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Duration of vaccine efficacy against malaria: 5th year of follow-up in children vaccinated with RTS,S/AS02 in Mozambique

Joseph J. Campo[a,b,*], Jahit Sacarlal[b,c], John J. Aponte[a,b], Pedro Aide[a,d], Augusto J. Nhabomba[b], Carlota Dobaño[a,b], Pedro L. Alonso[a,b]

[a] Barcelona Centre for International Health Research CRESIB, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain
[b] Centro de Investigaçao em Saúde de Manhiça (CISM), Manhiça, Mozambique
[c] Faculdade de Medicina, Universidade Eduardo Mondlane, Maputo, Mozambique
[d] Instituto Nacional de Saúde, Ministério de Saúde, Maputo, Mozambique

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ABSTRACT

A primary concern for the RTS,S malaria vaccine candidate is duration of protection. The ongoing Phase III trial reported evidence of waning efficacy within the first year following vaccination. Multiple Phase IIb trials demonstrated early waning of efficacy. The longest duration of protection for RTS,S recorded to date was in a trial of a cohort of 1605 Mozambican children age 1–4 yr at the time of immunization (C1), which showed an overall efficacy against clinical malaria of 30.5% over 43 subsequent months of surveillance. A significant reduction in parasite prevalence in RTS,S vaccinees indicated that the vaccine continued to protect at the end of this period. Although follow-up for recording incident cases of clinical malaria was stopped at 45 months, we were interested in evidence of further durability of protection, and revisited the cohort at 63 months, recording the secondary trial endpoint, prevalence of asexual *Plasmodium falciparum* parasitemia, in the RTS,S and comparator vaccine groups as a proxy for efficacy. As a comparator, we also visited the contemporaneous cohort of 417 children (C2), which showed waning efficacy after 6 months of follow-up. We also assessed anti-circumsporozoite antibody titers. These results were compared with those of other Phase IIb trials. Prevalence of parasitemia was not significantly lower in the RTS,S/AS02 group compared to comparator groups in C1 (57 [119%] Vs 62 [128%]; $p = 0.696$) or C2 (30 [226%] Vs 35 [276%]; $p = 0.391$), despite elevated antibody titers, suggesting that protection did not extend to 5 years after vaccination. This is in contrast to the earlier assessment of parasitemia in C1, where a 34% lower prevalence of parasitemia was observed in the RTS,S/AS02 group at month 45. Comparison with other Phase II trials highlights a complex relationship between efficacy, age and transmission intensity. RTS,S/AS02 provided partial protection from clinical malaria for at least 3.5 years in C1. Duration of protection may depend on environmental circumstances, such as changing malaria transmission, and special attention should be given in the Phase III trial to identifying factors that modify longevity of protection.

1. Introduction

Malaria remains one of the most pressing issues in global health, with approximately 216 million cases and 655,000 malaria-related deaths worldwide in 2010, mostly in Africa [1]. This represents a 17% reduction in malaria incidence and 26% reduction of malaria mortality since 2000, but still short of the 50% reduction targeted in the Global Malaria Action Plan [2]. An efficacious malaria vaccine is widely believed to be an important tool in meeting these targets and eventually participating in eliminating malaria when used along with other measures [3]. A minimally 50% efficacious malaria vaccine that lasts at least a year is critical to the 2015 landmark goals of the Malaria Vaccine Technology Roadmap (http://www.malariavaccine.org/malvac-roadmap.php).

A vaccine's duration of protection can be considered the length of time during whichvaccination provides efficacy above zero. However, definitions vary and can include significance in efficacy over the entire follow-up period [4], point efficacy estimates above zero [5] or lower 95% confidence interval bounds above zero. Loss of vaccine efficacy over time is referred to as "waning", although assessment of waning remains a complex problem [6].

* Corresponding author at: C/ Rosselló, 153, Centro Ester Koplowitz 1ª Planta, Barcelona, 08036, Spain. Tel.: +34 932275400x4584; fax: +34 933129410.
E-mail addresses: joe.campo@cresib.cat, campoj@gmail.com (J.J. Campo).

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The common approach for determining waning efficacy has been to test the proportionality of the hazard (PH) assumption (the assumption that the relative hazard of two groups is constant over time) using one or more methods, such as sequential point efficacy estimates, graphical interpretation of efficacy over time or time-dependent Cox regression models.

The leading candidate, GlaxoSmithKline's RTS,S/AS01E, is in a Phase III clinical trial and has thus far demonstrated 55.8% efficacy (97.5% CI 50.6–60.4) against clinical malaria during the first year of follow-up after 3 doses in children aged 5–17 months at the age of first vaccination [7]. In infants aged 6–12 weeks of age at time of first vaccination, vaccine efficacy was 31.3% (97.5% CI 23.6–38.3) during the first year of follow-up [5]. However, the study may have already shown signs of waning efficacy. For both age groups, children and infants, evaluation of the proportionality of the hazard assumption showed that vaccine efficacy was not constant over time, with efficacy higher at the beginning than at the end of the follow-up period [7,8].

RTS,S is a recombinant antigen based upon a single polypeptide chain of the *P. falciparum* circumsporozoite protein (CSP), which contains both B cell and T cell epitopes, fused to hepatitis B surface antigen [9] and formulated with either an oil-in-water emulsion (AS02) or liposomes (AS01) plus immunostimulants QS21 and 3D-MPL [10]. Phase II clinical development of RTS,S has consistently shown moderate protective efficacy (30–65%) in both naïve adults and endemic adults and children, but has given varying results on the duration of protection [9,11–18]. A Phase IIb trial in Mozambican children reported significant efficacy against risk of clinical malaria, extending to at least a 45 month surveillance period [4], and further follow-up of other Phase IIb trials is expected [16]. Additionally, the age of children at vaccination has varied between trials and, with it, estimates of efficacy and duration.

Vaccination with RTS,S generates high antibody titers to the NANP repeat region of the CSP that decline precipitously to 25% of peak antibody titers over 6 months, but have remained significantly higher than that of comparator vaccinated individuals for up to 3.5 years after vaccination [19]. Antibody titers at 1 month post-vaccination have been correlated with risk of infection, but have not consistently shown correlation with prospective risk of clinical malaria episodes [15,18,20], although titers at 6.5 months post vaccination were correlated with risk of malaria cases in one study [21]. To date, no immune determinant has correlated well enough with risk of malaria to serve as a surrogate marker of protection.

The largest Phase IIb trial of RTS,S was a proof-of-concept trial of 2022 Mozambican children, designed with two cohorts for different efficacy endpoints: Cohort 1 (C1) in Manhiça town with 1605 children followed by passive case detection for assessment of efficacy against risk of clinical malaria and Cohort 2 (C2) in Ilha Josina with 417 children followed by active detection of infection for assessment of efficacy against risk of infection [22]. Vaccine efficacy against clinical malaria in C1 during 45 months of surveillance was 30.5%, and tests based on Schoenfeld residuals and time-dependent Cox regression models, which test the PH assumption, found no evidence of waning [4,15,23]. Importantly, efficacy against malaria infection waned in C2. Point prevalence of parasitemia at cross-sectional timepoints, a secondary trial endpoint, was also measured as an indicator of protection. Strong supporting evidence for the duration of protection was found in the marked difference in cross-sectional parasitemia between RTS,S and comparator vaccine groups in C1 up to study month 45 [4].

Given that waning efficacy has been observed in other trials of RTS,S [5,18,23], including the Phase III study [7,8], it is important to validate the findings in C1 and duration of protection. In this study, we revisit the two Manhiça cohorts at 63 months after first dose of vaccine to determine if evidence exists for protection beyond 3.5 years after vaccination, and we compare this trial with other trials of RTS,S to identify factors that influence duration of protection.

2. Materials and methods

2.1. Study population

The study was conducted at the Centro de Investigação em Saúde de Manhiça (CISM) in Manhiça District, Mozambique. The trial began in April 2003, and the currently reported cross-sectional survey was carried out from August to September of 2008. The characteristics of the study area have been described elsewhere [24]. The study participants were enrolled as part of a Phase IIb proof-of-concept randomized, controlled trial of the RTS,S/AS02 vaccine (GSK Biologicals, Rixensart, Belgium), administered intramuscularly in the deltoid region of alternating arms in three monthly doses to 1–4 year-old Mozambican children (ClinicalTrials.gov registry number NCT00197041), and the trial study design and population demographic and baseline clinical characteristics have been described in detail in multiple primary research articles [4,15,22,23].

The study was approved by the Mozambican National Health and Bioethics Committee, the Hospital Clínic of Barcelona Ethics Committee and the PATH Research Ethics Committee. Written informed consent was obtained from parents/guardians using standard ICH guidelines. All study participants invited to the study month 63 cross-sectional survey were those that had previously given written informed consent, obtained from the parents/guardians, for participation in the trial up to study month 45.

2.2. Study procedures

A new study protocol was developed to conduct an additional cross-sectional survey at 63 months post-first vaccination, or approximately 5 years after receiving a full course of RTS,S/AS02 or comparator (seven-valent pneumococcal conjugate vaccine and Haemophilus influenzae type b vaccine in children under 24 months of age; pediatric hepatitis B vaccine in children 24 months of age or older) vaccine. Passive detection of clinical malaria was registered as per protocol until study month 45, after which children followed standard care provided by the National Ministry of Health. During the period between study month 45 and the new protocol at study month 63, no monitoring of participants for the trial primary endpoint of clinical malaria or loss to follow-up occurred. The study was formally unblinded after 6 months of follow-up after dose 3 (study month 8.5), but study participants and case ascertainment mechanisms remained blinded, as described previously [4]. All children originally enrolled in the trial that had not withdrawn from the study, migrated, or died were invited to participate in the cross-sectional clinic-based survey. Children and accompanying family members or guardians were taken to the Manhiça District Hospital or Ilha Josina Health Post for the survey. Information on use of malaria preventive measures was gathered by completing a questionnaire. Hematocrit was assessed by measurement of packed cell volume in capillary tubes. Thick and thin blood smears were prepared and stained with Giemsa's azure eosin methylene blue (Merck & Co., Inc., Whitehouse Station, NJ) for assessment of asexual *P. falciparum* parasitemia. A blood sample was collected in a BD microtainer tube with lithium-heparin (Becton Dickinson, Franklin Lakes, NJ) for collection of plasma. A subset of 220 C1 and 180 C2 plasma samples were randomly selected within cohorts for assessment of antibodies. Levels of IgG antibodies to the NANP repeat region of the CSP were measured by an ELISA coated with recombinant R32LR antigen as previously described [19]. The assays were performed using a validated assay at CEVAC,

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Table 1
Common approaches for assessing waning of vaccine efficacy.

Method	Result of test	Limitation
Schoenfeld residuals	Significance equals rejection of the null hypothesis of no change in PH over time, indicating change in efficacy	Does not evaluate the path of change in efficacy
Time-dependent Cox regression models	Identifies if models with time interactions provide better fits; Graphs of efficacy over time show PH and trend (zero slope: no change in efficacy; positive slope: increasing efficacy; negative slope: waning efficacy)	Unclear criteria on interpretation of slope and confidence intervals
Efficacy in sequential follow-up intervals	Multiple point efficacy measurements show trend in efficacy	Selection of follow-up intervals is subjective; power of estimates may change over time
Kaplan–Meier curves	Visual assessment of curves shows PH by trend of lines, e.g. separating to parallel lines denote change in efficacy	Subjective assessment of trend; difficult to determine points of change

University of Ghent, and antibody concentrations were expressed in EU/mL, with a lower limit of quantification of 0.25 EU/mL [25].

2.3. Statistical methods

The primary outcome was prevalence of asexual *P. falciparum* parasitemia and was compared between RTS,S/AS02 and comparator vaccine group by Fisher's exact test in each study cohort. Antibody levels were summarized by vaccine group as geometric mean titers (GMT), and seropositivity was defined as anti-R32LR titers $\geq$0.5 EU/mL. For means and proportions, 95% confidence intervals were calculated. Prevalence of anemia and fever and use of bednets and indoor residual spraying were assessed by Fisher's exact test. Continuous data for parasite density, antibody levels, axillary temperature and hematocrit were assessed by Wilcoxon rank-sum test. For each cohort (C1 and C2), both the children fulfilling According-To-Protocol (ATP) and the Intention-To-Treat (ITT) criteria, as previously defined [4], were included in analysis, separately. Information from reported Phase IIb and Phase III RTS,S/AS clinical trial results were tabulated for comparison purposes, including reported tests on the PH assumption (Table 1). All analyses were performed using STATA statistical software, version 12 (College Station, TX).

3. Results

3.1. Study subjects

All children that had completed follow-up previously were invited to participate in this cross-sectional survey, on average 63 months after the first dose of RTS,S/AS02 or comparator vaccine. A total of 1,375 children in C1 and 321 children in C2 were invited to participate. Of these, 1,011 children (74%) in C1 (966 from ATP cohort) and 291 children (91%) from C2 (260 from ATP cohort) were enrolled. The survey took place at the end of August through September 2008, at the beginning of the high malaria transmission season. At the time of the survey, children in both cohorts were from 6 to 10 years of age. Additional characteristics of the study population in each treatment group are provided in Table 2. The ATP and ITT cohorts were similar in all comparisons, and thus only the ATP cohort is discussed further.

3.2. Protection and immunogenicity in Mozambican cohorts 1 & 2

In C1, the RTS,S/AS02 and comparator vaccine groups had similar prevalence of fever (6 [13%] Vs 6 [12%], respectively; $p = 1.0$) and anemia (23 [48%] Vs 19 [39%], respectively; $p = 0.533$) at time

Table 2
Characteristics of the ATP study cohorts between RTS,S/AS02 and Comparator vaccine groups at 63 months after first vaccine dose.

	RTS,S/AS02A vaccine group (SD) (n/N)	Comparator vaccine group (SD) (n/N)	*P* value
Cohort 1			
Axillary temperature (°C)[a]	36.6 (0.41)	36.6 (0.45)	0.087[b]
Fever (%)	1.3 [6/479]	1.2 [6/485]	1.000[c]
Hematocrit (% PCV)[a]	34.3 (8.21)	34.3 (7.54)	0.224[b]
Anemia (%)	4.8 [23/481]	3.9 [19/483]	0.533[c]
Recent bednet use (%)	30.6 [147/481]	29.7 [144/485]	0.779[c]
Of which are ITN (%)	92.5 [137/151]	85.4 [123/146]	0.062[c]
Recent IRS treatment (%)	60.3 [290/481]	60.8 [295/485]	0.895[c]
Parasite density (parasites/μL)[d]	862 (6.60)	983 (6.73)	0.693[b]
Parasitemia (%)	11.9 [57/479]	12.8 [62/485]	0.696[c]
α-CSP GMT (EU/mL)[d]	6.6 (4.63)	0.3 (2.23)	<0.0001[b]
α-CSP seropositivity (%)	94.0 [94/100]	9.3 [10/108]	<0.001[c]
Cohort 2			
Axillary temperature (°C)[a]	36.6 (0.34)	36.6 (0.34)	0.593[b]
Fever (%)	1.5 [2/133]	1.6 [2/127]	1.000[c]
Hematocrit (% PCV)[a]	36.0 (5.20)	34.9 (5.16)	0.004[b]
Anemia (%)	1.5 [2/133]	1.6 [2/127]	1.000[c]
Recent bednet use (%)	35.3 [47/133]	31.5 [40/127]	0.599[c]
Of which are ITN (%)	89.4 [42/47]	85.0 [34/40]	0.748[c]
Recent IRS treatment (%)	29.3 [39/133]	29.9 [38/127]	1.000[c]
Parasite density (parasites/μL)[d]	390 (6.14)	230 (3.69)	0.244[b]
Parasitemia (%)	22.6 [30/133]	27.6 [35/127]	0.391[c]
α-CSP GMT (EU/mL)[d]	15.6 (3.02)	0.4 (2.19)	<0.0001[b]
α-CSP seropositivity (%)	100 [85/85]	31.6 [25/79]	<0.001[c]

[a] Estimated using arithmetic mean.
[b] Wilcoxon rank-sum test.
[c] Fisher's exact test.
[d] Estimated using geometric mean and geometric standard deviation.

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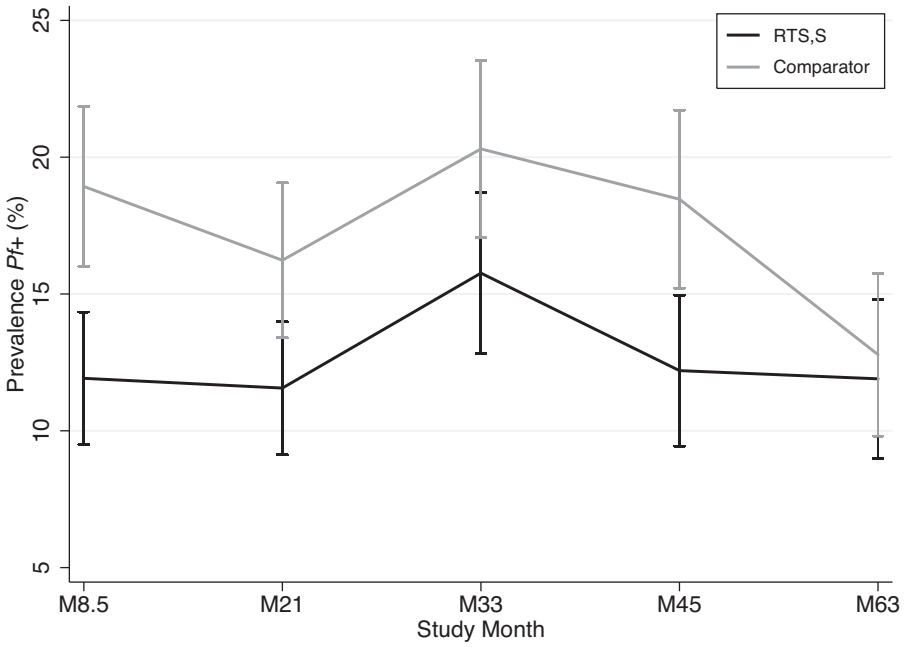

Fig. 1. Prevalence of asexual *P. falciparum* parasitemia in Cohort 1 (Manhiça). From study month 8.5 to study month 63, the RTS,S/AS02 group is shown in black lines with 95% confidence interval bars, and the comparator vaccine group is shown in grey lines with 95% confidence interval bars.

of blood sampling (Table 2). Bednet use was reported in 291 (30%) of participants, for whom 147 (31%) were from the RTS,S/AS02 group and 144 (30%) from the comparator vaccine group ($p = 0.779$). Of bednet users, 136 (93%) in the RTS,S/AS02 group and 123 (85%) in the comparator vaccine group reported use of an insecticide treated net ($p = 0.062$). IRS was reported as recently done at the homes of 290 (60%) participants in the RTS,S/AS02 group and 295 (61%) participants in the comparator vaccine group ($p = 0.895$). In the RTS,S/AS02 group, 57 (12%) participants were positive for asexual *P. falciparum* parasitemia, and 62 (13%) were positive in the comparator vaccine group ($p = 0.696$). The difference in parasitemia throughout follow-up in C1 is shown in Fig. 1 . Antibody titers were significantly higher in the RTS,S/AS02 group than in the comparator vaccine group, as was seroprevalence (94 [94%] Vs 10 [9%], respectively; $p < 0.001$). Fig. 2 shows antibody levels over the duration of follow-up.

In C2, the RTS,S/AS02 and comparator vaccine groups had similar prevalence of fever (2 [15%] Vs 2 [16%], respectively; $p = 1.0$), and there was no difference in anemia (2 [15%] Vs 2 [16%], respectively; $p = 1.0$) despite slightly lower hematocrit in the comparator vaccine group. Bednet use (47 [35%] Vs 40 [31%], respectively; $p = 0.599$), use of insecticide treated nets (42 [89%] Vs 34 [85%], respectively; $p = 0.748$) and IRS treatment (39 [29%] Vs 38 [30%], respectively; $p = 1.0$) were similar (Table 1). No difference was

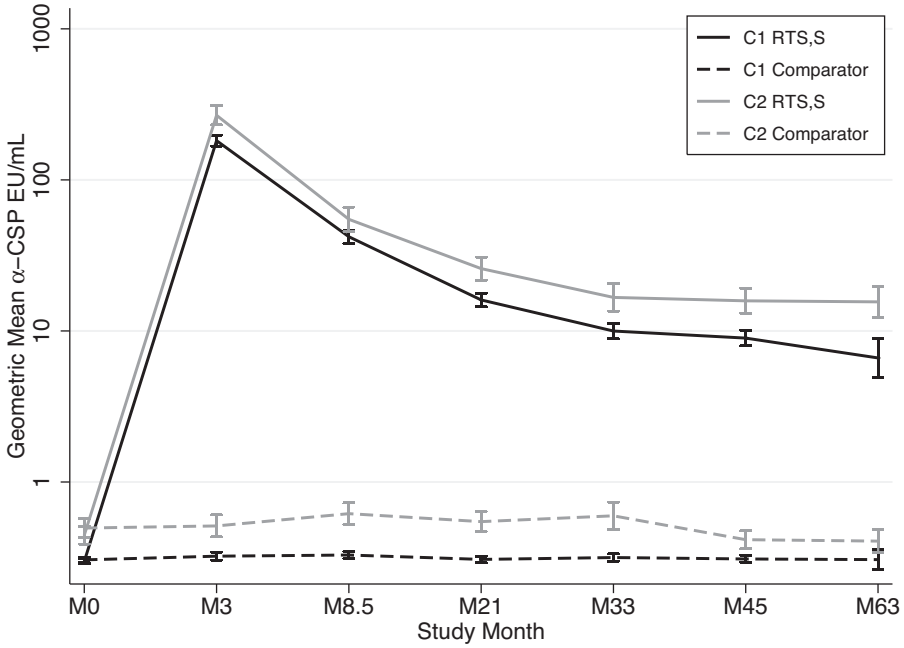

Fig. 2. Geometric mean titers of antibodies to the NANP repeat region of *P. falciparum* CSP. Beginning with pre-vaccination screening (study month 0) through study month 63, the RTS,S/AS02 group is represented by solid lines, and the comparator vaccine group by dashed lines. Cohort 1 (Manhiça) is shown in black, and Cohort 2 (Ilha Josina) is shown in grey, with 95% confidence interval bars included.

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Table 3
RTS,S Phase IIb & III clinical trial summary (According-To-Protocol analysis of clinical malaria using primary case definition).

Clinical cases/PYAR				Parasitemia at EOF				
Trial (age at 1st dose)	RTS,S	Comparator	Efficacy	*P* value	RTS,S	Comparator	*P* value	Reference
Mozambique (1–4 yr), C1[a,1]								
ATP $_{2.5–8.5}$	0.38	0.52	29.9 (11.0–44.8)	0.004	11.9	18.9	<0.001	[15]
ATP $_{8.5–21}$	0.18	0.24	28.9 (8.4–44.8)	0.008	11.6	16.2	0.017	[23]
ATP $_{21–33}$	0.32	0.37	16.8 (−2.5–32.4)	0.084	15.8	20.3	0.049	[4]
ATP $_{33–45}$	0.14	0.15	11.8 (−20.1–35.2)	0.426	12.2	18.5	0.004	[4]
ATP $_{2.5–45}$	0.19	0.26	30.5 (18.9–40.4)	<0.001	12.2	18.5	0.004	[4]
Mozambique (1–4 yr), C2[b,1]								
ATP $_{2.5–8.5}$	0.63	0.94	35.4 (4.5–56.3)	0.029	61.4	71.9	0.036	[22]
ATP $_{8.5–21}$	0.49	0.51	9.0 (−30.6–36.6)	0.609	73.5	72.5	0.906	[22]
Tanzania/Kenya (5–17 mo)[c,2]								
ATP $_{2.5\text{-EOF}\,(4.5–10.5)}$[3]	0.13	0.28	53 (28–69)	<0.001	2	3	–	[16]
ATP $_{2.5\text{-EOF}\,(49)}$[4]	0.24	0.36	32.1 (11.6–47.8)	0.004	7	5	0.21	[5]
Phase III (5–17 mo)[a,2]								
ATP $_{2.5–14}$	0.44	0.83	55.8 (51.3–59.8)	<0.001	–	–	–	[7]
Phase III (6–12 wk)[a,2]								
ATP $_{2.5–14}$	0.37	0.48	31.3 (23.6–38.3)	<0.001	–	–	–	[8]
Mozambique (8 wk)[b,1]								
ATP $_{3–6}$	0.4	1.1	65.8 (25.3–84.4)	0.007	5	8	0.536	[18]
ATP $_{3–14}$	0.58	0.88	33.0 (−4.3–56.9)	0.076	–	–	–	[20]
Tanzania (8 wk)[3,1]								
ATP $_{2.5–7}$	0.10	0.15	43.2 (−47.1–78.0)	0.24	–	–	–	[17]
ATP $_{2.5–14}$	0.11	0.20	53.6 (8.6–76.4	0.026	–	–	–	[26]
ATP $_{2.5–20}$	0.14	0.19	34.9 (−8.8–61.1)	0.101	–	–	–	[26]

[a] Passive case detection.
[1] AS02 adjuvant.
[b] Active detection of infection/passive case detection.
[c] Active case detection/passive case detection.
[2] AS01 adjuvant.
[3] The Tanzania/Kenya had a varying follow-up period, and the range or median end of follow-up is shown in parentheses.
[4] Only the Kilifi, Kenya cohort included in extended follow-up.

observed in prevalence of parasitemia (30 [23%] Vs 35 [28%], respectively; $p = 0.391$). As with C1, antibody titers and seroprevalence (85 [100%] Vs 25 [32%], respectively; $p < 0.001$) were significantly higher in the RTS,S/AS02 group.

3.3. Comparison of Phase III & IIb trials

In Table 3, we summarize the results of the Phase III and IIb efficacy trials of RTS,S in infants and children. Each trial in Table 3 has a different study design, whether by surveillance system (active detection of infection, active case detection or passive case detection), time period of follow-up, age group, adjuvant used or primary case definition (e.g. fever or history of fever and >5000 parasites/μL in the Phase III, >2500 parasites/μL for Phase IIb children or >500 parasites/μL for Phase IIb infants). In all studies except the Tanzanian infant trial, incidence rates in the comparator group were as high or higher than the Mozambique C1 cohort (the Tanzanian–Kenyan cohort had lower incidence than the first 6 months of follow-up in C1 but was similar thereafter). Reported tests on the PH assumption to detect constant or changing efficacy are summarized in Table 4. Waning vaccine efficacy, as determined by the non-proportionality of the hazard of clinical malaria, was observed in trials where the incidence rates in the comparator vaccine group, a proxy for transmission intensity, were higher than those in C1. These trials included C2 of the same study, the Mozambican infant trial, and the Phase III 5–17 month age group. Notably, the Phase III infants had higher incidence than later follow-up timepoints in C1, and the Phase IIb trial conducted in

Table 4
RTS,S Phase IIb & III reported tests of proportionality of the hazard assumption.

Trial (age at 1st dose)	Reported follow-up period from 1st dose	Test of Schoenfeld residuals	Time-dependent Cox regression models	Change in sequential point efficacy estimates	Reference
Mozambique (1–4 yr), C1[a,1]	45 months	Proportional	Proportional	Non-proportional	[23] [4,15]
Mozambique (1–4 yr), C2[b,1]	21 months	Non-proportional	Non-proportional	Non-proportional	[22]
Tanzania/Kenya (5–17 mo)[c,2,3]	47.5 months (median)	–	Non-proportional[4]	–	[5,16]
Phase III (5–17 mo)[a,2]	14 months	Non-proportional	Non-proportional	–	[7]
Phase III (6–12 wk)[a,2]	14 months	Non-proportional	Non-proportional	–	[8]
Mozambique (8 wk)[b,1]	14 months	Non-proportional	–	–	[18] [20]
Tanzania (8 wk)[b,1]	20 months	Proportional	–	–	[17] [26]

[a] Passive case detection.
[1] AS02 adjuvant.
[b] Active detection of infection/passive case detection.
[c] Active case detection/passive case detection.
[2] AS01 adjuvant.
[3] Only the Kilifi, Kenya cohort included in extended follow-up.
[4] Weak evidence of non-proportionality ($p = 0.07$) shown using time-dependent Cox regression.

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Kenya showed increasing incidence over time, both studies for which evidence of waning was observed. On the other hand, the test on Schoenfeld residuals tested in the Tanzanian infant trial did not reject the PH assumption, although the trial was small and may not have had power to detect changing efficacy [26].

4. Discussion

In this study, we measure prevalence of parasitemia, a secondary trial endpoint, instead of risk of clinical malaria, the primary endpoint, to gather evidence of ongoing protection. Prevalence of parasitemia is not a substitute for the hazard function, but we argued previously that it serves as an indirect indicator of recent incidence [4]. We found that the difference in parasitemia between the RTS,S/AS02 and comparator vaccine groups was diminished at 5 years after receiving a full vaccine course. These data should be interpreted cautiously with respect to protection. Whereas previously we justified a protective effect of vaccination with differences in prevalence [4], lack of a difference may not be evidence of a lack of effect, as was observed in the case of IPTi [27]. However, it is likely that similar prevalence between vaccine groups reflects similar levels of ongoing incidence, which we interpret as no evidence for protection afforded by the vaccine at 5 years. Assessment of incidence of clinical malaria between study months 45 and 63 was not performed, since data monitoring was discontinued after study month 45. It is unclear whether protracted protection reported during the first 45 months of follow-up in C1 was a chance finding or an indication of long-term duration of protection in some settings. The tests on the PH assumption give confidence that protection was sustained, although late follow-up point estimates of efficacy suggest waning and may indicate a lack of power to detect time-dependency. This limitation was mitigated by the significant difference in parasite prevalence at study month 45, which indicated that even with a decline in efficacy, some protection still occurred.

A limitation of this study was that the survey was performed in August-September, at the beginning of the high transmission season, whereas previous surveys from study month 8.5 to 45 were performed in April-May at the end of the high transmission season. Thus, we cannot discard the possible effect of seasonal changes in transmission on the absence of a difference, but it is unlikely that exposure during the high transmission season would increase the difference in parasitemia, given that there exists no evidence for natural boosting of anti-CSP antibodies in vaccinated children. Furthermore, the overall parasite prevalence in the study population was not disproportionately lower than that of earlier measurements at the end of transmission seasons (Fig. 1). Previous cross-sectional timepoints showed significant differences in parasitemia between treatment arms in C1, but not when efficacy had waned in C2, which supported vaccine efficacy estimates and illustrated the usefulness of the parasitemia endpoint. Importantly, the loss of this difference at study month 63 supports the previous assessments of parasitemia prevalence during the first 45 months of surveillance, in that it demonstrates no inherent differences in the randomization of the two treatment arms, and it follows the trend of lower efficacy estimates for the follow-up periods of study months 21–45 and study months 33–45 [4]. It seems unlikely that the change in this trend of different parasite prevalence would be due to selection bias, as the health-seeking behavior of the study participants was likely unchanged between study months 45 and 63. Furthermore, recruitment for the survey was even between vaccination groups in both C1 and C2 (50% RTS,S/50% Comparator), and no apparent differences were noted in demographics.

Level of anti-CSP antibodies in the RTS,S/AS02 group was significantly higher than in the comparator vaccine group, although titers were 20-fold lower than at the peak responses post-vaccination. The seropositivity of all children vaccinated with RTS,S/AS02 remained at 96%, whereas 89% and 68% of the comparator vaccine group remained seronegative in C1 and C2, respectively. As indicated in Fig. 2, it appears that the decline in antibody titers reached a steady state, likely due to a population of long-lived plasma cells residing in the bone marrow. Indeed, CSP-specific memory B cells have been detected a year after 3rd dose of RTS,S, lending support to the hypothesis of a long-term memory pool for antibody production [28]. Interestingly, there was no reduction in prevalence of parasitemia in the RTS,S vaccine group at study month 63, whereas the surveys at study months 21, 33 and 45 showed reduced prevalence in C1, albeit with similar antibody titers, although it is possible that the lack of effect is partly due to lack of power to demonstrate effects so long after vaccination. This illustrates a gap in knowledge of the role of antibodies to CSP in protection, particularly in terms of antibody quality, specificity and functionality. Although antibodies likely play a straightforward role in protection, the inconsistencies of NANP repeat antibodies as a surrogate marker of protection suggest a missing element. Efforts within the Phase III trial are underway to further characterize antibodies generated by RTS,S vaccination and how they may mediate protection.

Only 3 children out of 130 with positive blood slides in C1 presented with clinical malaria during the cross-sectional survey, using the primary case definition of axillary temperature $\geq$37.5 °C and asexual parasite density $\geq$2500 parasites/μL, and no child presented with clinical malaria in C2, implying that the study population had an already advanced level of immunity to clinical malaria. Throughout the first 45 months of follow-up, we found no evidence that the RTS,S group was at higher risk of clinical malaria if exposed to blood stage infection, as evidenced by a lack of rebound effect and no differences in fever among parasitemic children at cross-sectional surveys. Furthermore, we previously reported that levels of blood stage antibodies were not significantly lower in RTS,S vaccinated children, although there was a tendency for the younger children to have lower antibodies to some antigens [29], indicating a potential effect of differential acquisition of immunity as a factor in the PH assumption. This finding suggests that the first 3.5 years of protection conferred by RTS,S with no waning or rebound effects may have covered a critical period of susceptibility to clinical malaria. In C2, the finding of no difference in parasitemia between treatment groups was consistent with the previously described early waning of efficacy and lack of reduction in parasitemia at study month 21 [23].

The Phase IIb trial of RTS,S/AS02 in Mozambique showed moderate vaccine efficacy against clinical malaria and lower parasite prevalence in C1 that was sustained through 45 months of follow-up. However, the waning efficacy observed in C2, as well as in other trial cohorts [14,18,20], is of concern if the duration of protection falls short of the one-year minimum goal set by the Malaria Vaccine Technology Roadmap. Comparisons between trials (Table 4) should be interpreted with caution due to differences in the study designs.

Waning of vaccine efficacy in study populations with higher incidence rates might indicate that transmission intensity modifies duration of protection. A meta-analysis of the Phase IIb trial data reported to date, although limited by the same problem of non-standard study designs, showed clear evidence for an effect of transmission intensity on duration of protection [30]. In C1, the incidence rate declined during follow-up, which further complicates analysis on the effect of transmission intensity and could be a conditional factor in the longevity of protection. Recent modeling of anti-CSP antibodies in the controlled human challenge model suggests a concentration-mediated reduction in probability of breakthrough infections [31]. Perhaps, the duration of protection observed in C1 could be attributable to exposure declining in

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parallel to protective antibody decay. This hypothesis is not inconsistent with the results of the Kenyan Phase IIb study, which showed faster waning of efficacy in the high exposure group of children compared with the low exposure group, although both groups ended the 4 year follow-up with essentially zero efficacy [5]. Interestingly, incidence rates increased over time as antibody levels dropped in the Kenyan trial, and incidence in C2 remained high throughout follow-up, together suggesting that waning may occur faster in settings of high or increasing transmission intensity. From the trial results summarized in Tables 4 and 5, age at time of vaccination does not have a clear role in either efficacy or waning. However, lower titers of NANP-specific peak antibodies were measured in the infant age group of the Phase III trial [8], which has been reflected in other infant trials [17], indicating that age may also be an important factor in protection. Despite having the well-validated method of determining proportionality of the hazard function using Schoenfeld residuals [32,33], selection of the best time-dependent model is not standardized, and the interpretation of how long protection lasts, when waning occurs and when protection ends can be difficult.

In conclusion, we found no evidence of sustained protection to 5 years after vaccination with RTS,S/AS02 in Mozambican children, and we hypothesize that changing transmission patterns and age may affect the onset of waning efficacy. The most significant issue at hand is the duration of protection afforded by RTS,S vaccination, and the ongoing Phase III study will analyze long-term efficacy and factors associated with waning.

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