

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Abdulla S, Oberholzer R, Juma O, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. *N Engl J Med* 2008;359:2533-44. DOI: 10.1056/NEJMoa0807773.

## **Supplementary Methods**

### **Ethical considerations**

This Phase IIb, single-centre, double-blind, controlled trial was conducted between July 2006 and February 2008 by the Bagamoyo Research & Training Centre, a branch of the Ifakara Health Institute (IHI; formerly Ifakara Health Research & Development Centre, IHRDC) in Bagamoyo, Tanzania.

### **Investigator and sponsor roles**

The study was sponsored by GlaxoSmithKline Biologicals (GSK), the vaccine developer and manufacturer, and funded by the PATH Malaria Vaccine Initiative. The data were subject to a confidentiality agreement between the sponsor and investigators, which established full access to the study data by the investigators and included an obligation to permit publication without excessive delay.

### **Location and site details**

The study was conducted in and around Bagamoyo town, on the Tanzanian coast, 70 km north of Dar-es-Salaam. Approximately 61 000 people live in villages in the area. The main rainy season is from March to May, with a second period from November to December; the average rainfall is 1200 to 2100 mm per year. The majority of the residents are subsistence farmers and 65% of them are literate.

Malaria transmission in this area is perennial and almost entirely due to *P. falciparum*.

The main vectors are *Anopheles gambiae sensu stricto*, *An. arabiensis* and *An. funestus*. Insecticide Treated Bednets (ITN) are promoted through a national programme and Artemether/Lumefantrine (Coartem®) is currently the first line treatment in Tanzania.

Vaccination services are organized at Reproductive and Child Health clinics at dispensaries following the cold chain procedures established at the national level.

Vaccination coverage (fully vaccinated with BCG, DTP, HBV polio and measles) is estimated to be more than 90% in Bagamoyo.

### **Participants**

Trained Village Health Care Workers identified pregnant women in their third trimester at home and at antenatal clinics in the study area, provided them with the study information sheet about the study and invited those interested to attend Bagamoyo District Hospital (BDH) for formal consenting. A trained counselor provided further information and enrolled the women for screening after oral verification of the comprehension of the Swahili information sheet and a written consent. The screening procedures of antenatal mothers included medical history, physical examination, HIV, HBV and other routine antenatal tests. Consented mothers were screened for HIV (Determine HIV1-2, Abbott Laboratories, Tokyo, Japan, and Capillus HIV, Trinity Biotech, Bray, Ireland) and hepatitis B (OMEGA PATHOZYME, Diagnostics, Scotland, UK). Women identified with HIV were referred to BDH to get HIV care and treatment as per national guidelines. Babies born to mothers who were found to be HepB positive were offered vaccination against hepatitis B starting at birth. Pregnant women were provided with and trained to use an ITN at delivery.

All enrolled women were encouraged to deliver at the BDH and were followed up until delivery and vaccination of the infants with BCG and oral polio. The infants were invited for screening and vaccination at between 6 and 10 weeks of age. A separate written (witnessed thumbprint if illiterate) informed consent was asked from the parents/guardians of the infants after oral verification of comprehension of the information sheet. Exclusion criteria included: children born to HIV or HBV positive mothers; birth weight below 2.5 kg; malformations at birth; same sex twins; duration

of pregnancy outside 36 to 42 weeks. Screening involved a medical history, examination, and blood sampling for baseline hematology, biochemistry, and immunology.

### **Randomization and blinding**

If there were no contraindications to vaccination and all inclusion and exclusion criteria were fulfilled, the child was randomized by being assigned a sequential number. The vaccines were packaged in identical boxes, identified by treatment numbers corresponding to a randomization list generated at GSK Biologicals, Rixensart, and then shipped to the trial sites. Randomization was undertaken at GSK Biologicals (SAS version 9). A photoidentity card of the mother and child including information on their names, randomization number, emergency call numbers and important trial milestones, was provided to the caregivers of the participants.

To maintain blinding of the investigators responsible for endpoint evaluation and participants, an independent team (not blinded and not involved in any other study procedure) prepared and administered vaccines. Two restricted access rooms with a small interconnecting window were used for the vaccination process. Vaccines were administered to participants after verification of identity. Data pertaining to RTS,S/AS02D or hepatitis B vaccine were collected in an observer-blinded manner; data relating to DTP/Hib vaccine were collected in an open fashion. The code was released once databases had been monitored, checked for inconsistencies, and locked.

### **Study vaccines**

Each dose of RTS,S/AS02D (0.5 mL) contained 25 µg of RTS,S and the Adjuvant System AS02D, as previously described<sup>1</sup>. RTS,S is a hybrid recombinant protein consisting of the *P. falciparum* circumsporozoite protein central tandem repeat and carboxy-terminal regions fused to the amino-terminus of the S antigen of hepatitis B

virus (HBsAg). The proteins auto-assemble to form a particle that also includes unfused S antigen.

### Safety assessments

Blood for safety monitoring of hematology (hemoglobin, hematocrit, platelets, WBC), renal (creatinine, bilirubin) and hepatic (ALT) function was measured at screening, one week post-Dose 1 and one month post-Dose 3. The acceptable/normal ranges for blood testing are presented below:

	Acceptable	Toxicity grading scale			
	limit/ normal range	Grade 1	Grade 2	Grade 3	Grade 4
Hemoglobin	$\geq 8.0$ g/dL	< LLN	< 6.0 g/dL	< 5.0 g/dL	< 5.0 g/dL & clinical signs of heart failure
Total white cell count	$\geq 4.0 \times 10^3$ / $\mu$ L < 17 x 10 <sup>3</sup> / $\mu$ L	2.5 to LLN	1.5 to 2.4 x 10 <sup>3</sup> / $\mu$ L	1.0 to 1.4 x 10 <sup>3</sup> / $\mu$ L	< 1.0 x 10 <sup>3</sup> / $\mu$ L
Platelets	$\geq 100 \times 10^3$ / $\mu$ L	50 to 99 x 10 <sup>3</sup> / $\mu$ L	25 to 49 x 10 <sup>3</sup> / $\mu$ L	< 25 x 10 <sup>3</sup> / $\mu$ L	< 25 x 10 <sup>3</sup> / $\mu$ L & clinical signs of bleeding
ALT	$\leq 60$ IU/L	1.1 to 2.5 x ULN	2.6 to 5.0 x ULN	5.1 to 10.0 x ULN	> 10.0 x ULN
Creatinine	$\leq 60$ $\mu$ mol/L (or 0.6 mg/dL)	1.1 to 1.5 x ULN	1.6 to 3.0 x ULN	3.1 to 6.0 x ULN	> 6.0 ULN or requires dialysis

ULN: Upper Limit of Normal LLN: Lower Limit of Normal

The intensity of adverse events was graded on a scale of 0–3, as defined below:

<b>Adverse event</b>	<b>Intensity grade</b>	<b>Parameter</b>
Pain at injection site	0	Absent
	1	Minor reaction to touch
	2	Cries/protests on touch
	3	Cries when limb is moved/spontaneously painful
Swelling at injection site	0	None
	1	< 5 mm
	2	5 to 20 mm
	3	> 20 mm
Fever*	0	< 37.5°C
	1	37.5-38.0°C
	2	> 38-39.0°C
	3	> 39.0°C
Irritability/Fussiness	0	Behaviour as usual
	1	Crying more than usual/ no effect on normal activity
	2	Crying more than usual/ interferes with normal activity
	3	Crying that cannot be comforted/ prevents normal activity
Drowsiness	0	Behaviour as usual
	1	Drowsiness easily tolerated
	2	Drowsiness that interferes with normal activity
	3	Drowsiness that prevents normal activity
Loss of appetite	0	Appetite as usual
	1	Eating less than usual/ no effect on normal activity
	2	Eating less than usual/ interferes with normal activity
	3	Not eating at all

\*Fever is defined as axillary temperature  $\geq 37.5^{\circ}\text{C}$

## **Monitoring for clinical malaria episodes**

The active surveillance period for malaria infection began 14 days after Dose 3 and continued every 2 weeks for approximately 7 months. Blood samples were taken for examination of malaria parasitaemia at each ADI visit. No further visits for surveillance of infection were undertaken after the first detection of asexual *P. falciparum* infection. The current clinical practice at BDH, to treat malaria if history of fever within the previous 24 hours or documented fever (temperature  $\geq 37.5^{\circ}\text{C}$ ) at the time of presentation and any level of parasitaemia, was not changed. All breakthrough infections detected by ADI or PCD were treated. Treatment for episodes of malaria was with artemether-lumefantrine. Children requiring admission and too unwell to take oral medication were treated with intravenous quinine. Blood slides were collected during cross-sectional assessment after Coartem® treatment, active detection of infection and passive follow up of illnesses at the health facility for the determination of parasitaemia. Duplicate blood slides were taken and labeled with the same unique laboratory ID number, which was also recorded on the IHRDC clinic morbidity surveillance questionnaire. Both slides were taken to the IHRDC Laboratory and stained with Giemsa Stain. One was read immediately (within 2 hours) and the result reported to guide diagnosis and management. The second slide was kept for a later reading, according to IHRDC SOPs, and determined parasite density for data analysis. Parasite counts were made relative to a concomitantly measured white cell count<sup>2</sup>. Well trained and accredited laboratory technicians read the giemsa stained thick and thin films in the Bagamoyo research laboratory. Films were read in duplicate and third read if there was discordance of the first two. Discordance was defined as difference of malaria positivity or the ratio of their natural log of counts not to be between 0.67 and 1.50 when absolute counts were

more than 10. For the discordance of positivity the majority of either positive or negative was taken as the final results and for the final results the geometric mean of the three was taken as the final count. Final densities were undertaken for all slides and included into the database before unblinding was done. The majority of the slides (98%) were negative. A 3<sup>rd</sup> read was required in 0.8% of all slides and 37% of the positive slides. For the slides that required a 3<sup>rd</sup> read, 32% were from RTS,S/AS02 group. No adjustment was made in analysis due to the small number of events involved and the fact that the main efficacy outcome interest was incidence of any density of malaria infection.

Biochemical parameters were measured using a dry biochemistry photometer VITROS DT II (Orto Clinical Diagnostics, Johnson & Johnson Company, Rochester, NY, USA). Hematological tests were performed using a Sysmex KX-21N cell counter (Sysmex Corporation Kobe, Japan).

### **Laboratory analysis**

Antibodies to the circumsporozoite protein (CS) tandem repeat epitope were assessed by ELISA. The antibody response to the *P. falciparum* CS repeat region (designated anti-CS) was measured at CEVAC, Ghent, Belgium and results were reported in EU/mL. Plates were adsorbed with the recombinant antigen R32LR that contains the sequence [NVDP(NANP)<sub>15</sub>]<sub>2</sub>LR.

Anti-HBs antibody levels were measured with a commercial radioimmunoassay (AUSAB, Abbott, IL, USA) with an assay cut-off of 10 mIU/mL. Anti-PRP antibodies were measured by ELISA with a cut-off of 0.15 µg/mL. Anti-diphtheria and anti-tetanus antibodies titers were measured by ELISA with an assay cut-off of 0.10 IU/mL. Anti-whole-cell-B pertussis (Pw) antibody titers were determined by ELISA (Labsystems, Vantaa, Finland) with an assay cut-off of 15 EU/mL<sup>3</sup>.

### **Statistical analysis**

Anti-CS and anti-HBs antibody data were summarized by geometric mean titers (GMTs) with 95% confidence intervals (CIs). Anti-CS seropositivity was defined as  $\geq 0.5$  EU/mL and seroprotection against hepatitis B was defined as  $\geq 10$  IU/mL.

The effect of anti-CS antibody titers on the risk of malaria infection was assessed by estimating the hazard ratio per doubling of anti-CS antibody titer with Cox regression models. Also, we compared the GMT in RTS,S/AS02D recipients who had at least one episode of malaria infection against those without documented malaria infection using a Wilcoxon Rank Sum test.

### **Study cohorts**

The intent-to-treat (ITT) cohort included all randomized children; for the ITT cohort time at risk started at dose 1 and ended at the cross-sectional visit at study month 9 or drop out, whichever occurred first. The according to protocol (ATP) immunogenicity cohort included all subjects receiving 3 doses of study vaccine according to the randomization list. Subjects were only included if doses and blood samplings occurred within protocol-specified timeframes and no immune-modifying drugs/blood transfusions or underlying medical conditions influencing immune responses had been reported. The ATP efficacy cohort included subjects receiving 3 doses of study vaccine according to randomization list, within protocol specified ranges. Subjects not receiving clearance drug or not contributing time at risk and subjects with underlying medical conditions influencing immune responses were excluded. Time at risk for the ATP efficacy cohort started 14 days post dose 3 and ended at the cross-sectional visit at month 9 or withdrawal. Time at risk was corrected for absences more than 2 weeks from the study area and antimalarial drug use.

## References

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