has been demonstrated to be an excellent measure of malaria epidemiology and an informative parameter to measure in intervention studies [Mueller et al., 2012]. Molecular FOI can be difficult to measure outside the context of a longitudinal trial, but the structure of the MALARIA-094 trial offers the perfect opportunity to study mol(FOI). In this study mol(FOI) will be calculated for the individual participants of the trial using amplicon sequence data generated for the Primary Objective from cross-sectional and clinical malaria samples, and used to explore the relationship between that parameter and subsequent infection risk across the arms of the trial. By capturing variation among individuals in mol(FOI), understanding the impact of various booster dosages and scheduling regimens on VE will be refined and improved.

In contrast to molecular studies utilizing 18S PCR or other non-antigenic markers for infection detection, amplicon sequencing data will be taken a step further to evaluate whether prior infection by a given haplotype reduces the likelihood of subsequent infection by the same haplotype. Though only two antigens out of hundreds in the *P. falciparum* proteome with amplicon sequencing data will be surveyed, the CS and SERA2 are assumed to be highly polymorphic due to selection to evade an acquired immune response. Each locus exhibits high haplotype diversity, but harbors at least four common haplotypes with a population frequency of at least 10% at most of the MALARIA-055 study sites. Given the density of cross-sectional sampling in the MALARIA-094 trial design and the expectation that 10 or more infections will be detected from many participants, reasonable power to detect decreased susceptibility to infection by parasite strains exhibiting common amplicon haplotypes given prior infection by the same haplotype is anticipated.

One of the major unknowns in the development of strategies to interrupt malaria transmission using vaccine approaches that target incident infections is how high VE needs to be in order to drive R_0 to < 1. This study will capture both the incidence and prevalence of malaria infection using two accepted methodologies that differ in the sensitivity of parasite detection by several log fold, namely positive blood smear (asexual stage and gametocyte parasites) determined by expert microscopy and molecular genetic sequence-based technology. This study will provide the first ever comparison of malaria incidence based both on smear-positive (asymptomatic and clinical) infection in non-immune young children and also VE based on sub-microscopic parasite prevalence in asymptomatic children. Whether one believes that all parasites in any single individual need to be eliminated to achieve malaria elimination for a population or community is a matter of debate. Malaria has been eliminated from dozens of countries based solely on detection of new smear-positive clinical infections when the FOI was exceedingly low. The data collected here in conjunction with mathematical modeling (Gates Foundation Malaria Modeling Consortium) will provide for the first time an indication of the incidence of asymptomatic malaria infection after RTS,S immunization, the prolongation of VE with annual booster immunizations, the relative difference in the sensitivity of detection of parasites in the population, and VE as a measure of escape mutations in circulating strains that differ from vaccine strain.

2.4.4. Exploratory objective 3

- To determine whether prior infection by a particular parasite genotype, not necessarily the vaccine strain, reduces the likelihood of re-infection by a parasite with the same genotype.

Since the first new infection in young children may be a clinical malaria episode (uncomplicated or severe malaria), it is presently not possible to predict how antimalarial therapy impacts the clearance of blood stage parasitemia in children. We assume that asexual parasites are cleared from the blood as typically defined by the absence of parasites on subsequent thick blood smears. However, we have no knowledge of whether parasites at levels only detectable by ultrasensitive molecular methodologies are persistently present following treatment. Furthermore, we know that stage 5 gametocytes are not affected by antimalarial treatments that are aimed at clearing parasites in clinical malaria, and therefore we cannot exclude the possibility that such low levels of circulating gametocytes may be detected by the DNA amplification methodology used in this study. Hence we aim to understand how antimalarial treatment impacts the detection and definition of a new genotype in a subject previously identified as having an identical haplotype detected at a previous time point prior to antimalarial treatment. In geographic areas that have low parasite transmission, we predict that the complexity of infection will be reduced to less than 2 genotypes and therefore any recurrent haplotype may indeed be the basis for a new infection from a new mosquito bite (we do not assume that this is operative in Siaya, Kenya or Agogo, Ghana).

2.4.5. Exploratory objective 4

- To understand how gaps in time as measured by consecutive monthly dried blood spots impact VE against new infections.

Since parasites are not necessarily present in the peripheral circulation continuously over time because of parasite sequestration in the deep vascular beds, the absence of the same genotype(s) detected on successive monthly blood sampling does not exclude the possibility that the same parasite genotype may be still present in the subject, although undetected. For example, genotype A is present at Month 1, absent at Month 2, but genotype A is detected again at the Month 3 time point and may represent either persistent parasites or a new infection from a mosquito bite occurring in the interval between Month 2 and Month 3. We will explore whether such genotypes represent a new infection or represent persistent but undetected parasites from an antecedent infection.

3. RESEARCH DESIGN AND METHODS

3.1. Study Design Overview

This study is proposed to be an ancillary of the GSK RTS,S/AS01$_E$ phase IIb randomized trial MALARIA-094 (Study Number 204889) to be performed across all study sites. The incidence of *P. falciparum* infection defined by positive amplicon sequencing detection, and subsequent parasite genotyping, will be evaluated using samples collected as described in the MALARIA-094 trial.

The following hierarchical approach is proposed for sample evaluation to investigate the study objectives, with Tier 1 sampling essential to addressing the Primary Objective, and Tier 2 sampling to be performed after the primary immunization series and booster immunization series. Two analyses will be performed:

Tier 1: All cross-sectional dried blood spot samples collected monthly from all subjects during the first 20 months (approximately 31,500 samples) and clinical malaria cases (approximately 14,400) of the primary series from all arms of the study.

Tier 2: All cross-sectional dried blood spot samples collected for three consecutive months and then quarterly thereafter for one year following the first booster RTS,S dose (approximately 9,000 samples) and clinical malaria cases in all arms of the study up through Month 32 (approximately 8,640 samples).

In this genomic study interests include: i) assessing VE using molecular genetic data to identify first infections following vaccination, and to distinguish new from existing infections, ii) determining parasite positivity in cross-sectional samples from all children and distinguish new versus existing infections using an amplicon sequencing-based strategy: deep sequencing of small, highly variable regions of the parasite genome to allow for highly sensitive detection of parasitemia (analogous to conventional PCR-based detection), and iii) identification of genetically distinct parasite populations within or between affected individuals.

3.2. Outcomes, Predictors, and Other Covariates of Interest

3.2.1. Outcomes

The outcomes of interest will be: a) amplicon sequence-based parasite positive/negative status - positive status will be defined as ≥200 sequence reads aligning to at least one amplicon; and b) genotype data for the CS C-terminus and SERA2 loci – haplotypes observed at each amplicon following filtration of PCR and sequencing errors must be supported by 1% and at least 5 sequence reads aligned.

3.2.2. Main exposures

The main predictor of interest will be vaccination group as defined in MALARIA 094.

3.2.3. Candidate confounders

When studying all aims, additional predictors may be considered such as study site, age, date of infection or disease, parasitemia, time of detection from baseline, bednet use, number of antimalarial treatments, and relevant clinical characteristics, as available.

3.3. Study Cohort

3.3.1. Overview

Information about the study cohort(s) can be found in the main trial protocol MALARIA-094 (Study Number 204889).

3.3.2. Endpoints

Cohorts of analyses include According-To-Protocol (ATP) and Total Vaccinated Cohorts (TVC) which are described in the MALARIA-094 protocol. Analyses as per this study will be conducted on the same cohort of study participants with the following **Primary Endpoints**:

1. Time to the first new malaria infection
2. Number of new malaria infections during a specified follow-up period

A new malaria infection endpoint is defined by a detected new infection from genomic analysis of blood spots detected from active screening of infection or passive detection of clinical malaria (secondary case definition [no parasitemia threshold] as defined in the MALARIA-094 protocol); thus it measures either an asymptomatic or clinical malaria infection of a new genotype (primary objective) not previously detected either at baseline or in a prior sampling timepoint. Exploratory endpoints may define a “new” genotype less conservatively than the definition above.

Any participant may be excluded from the ATP analysis according to the criteria outlined in the main trial protocol (204889; MALARIA-094). Furthermore, exclusion of samples from the final analysis may occur if the sample: is not collected; is not collected with full or clear identification; does not yield any parasite genetic material upon process; or does not yield a clear and unambiguous genotyping result for any reason.

3.3.3. Total vaccinated cohort (TVC) and according-to-protocol (ATP) analyses

VE against each primary endpoint will be assessed using both a TVC and ATP analysis. For the primary endpoints these analyses will be based on data collected up to Month 20. For primary endpoint 1, the time to event is measured from the date of first vaccination in the TVC analysis and from the date of Dose 3 plus 14 days in the ATP analysis. For primary endpoint 2, the TVC analysis estimates the number of new malaria infections occurring from the date of first vaccination until Month 20, and the ATP analysis estimates the number of new malaria infections occurring from the date of Dose 3 plus 14 days until Month 20.

3.4. Parasite Genotyping Methodology

The Broad Institute Genomics Platform implements strict quality control guidelines to reduce the likelihood of sample contamination. Appropriate and rigorous data cleaning for all single nucleotide polymorphisms (SNPs) called will be performed. Unreliable

sequencing or genotyping data will be excluded from the analysis. All unused or discarded samples will be noted and reported as described in the main trial protocol MALARIA-094.

Current technology exists to accomplish the specific aims of this proposal and has been optimized and validated on samples from the MALARIA-055 phase 3 study (see Supplementary Appendix, Neafsey et al., 2015 for details). Briefly, custom workflows developed at the Broad Institute will be used to extract DNA from filter paper samples, PCR amplify the DNA, and sequence it via the Illumina MiSeq platform. Whatman FTA blood spot cards will be batched and shipped according to an agreed schedule from the GSK-designated clinical research organization (CRO) in Africa to the Broad Institute Genomics Platform (GP) under a Center for Disease Control (CDC) import permit. GSK barcodes will be registered in the GP laboratory information management system (LIMS), and the cards will be stored securely in desiccator cabinets at room temperature until further processing.

Genomic DNA extraction will be performed in batches of 96 FTA cards, including one blank FTA card as a control. For each FTA card, disks will be punched out of the blood spot, using an automated laser guided hole puncher, into a distinct well of 96 well plates. Genomic DNA will be extracted from the punches using the automated Chemagen Chemagic bead-based DNA extraction platform using standard protocols. DNA samples will be registered in LIMS and stored in barcoded tubes. The DNA concentration of each sample will be quantified using standard automated PicoGreen quantification. *Plasmodium* PCR amplicons; “CS C-terminus” and “SERA2”, will be amplified as previously described [Neafsey et al., 2015]. Samples will be batched into groups, typically 384 (4 sets of 96), with a negative control sample (originating from a blank blood spot card) included within each set of 96. For each batch, CS C-terminus and SERA2 PCR2 product pools will be normalized and combined into a single pool for MiSeq sequencing. Sequencing data will be processed through the Broad Picard sequencing analysis pipeline generating standard sequencing metrics (e.g. read counts) and demultiplexed sample specific sequencing read Binary Alignment/Map (BAM) files. BAM files will be screened and filtered for human contaminating sequences, and packaged for submission to the National Center for Biotechnology Information (NCBI) short read archive database. All steps will be tracked in the LIMS.

3.5. Conduct of the Study

3.5.1. Follow-up and identification of cases

Study visits, cross-sectional surveys and surveillance for clinical and severe malaria cases are detailed in the main trial protocol (204889; MALARIA-094). Blood smears and filter paper materials (Whatman FTA Cards) will be obtained within the current protocol as detailed in the main trial protocol (204889; MALARIA-094). Additionally, blood smears and filter paper materials will be collected from any participant diagnosed with clinical malaria disease or severe malaria disease throughout the trial period, according to the case definitions defined in the main trial protocol (204889; MALARIA-094).

3.5.2. Laboratory assays

All parasitological and clinical patient assessments will be performed as part of the vaccine trial procedures in compliance with the main trial protocol (204889; MALARIA-094).

3.5.3. Additional study procedures

Subject identification, sample handling, and safety monitoring will be performed in compliance with the main trial protocol (204889; MALARIA-094). All processes used to transfer information to analysis sites will be defined in collaboration with GSK.

4. INVESTIGATIONAL PRODUCTS AND ADVERSE EVENTS

This study will be using the same investigational products as the main trial protocol (204889; MALARIA-094) and adverse events will be collected under the MALARIA-094 protocol.

5. STUDY TIMELINE

The study timeline will follow the main trial protocol (204889; MALARIA-094) for collection times; parasite genotyping and sequence analysis will occur during the trial period. Results of genotyping will be kept in a database in Boston. Only the Principal Investigators (PIs) listed in this protocol, sub-Investigators and other staff members of the Broad Institute and Harvard T. H. Chan School of Public Health, designated by the PIs, will have access to the genotyping database before the end of the study.

6. STATISTICAL CONSIDERATIONS

Primary endpoints, TVC and ATP analyses are presented in Section 3.3.2 and Section 3.3.3.

6.1. Analytical Plan

A formal Statistical Analysis Plan (SAP) will be finalized and approved by all parties prior to data un-blinding. All genetic material and sequence analysis will be performed through the study period in a schedule agreed upon by GSK.

Briefly, an alpha level of 0.05 will be used to test statistical significance. All statistical tests will be two-sided. Baseline characteristics of interest will be summarized by the appropriate comparison group. The number of filter paper samples lost or not collected will also be tabulated by comparison groups, as well as PCR negative results or any other reason for excluding a sample from the analysis. In mixed infections, it is expected that sequencing will yield the genotype of the more prevalent strain(s) or a heterozygous call. All analyses across study sites will be adjusted for study site. Analysis stratified by study site will also be presented.

Primary Endpoint 1. Instantaneous VE against the first new malaria infection endpoint observed during 12 months after the third immunization in each vaccine arm vs. the common control vaccine arm will be assessed using a proportional hazards model with vaccine group indicator as the lone covariate, with the model stratified by study site (i.e., allowing a separate unknown baseline hazard of the first new malaria infection at each study site). The same proportional hazards modeling approach will be used for comparing the hazard of the first new malaria infection between pairs of vaccine arms, except hazard ratios will be assessed instead of VE parameters. A secondary analysis of the first new malaria infection endpoint will estimate instantaneous VE over time for each vaccine arm versus the control vaccine arm using nonparametric kernel smoothing with pointwise and simultaneous confidence bands [Gilbert et al., 2002] and using flexible partial-likelihood based proportional hazards modeling, for example using time-varying coefficients in the Cox model modeled with smoothing splines or using smoothed Schoenfeld residuals [Durham et al., 1998].

Overall VE against the first new malaria infection (Primary Endpoint 1) and VE against genotype-specific new malaria infection (with genotype defined by full vaccine match, a mismatch, or a genetic distance) (Exploratory Endpoint 1) will be estimated using both cumulative incidence and hazard ratio methodologies, with multiple outputation [Follmann et al., 2003] to analyze data from complex infections, as in MALARIA-066.

Primary Endpoint 2. Plots of estimates of reverse cumulative distribution functions (rcdfs) of the number of new malaria infections by study arm will be used to summarize the distributions of the number of new infections. VE against all new infections for a given vaccine arm vs. the control arm will be assessed in two ways. First, VE will be analyzed by negative binomial regression allowing for interdependence between episodes within the same participant (non-linear mixed model with overdispersion parameter estimated from the random effect and follow-up time entered into the model as an offset) and adjusting for study site. Second, VE against all new infections will be assessed by the additive difference in the mean number of all new malaria infections in each vaccine arm vs. the mean number of all new malaria infections in the control vaccine arm.

6.2. Sample Size and Power

For the MALARIA-094 study's primary endpoint, demonstrating incremental VE against clinical malaria of a Fx dose schedule over the standard schedule, the study has at least 90% power to show a significant incremental VE (lower limit [LL] of 95% CI above 0), assuming 250 evaluable subjects per group and an incidence of at least 0.5 episodes/per year (pyr) in the Control group, 0.29 episodes/pyr in the standard schedule groups and 0.16 episodes/pyr in the Fx dose schedule. This corresponds to an incremental efficacy (1-Hazard Rate) of 50% of the Fx dose schedule over the standard schedule. The MALARIA-094 study's primary endpoint compares the R012-20+R012-14-mD groups versus the Fx012-14-mFxD group (2:1). For the MALARIA-094 study's secondary endpoints evaluating efficacy against *P. falciparum* infections, it is anticipated that incidence of infection in the study will be higher than incidence of clinical malaria resulting in adequate power.

7. DATA MANAGEMENT METHODS

All data collected for this study will be part of the case report forms (CRFs) originally designed for the main trial protocol MALARIA-094. Samples will be tracked using the LIMS system in the Broad Genomics Platform; raw and filtered data will be stored in a Structured Query Language (SQL) database on a secure Broad server with user permissions restricted to designated study personnel. The clinical data management system will be overseen by GSK. Exchanges of clinical and genetic data will be performed according to a mutually agreed schedule and terms.

8. PROTECTION OF HUMAN RESEARCH SUBJECTS

No analysis of human DNA will be performed in this study. Samples will be collected exclusively for parasite genotyping. Therefore, this study will not result in any risks, legal or ethical concerns for subjects other than the ones discussed in the original study protocol (MALARIA-094). Subjects' discontinuation of the study will also follow procedures specified in the main protocol. Informed consent for the genotyping study will be included in the informed consent of the main protocol.

9. STUDY ORGANIZATION

- Dr. PPD , the leader of the Harvard/Broad Malaria Genome project will oversee the whole project, play a supervisory role and provide scientific guidance.
- Dr. PPD will have a key role in the development and choice of appropriate technology for evaluating parasite genetics and in both parasite genotyping and statistical analysis.
- Dr. PPD will oversee all parasite genotyping and will be responsible for communications with other investigators.
- Dr. PPD will provide consultation on laboratory processes and scientific guidance.
- Dr. PPD , Dr. PPD and Dr. PPD will design and oversee the statistical analysis of the genotyping data.
- Mr. PPD will contribute to scientific discussions, as needed.
- Dr. PPD will facilitate communications, and exchange of material and data among site investigators, GSK members, the Harvard T. H. Chan School of Public Health and Broad Institute, and the Fred Hutchinson Cancer Research Center.
- Mr. PPD will have an advisory role in the Genotyping study that involves statistical design and analysis of the genotyping infection endpoints.

10. REFERENCES

Alloueche A, Milligan P, Conway DJ, Pinder M, Bojang K, Doherty T, Tornieporth N, Cohen J, Greenwood BM. (2003) Protective efficacy of the RTS,S/AS02 Plasmodium falciparum malaria vaccine is not strain specific. Am J Trop Med Hyg, 68, 97-101.

Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, Mandomando I, Spiessens B, Guinovart C, Espasa M, Bassat Q, Aide P, Ofori-Anyinam O, Navia MM, Corachan S, Ceuppens M, Dubois MC, Demoitié MA, Dubovsky F, Menéndez C, Tornieporth N, Ballou WR, Thompson R, Cohen J. (2004) Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet, 364, 1411-20.

Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, Ballou WR, Conway DJ, Reece WH, Gothard P, Yamuah L, Delchambre M, Voss G, Greenwood BM, Hill A, McAdam KP, Tornieporth N, Cohen JD, Doherty T; RTS, S Malaria Vaccine Trial Team. (2001) Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 358, 1927-1934.

Durham LK, Longini IM Jr, Halloran ME, Clemens JD, Nizam A, Rao M. (1998) Estimation of vaccine efficacy in the presence of waning: application to cholera vaccines. Am J Epidemiol, 147, 948-595.

Enosse S, Dobaño C, Quelhas D, Aponte JJ, Lievens M, Leach A, Sacarlal J, Greenwood B, Milman J, Dubovsky F, Cohen J, Thompson R, Ballou WR, Alonso PL, Conway DJ, Sutherland CJ. (2006) RTS,S/AS02A malaria vaccine does not induce parasite CSP T cell epitope selection and reduces multiplicity of infection. PLoS Clin Trials, 1, e5.

Follmann D, Proschan M, Leifer E. (2003) Multiple outputation: inference for complex clustered data by averaging analyses from independent data. Biometrics, 59, 420-429.

Gilbert PB, Wei LJ, Kosorok MR, Clemens JD. (2002) Estimation of vaccine efficacy in the presence of waning: application to cholera vaccines. Biometrics, 58, 773-780.

Kester KE, McKinney DA, Tornieporth N, Ockenhouse CF, Heppner DG, Hall T, Krzych U, Delchambre M, Voss G, Dowler MG, Palensky J, Wittes J, Cohen J, Ballou WR; RTS,S Malaria Vaccine Evaluation Group. (2001) Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria. J Infect Dis, 183, 640-7.

Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, Schwenk R, Nielsen RA, Debebe Z, Pinelis E, Juompan L, Williams J, Dowler M, Stewart VA, Wirtz RA, Dubois MC, Lievens M, Cohen J, Ballou WR, Heppner DG Jr; RTS,S Vaccine Evaluation Group. (2009) Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01 and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis, 200, 337-46.

Lalvani A, Moris P, Voss G, Pathan AA, Kester KE, Brookes R, Lee E, Koutsoukos M, Plebanski M, Delchambre M, Flanagan KL, Carton C, Slaoui M, Van Hoecke C, Ballou WR, Hill AV, Cohen J. (1999) Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine. J Infect Dis, 180, 1656-1664.

Mueller I, Schoepflin S, Smith TA, Benton KL, Bretscher MT, Lin E, Kiniboro B, Zimmerman PA, Speed TP, Siba P, Felger I. (2012) Force of infection is key to understanding the epidemiology of Plasmodium falciparum malaria in Papua New Guinean children. Proc Natl Acad Sci USA, 109, 10030-10035.

Nardin EH, Nussenzweig RS. (1993) T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. Annu Rev Immunol, 11, 687-727.

Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, Lievens M, Abdulla S, Adjei S, Agbenyega T, Agnandji ST, Aide P, Anderson S, Ansong D, Aponte JJ, Asante KP, Bejon P, Birkett AJ, Bruls M, Connolly KM, D'Alessandro U, Dobaño C, Gesase S, Greenwood B, Grimsby J, Tinto H, Hamel MJ, Hoffman I, Kamthunzi P, Kariuki S, Kremsner PG, Leach A, Lell B, Lennon NJ, Lusingu J, Marsh K, Martinson F, Molel JT, Moss EL, Njuguna P, Ockenhouse CF, Ogutu BR, Otieno W, Otieno L, Otieno K, Owusu-Agyei S, Park DJ, Pellé K, Robbins D, Russ C, Ryan EM, Sacarlal J, Sogoloff B, Sorgho H, Tanner M, Theander T, Valea I, Volkman SK, Yu Q, Lapierre D, Birren BW, Gilbert PB, Wirth DF. (2015) Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. N Engl J Med, 373, 2025-2037.

Ockenhouse CF, Regules J, Tosh D, Cowden J, Kathcart A, Cummings J, Paolino K, Moon J, Komisar J, Kamau E, Oliver T, Chhoeu A, Murphy J, Lyke K, Laurens M, Birkett A, Lee C, Weltzin R, Wille-Reece U, Sedegah M, Hendriks J, Versteege I, Pau MG, Sadoff J, Vanloubbeeck Y, Lievens M, Heerwegh D, Moris P, Guerra Mendoza Y, Jongert E, Cohen J, Voss G, Ballou WR, Vekemans J. (2015) Ad35.CS.01 - RTS,S/AS01 heterologous prime boost vaccine efficacy against sporozoite challenge in healthy malaria-naïve adults. PLoS One, 10(7), e0131571.

Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, Lang T, Gould J, Dubois MC, Jongert E, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S, Mturi N, Drakeley CJ, Savarese B, Villafana T, Lapierre D, Ballou WR, Cohen J, Lemnge MM, Peshu N, Marsh K, Riley EM, von Seidlein L, Bejon P. (2011) Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5–17 months in Kenya and Tanzania: a randomised controlled trial. Lancet Infect Dis, 11, 102-9.

Pinder M, Reece WH, Plebanski M, Akinwunmi P, Flanagan KL, Lee EA, Doherty T, Milligan P, Jaye A, Tornieporth N, Ballou R, McAdam KP, Cohen J, Hill AV. (2004) Cellular immunity induced by the recombinant Plasmodium falciparum malaria vaccine, RTS,S/AS02, in semi-immune adults in The Gambia. Clin Exp Immunol, 135 (2), 286-93.

Stoute JA, Kester KE, Krzych U, Wellde BT, Hall T, White K, Glenn G, Ockenhouse CF, Garcon N, Schwenk R, Lanar DE, Sun P, Momin P, Wirtz RA, Golenda C, Slaoui M, Wortmann G, Holland C, Dowler M, Cohen J, Ballou WR. (1998) Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. J Infect Dis, 178, 1139-44.

Waitumbi JN, Anyona SB, Hunja CW, Kifude CM, Polhemus ME, Walsh DS, Ockenhouse CF, Heppner DG, Leach A, Lievens M, Ballou WR, Cohen JD, Sutherland CJ. (2009) Impact of RTS,S/AS02A and RTS,S/AS01B on genotypes of P. falciparum in adults participating in a malaria vaccine clinical trial. PLoS One, 4(11), e7849.

Weedall GD, Preston BM, Thomas AW, Sutherland CJ, Conway DJ. (2007) Differential evidence of natural selection on two leading sporozoite stage malaria vaccine candidate antigens. Int J Parasitol, 37, 77-85.

White MT, Bejon P, Olotu A, Griffin JT, Riley EM, Kester KE, Ockenhouse CF, Ghani AC. (2013) The relationship between RTS,S vaccine-induced antibodies, CD4 T cell responses and protection against Plasmodium falciparum infection. PLoS One, 8(4), e61395.

WHO World Malaria Report 2015. http://who.int/malaria/publications/world-malaria-report-2015/report/en/. Accessed 30 January 2017.

Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V. (1983) Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J Exp Med, 157, 1947-1957.