







## RESEARCH ARTICLE

# A seven-year study on the effect of the pre-erythrocytic malaria vaccine candidate RTS,S/AS01<sub>E</sub> on blood stage immunity in young Kenyan children [version 1; peer review: 1 approved, 2 approved with reservations]

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## Abstract

**Background:** RTS,S/AS01<sub>E</sub>, the most advanced malaria vaccine confers partial immunity. The vaccine-induced pre-erythrocytic immunity reduces exposure to blood-stage parasites, delaying acquisition of antibodies to blood-stage antigens. However, the duration of this effect is unknown.

**Methods:** We measured, by enzyme-linked immunosorbent assay, IgG-antibodies to 4 *Plasmodium falciparum* blood-stage antigens (AMA1, MSP1<sub>42</sub>, EBA175, and MSP3) on 314 children randomized to receive RTS,S/AS01<sub>E</sub> or Rabies vaccine at 5 – 17 months of age in a phase 2b trial in Kenya, and thereafter participated in a 7-year study of the duration of vaccine immunity.

**Results:** Antibody levels to MSP1<sub>42</sub>, AMA1 and EBA175 were slightly lower among the RTS,S/AS01<sub>E</sub> recipients, relative to the Rabies-control vaccinees, during the first 48 months of surveillance. Irrespective of vaccine arm, antibody levels to merozoite antigens were positively associated with the risk for malaria. However, this was only apparent at high levels for EBA175 and AMA1 and was not evident after adjusting for heterogeneity in malaria-exposure. Among children with asymptomatic parasitaemia, antibody levels were associated with reduced clinical malaria.



**Conclusions:** The reduction in levels of antibodies to blood-stage antigens induced by vaccination with RTS,S/AS01<sub>E</sub> can last for several years. In absence of asymptomatic infection, anti-merozoite antibody levels were unreliable correlates of clinical immunity.

## Keywords

*Plasmodium falciparum*, malaria, RTS, S/AS01 E, Vaccines, immunity, pre-erythrocytic, blood stages

## Open Peer Review

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## Introduction

Despite the recent gains in malaria control, the disease remains a major public health risk, with 216 million cases and 445,000 deaths associated with malaria in 2016<sup>1</sup>. Progress in malaria control has stalled and may have reversed in some areas<sup>2</sup>.

RTS,S/AS01<sub>E</sub> is the most advanced candidate malaria vaccine and is based on the circumsporozoite protein (CSP) that targets the pre-erythrocytic cycle of *Plasmodium falciparum* in humans. Vaccination with RTS,S/AS01<sub>E</sub> has been partially efficacious against malaria in phases II and III trials in Africa<sup>3,4</sup>. RTS,S/AS01<sub>E</sub> induces pre-erythrocytic immunity. In contrast, naturally acquired immunity to malaria is largely dependent on antibodies to blood-stage parasites including the merozoite stage. Although there are no unambiguous correlates of natural immunity<sup>5</sup>, antibodies to merozoite antigens have been associated with protection through multiple mechanisms including the inhibition of erythrocyte invasion and replication<sup>6</sup>, complement-dependent mechanisms<sup>7</sup>, and enhancement of uptake and clearance by circulating phagocytes<sup>8,9</sup>. Antibodies to antigens expressed on the surface of infected red blood cells (iRBCs) have also been associated with immunity, which could inhibit or reverse sequestration of iRBCs, inhibit formation of rosettes, and promote opsonization of iRBCs for uptake by phagocytes<sup>10–12</sup>.

Antibodies to malaria parasites are acquired as a result of exposure. As such, interventions like insecticide impregnated bed nets and RTS,S/AS01<sub>E</sub> -vaccination that reduce exposure to blood-stage antigen will affect the rate at which antibodies to merozoite and other blood-stage antigens are acquired. Previously, we and others demonstrated that RTS,S/AS01<sub>E</sub> and RTS,S/AS02 vaccinations reduced blood stage antibody levels, likely as a result of reducing the exposure to blood stage parasites due to induction of partial pre-erythrocytic immunity<sup>13,14</sup>. However, the duration of this effect remains unknown. It is important to determine the duration of this effect as RTS,S/AS01<sub>E</sub> vaccination could delay the development of naturally acquired immunity, increasing the possibility of continued susceptibility in older children after the waning of the vaccine induced immunity<sup>15</sup>.

In this study, we aimed to determine the durability of the previously reported reduction in antibody levels to merozoite antigens in children receiving RTS,S/AS01<sub>E</sub> vaccination, relative to Rabies control vaccines<sup>13</sup>. We analysed plasma samples collected from children during a seven-year extended follow up of a phase IIb randomized, controlled trial of RTS,S/AS01<sub>E</sub> among young children in Kilifi, Kenya, examining antibodies to 4 different merozoite antigens by enzyme-linked immunosorbent assay (ELISA). We then analysed the effect of RTS,S/AS01<sub>E</sub> vaccination on the acquisition of these antibodies and tested for potential correlations between antibody levels and protection from clinical malaria episodes.

## Methods

### Study design

447 healthy Kenyan children aged 5–17 months were randomized in a 1:1 ratio to receive 3 doses at monthly intervals of either RTS,S/AS01<sub>E</sub> or Rabies vaccine in a phase 2b trial, to evaluate the

efficacy and safety of RTS,S/AS01<sub>E</sub> against clinical malaria episodes by *P. falciparum* infection. Details have been published elsewhere<sup>3</sup>.

### Monitoring for episodes of clinical malaria

The primary end point was a clinical episode of malaria, defined as an axillary temperature of  $>37.5^{\circ}\text{C}$ , with a *P. falciparum* parasite density of 2500 parasites/microlitre of blood. Active surveillance was implemented with weekly home visits, where children were screened for fevers associated with *P. falciparum* parasites, both during the trial and the extended follow up period. A parallel passive surveillance was implemented by field workers residing in the study villages and health care staff in local health facilities.

Asymptomatic infections were detected by both microscopy and blood-smears during the cross-sectional data and sample collection surveys described below.

### Blood samples

Vaccine doses were given at month 1, 2 and 3. Blood samples were initially taken (1) before vaccination (in March 2007), (2) 1 month after dose 3, (3) in March 2008 (i.e., mean, 8 months; range, 4–10 months after dose 3), and (4) 12 months after dose 3. Subsequently, the study was extended to test the duration of vaccine induced immunity, and further blood samples were collected in March (5) 2009, (6) 2010, (7) 2011, (8) 2012, (9) 2013, and (10) 2014. Separated plasma was aliquoted and stored at  $80^{\circ}\text{C}$  until assayed.

Previously, we reported anti-merozoite antibody responses for the samples collected from four time points during the first 14 months of follow up<sup>13</sup>. In the current study, we extend the analysis to samples collected during the extended study, including 10 timepoints taken over 7 years; i.e. pre-vaccination (i.e. month 0), then at 4, 6.5, 8, 14, 24, 36, 48, 60, 72, and 84 months.

### ELISA

Samples were tested by ELISA for the presence of human IgG against the following *P. falciparum* antigens as described elsewhere<sup>16</sup>: MSP1<sub>42</sub>, 3D7 sequence expressed in *Escherichia coli*<sup>17</sup>; MSP3, FVO sequence, expressed in *E. coli*<sup>18</sup>; the receptor-binding domain II (PfEBA175RII) of EBA175, 3D7 sequence, expressed in *P. pastoris*<sup>19</sup>; and AMA1, 3D7 sequence expressed in *E. coli*<sup>20</sup>. In brief, each antigen was coated onto high absorbance plates (Immulon4 HBX) at a concentration of 0.5 micrograms/mL and stored at  $4^{\circ}\text{C}$  overnight. The plates were washed 3 times in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T) and blocked for 3 h with blocking buffer (1% w/v dried skimmed milk powder in PBS-T). After 3 additional washes, 100 microlitre of each plasma sample were added to duplicate wells at a final dilution of 1/1000 in PBS-T. The next day, after 5 washes, 100 microlitre of horse radish peroxidase-conjugated antihuman IgG (DAKO) at a dilution of 1:5000 in blocking buffer was added to each well, and plates were incubated for 3 h. The plates were then developed using  $\text{H}_2\text{O}_2$  as substrate and OPD (Sigma) as the colorimetric indicator for 20 min in the dark. Plates were read at 492 nm on a Molecular Devices

Versa Max ELISA reader. Tests were repeated if duplicate optical density (OD) values for an individual plasma sample varied by more than a factor of 1.5. A pool of serum samples from an area in Africa where malaria is highly endemic was titrated on each plate and acted both as a positive control and provided values for a standard curve for converting optical density (OD) readings into arbitrary units, minimizing inter-plate and inter-day variations. A 3-parameter sigmoid ligand binding model was used to least-squares fit a curve to the values of the hyperendemic serum sample pool, and this was used to calculate sample antibody levels on each plate.

### Statistical analysis

Antibody scores from ELISAs were expressed relative to the OD readings obtained from the hyperimmune standard, with a score of 1000 scaled to be the maximum reactivity seen at the lowest dilution used in the hyperimmune standard curve, and then log-transformed before analysis. Student's T-test with comparison of means and non-parametric analyses with comparisons of medians and rank sum tests were used to compare groups. For the prospective association with malaria risk, the antibody levels were split into deciles, and a Poisson regression analysis was conducted with the unit of analysis being the period of time after each antibody level was estimated, hence including up to 10 observations per child, using the clustered sandwich estimate in Stata 15 (StataCorp LLC). We used the exposure index, as previously described<sup>21</sup>, to estimate exposure to malaria based on geographical location.

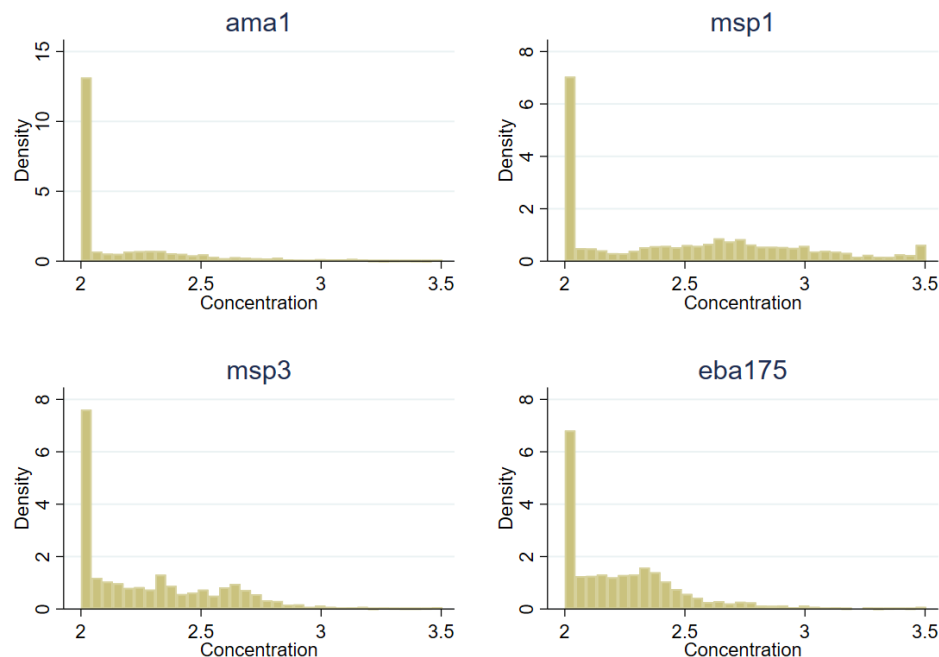
### Results

1735 plasma samples collected from 10 time points: at 0 (i.e. pre-vaccination), 4, 6.5, 8, 14, 24 (March 2009; n = 314), 36 (March 2010; n = 303), 48 (March 2011; n=295), 60 (March

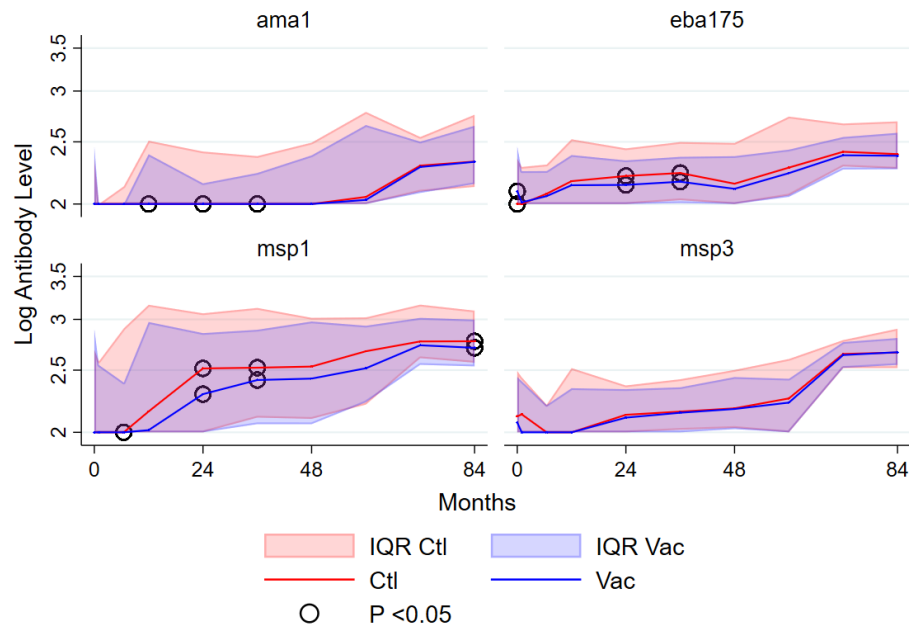
2012; n=276), (72) (March 2013; n = 269), and 84 (March 2014; n=278) months of the third dose of vaccination were tested for antibody levels. The antibody levels varied widely, with the majority of the children being unresponsive (i.e. lower than the lowest value on the straight part of the sigmoid curve based on the dilution of the hyperimmune standard serum), while the rest had values lying within the straight part of the hyperimmune standard curve (Figure 1).

### Anti-merozoite antigen antibody levels split by RTS,S/AS01<sub>E</sub> vaccination

Geometric mean antibody levels for all the 4 merozoite antigens increased with age, irrespective of vaccination group, but this was more apparent for 3 of the 4 antigens, and less apparent for AMA1 (Figure 2). There were indications of seasonal variation during the first year of sampling when 4 samples were collected per child, as previously described<sup>13</sup>, but it was not possible to assess seasonality once sampling was scaled back to 1 sample per child per year, timed to occur in the dry period just before the main transmission season. Antibody levels for AMA1, EBA175 and MSP1<sub>42</sub> diverged after vaccination, with levels being higher among the Rabies control vaccinees than the RTS,S/AS01<sub>E</sub> vaccinees at months 12, 24 and 36, 24 and 36, and 6, 12, 24, 36 and 84 of the third dose of vaccination for AMA1, EBA175 and MSP1<sub>42</sub>, respectively (Figure 2). Thus, the divergence was temporal for AMA1 and EBA175, as the differences in the median antibody levels reduced with time and were similar by 48 months of the third dose of vaccination. In contrast, anti-MSP1<sub>42</sub> antibody levels were still higher among the Rabies-control than the RTS,S/AS01<sub>E</sub> vaccinees at 84 months of the third dose (the last time point of sampling) with statistical significance (Table 1). Similar patterns were seen on non-parametric analyses with medians.



**Figure 1. Distribution of anti-merozoite antibody levels.** Antibody levels were measured by ELISA.



**Figure 2. Comparison of the mean levels of anti-merozoite antigen-specific antibodies between RTS,S/AS01<sub>E</sub> and Rabies control vaccinees.** Antibody levels were determined by ELISA and the values log-transformed to achieve normal distributions. Student's T-test with comparison of means and non-parametric analyses with comparisons of medians and rank sum tests were used to compare groups. The read blue and red lines indicate the mean levels of RTS,S/AS01<sub>E</sub> and Rabies control vaccinations, respectively. The shaded regions indicate the 95% confidence intervals.

**Table 1. Comparisons of the geometric mean antibody levels of antibody levels between RTS,S/AS01<sub>E</sub> and Rabies control vaccines at different time points.**

Antigen	Month	RTS,S/AS01 <sub>E</sub>	Rabies Control	P value
ama1	0	1.61 (1.56-1.66)	1.66 (1.61-1.71)	0.156
ama1	4	1.56 (1.53-1.59)	1.55 (1.53-1.58)	0.705
ama1	8.5	1.59 (1.56-1.63)	1.55 (1.53-1.58)	0.057
ama1	12	1.67 (1.61-1.73)	1.61 (1.56-1.66)	0.116
ama1	24	1.64 (1.6-1.69)	1.58 (1.55-1.62)	0.031
ama1	36	1.68 (1.62-1.73)	1.62 (1.58-1.67)	0.12
ama1	48	1.7 (1.65-1.76)	1.67 (1.62-1.72)	0.311
ama1	60	1.8 (1.73-1.87)	1.78 (1.71-1.84)	0.628
ama1	72	1.85 (1.8-1.9)	1.83 (1.78-1.88)	0.588
ama1	84	1.92 (1.86-1.98)	1.9 (1.85-1.96)	0.605
eba175	0	1.66 (1.6-1.71)	1.71 (1.66-1.76)	0.114
eba175	4	1.61 (1.58-1.63)	1.61 (1.58-1.63)	0.939
eba175	8.5	1.65 (1.62-1.68)	1.62 (1.6-1.65)	0.208
eba175	12	1.75 (1.69-1.81)	1.69 (1.65-1.73)	0.095
eba175	24	1.74 (1.7-1.79)	1.68 (1.65-1.71)	0.016
eba175	36	1.79 (1.74-1.84)	1.71 (1.68-1.75)	0.008
eba175	48	1.76 (1.7-1.82)	1.68 (1.65-1.72)	0.023
eba175	60	1.88 (1.82-1.95)	1.78 (1.73-1.83)	0.011
eba175	72	1.98 (1.93-2.03)	1.92 (1.88-1.96)	0.068

Antigen	Month	RTS,S/AS01 <sub>E</sub>	Rabies Control	P value
eba175	84	1.97 (1.92-2.02)	1.94 (1.9-1.98)	0.435
msp1 <sub>42</sub>	0	1.75 (1.67-1.84)	1.81 (1.72-1.9)	0.36
msp1 <sub>42</sub>	4	1.72 (1.65-1.79)	1.7 (1.64-1.76)	0.687
msp1 <sub>42</sub>	8.5	1.81 (1.74-1.88)	1.69 (1.63-1.74)	0.006
msp1 <sub>42</sub>	12	1.99 (1.87-2.11)	1.86 (1.76-1.95)	0.083
msp1 <sub>42</sub>	24	2.08 (1.99-2.16)	1.93 (1.85-2)	0.009
msp1 <sub>42</sub>	36	2.12 (2.04-2.2)	1.99 (1.92-2.07)	0.016
msp1 <sub>42</sub>	48	2.07 (1.99-2.14)	2 (1.93-2.07)	0.186
msp1 <sub>42</sub>	60	2.15 (2.08-2.22)	2.08 (2.01-2.14)	0.116
msp1 <sub>42</sub>	72	2.34 (2.29-2.4)	2.28 (2.23-2.32)	0.069
msp1 <sub>42</sub>	84	2.32 (2.27-2.38)	2.25 (2.2-2.29)	0.019
msp3	0	1.73 (1.67-1.78)	1.7 (1.65-1.75)	0.412
msp3	4	1.71 (1.67-1.75)	1.66 (1.62-1.7)	0.084
msp3	8.5	1.59 (1.56-1.62)	1.58 (1.56-1.6)	0.563
msp3	12	1.7 (1.63-1.76)	1.64 (1.59-1.69)	0.134
msp3	24	1.7 (1.66-1.74)	1.66 (1.63-1.69)	0.191
msp3	36	1.75 (1.7-1.8)	1.7 (1.66-1.74)	0.126
msp3	48	1.75 (1.71-1.8)	1.73 (1.69-1.77)	0.476
msp3	60	1.82 (1.76-1.88)	1.75 (1.7-1.79)	0.062
msp3	72	2.18 (2.13-2.22)	2.16 (2.13-2.19)	0.444
msp3	84	2.2 (2.16-2.24)	2.17 (2.14-2.21)	0.396

### Antibody levels and subsequent risk of clinical malaria

Antibody levels were split into deciles, which were then tested for prospective associations with protection from malaria in the transmission period after each, but before, the next sampling time-point. Pre-existing antibody levels for the 4 different merozoite proteins were not associated with clinical immunity (Figure 3). Instead, the incident rate ratio for clinical malaria increased with rising antibody levels. This relationship was most apparent at higher antibody levels (>5<sup>th</sup> decile) for AMA1 and EBA175 (irrespective of vaccine arm). However, the incident rate ratios for the effect of antibodies on clinical malaria reduced after controlling for heterogeneity in malaria exposure using an exposure index, suggesting that these anti-merozoite antibodies are markers of exposure, rather than immunity.

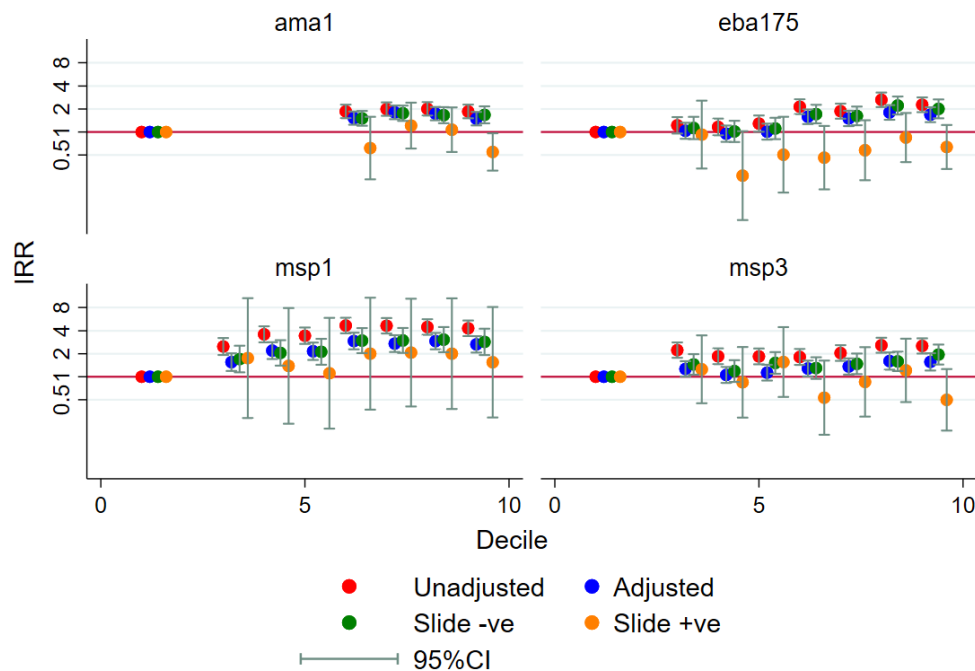
Furthermore, when all the data were stratified by asymptomatic-parasite positivity at sampling by microscopy, the highest levels for AMA1 and MSP3, and all the of levels for EBA175 above the non-reactive group, were associated with reduced rate ratios for clinical malaria, among the children with asymptomatic parasitaemia at the time of sampling. Associations between higher antibody levels and increased incident rate ratios were maintained among the children without asymptomatic parasitaemia (Figure 3).

### Discussion

We and others reported previously that RTS,S/AS01<sub>E</sub> vaccination resulted in a reduction in antibody levels to blood stage malaria antigens<sup>13,14</sup>. However, the duration of this effect is

unknown. Here, we investigated the longevity of the reduction of antibody levels to four blood stage antigens after an extended follow up of the vaccines and controls for up to 7 years post-vaccination. We found that immunization with RTS,S/AS01<sub>E</sub> and the associated clinical protection resulted in the reduction of antibody response to MSP1<sub>42</sub>, AMA1 and EBA175 antibody levels but not for MSP3. While the antibody levels for AMA1 and EBA175 among RTS,S/AS01<sub>E</sub> vaccinees were below those measured in the Rabies control vaccinees during the first 48 months of monitoring, antibodies to MSP1<sub>24</sub> remained lower in the RTS,S/AS01<sub>E</sub> vaccinees than in the controls throughout the study period. This latter, persistent difference was statistically significant except at the very last timepoint, when statistical significance was only marginal, considering that there are multiple comparisons by timepoint and by adjuvant ( $p=0.019$ ).

In this study, antibody levels to four specific merozoite antigens were not associated with clinical protection. Rather anti-merozoite antibody levels were positively associated with the risk of clinical malaria for the group as a whole. The most likely explanation for this is that antibody responses are markers of exposure, and therefore represent ongoing risk of future exposure to malaria, and this interpretation is supported by the fact that the positive association was reduced after controlling for the exposure index. It is possible that higher antibody titers might have been protective (i.e. those above a protective threshold<sup>22</sup>). In the presence of asymptomatic infection, anti-EBA175 antibodies at all the deciles were higher than the lowest (i.e. non-reactive) decile, and some of the higher deciles for AMA1 and



**Figure 3. Prospective association of antibodies with immunity to malaria.** Antibody levels were split into deciles and tested for association with the numbers of malaria episodes in the ensuing malaria transmission period, but before the next sampling time point. Poisson regression analysis was conducted with the unit of analysis being the period of time after each antibody level was estimated, hence including up to 7 observations per child, using the clustered sandwich estimate. The analysis adjusted for age, exposure index, vaccine arm, and bed net usage. The red and blue dots indicate unadjusted and adjusted analyses. The green and orange dots indicate analysis for parasite negative and positive samples.



MSP3 antibodies were associated with protection from clinical malaria (irrespective of the vaccine arm). This finding is consistent with several previous studies where analyses of single antigen-specific antibody responses within whole populations demonstrated no protective effect of antimalarial antibodies, but the same antibodies were associated with clinical immunity when parasite-positive individuals were analysed separately<sup>22,23</sup>. Our analysis involves only four antigens and there is evidence that the breadth of antibody positivity is also important for protection<sup>24,25</sup>.

An RTS,S/AS01<sub>E</sub> induced reduction in blood stage immunity will have implications for the outcomes of vaccination if the vaccine is deployed for routine use among African children. If vaccination resulted in delayed development of natural immunity, then some of the gains of the vaccination may be offset by delayed susceptibility as the vaccine induced immunity wears off. Studies done to date on Phase II trials have suggested this possibility with a three-dose vaccine regimen<sup>15</sup>, although the effect may be countered by a fourth dose<sup>26</sup>. We show here that antibodies to blood stage immunity are reduced after vaccination with RTS,S/AS01<sub>E</sub>, which is consistent with induction of pre-erythrocytic immunity leading to a reduced incidence of blood-stage parasitaemia. However, antibodies induced by natural exposure to the four blood stage antigens tested were not consistently associated with immunity to malaria and there is no widely accepted or consistent immunological marker for immunity to malaria. It will be important to combine RTS,S/AS01<sub>E</sub> with other malaria control measures like insecticide treated nets for protecting individuals from malaria, and further clinical evaluations of the four-dose vaccine regimen should include long-term follow up in the implementation trials.

## Data availability

### Underlying data

Havard Dataverse: Replication Data for: Effect of rtss vaccination on blood stage immunity data, <https://doi.org/10.7910/DVN/KH9ESP><sup>27</sup>.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

## Ethical considerations

The study protocol and its subsequent amendments received ethical and scientific approval from the Kenyan Medical Research Institute National Ethics Committee. The study was overseen

by an independent data-monitoring committee and local safety monitors and was conducted in accordance with the Helsinki Declaration of 1964 (revised 1996) and Good Clinical Practice guidelines. Written informed consent in the local languages (Swahili or Giriama) was required from parents/guardians for participation.

## Author information

FMN and PB conceptualized the study, supervised and managed the collection of immunology data, analysed and interpreted the data, and wrote the paper. JM performed the antibody measurements, JW conducted and supervised surveillance for malaria, and sample collection, PN conducted and supervised the vaccine trial, supervised and sample collection. KM and PB obtained the funding, and supervised the overall conduct of research. CD was involved in antibody measurements and interpretation of the data. PB conceptualized and provided supervision for the study, supervised and managed the clinical trial, analysed and interpreted the data, prepared the metadata and wrote the paper. All the authors reviewed the manuscript.

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*The Wellcome Trust had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. MVI had no other role in data collection or analysis, and the decision to publish was covered by a pre-study agreement.*

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# Open Peer Review

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## Version 1

Reviewer Report 31 May 2019

<https://doi.org/10.21956/wellcomeopenres.16366.r35552>

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**Tuan M. Tran**

Division of Infectious Diseases, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

### General Comments

In this study, the authors take advantage of a rich collection of longitudinally sampled plasma from an RTS,S malaria vaccine trial to address questions of blood-stage antibody responses after vaccination. In their prior work, they showed that RTS,S vaccinees have reduced antibodies against four merozoite antigens during the 12 months after vaccination (Bejon JID 2013). Here, as a follow-up, they compare antibody levels in RTS,S and control vaccinees at multiple intervals up to 84 months and assess the relationship between Pf-specific antibodies and prospective malaria risk during the interval prior to the following antibody time point.

Overall, the unadjusted data supports (albeit with modest significance at most time points) the main conclusion that humoral immunity to tested blood-stage antigens is hampered or delayed by RTS,S vaccination, and this reduction in immunity is extended for years after vaccination. As the authors mention in the Discussion, they only test four merozoite antigens that may not elicit naturally protective antibody responses, and so the connection to RTS,S-associated long-term reductions in protective blood-stage immunity still remains a question.

As shown in their previous work, the differences in antibody responses after vaccination was affected by RTS,S vaccine-induced differences in prior malaria episodes. Thus, it would be important to show if the significant difference between comparisons in Fig 2 and Table 1 still hold after adjusting for prior episodes. Related to this, and to provide more conservative interpretations of their results, if one were to account for multiple comparisons (three comparisons per time point), only EBA175 and MSP1 have significant differences at 24, 36, and 60; and at 8.5, 24, 36, respectively.

In the Discussion, the statement “In the presence of asymptomatic infection, anti-EBA175 antibodies at all the deciles were higher than the lowest (i.e. nonreactive) decile” is redundant and unclear as written. Was the intention to state “...[malaria protection] for anti-EBA175 antibodies at all the deciles [was] higher...,” given the lower IRR in the higher deciles for EBA175? The subsequent clause, “and some of the higher

deciles for AMA1 and MSP3 antibodies were associated with protection from clinical malaria (irrespective of the vaccine arm)" is not supported by the plots in Fig. 3 given most of the 95%CI bars cross unity (the lone exception being the highest decile for AMA1).

It is notable that the lone antigen that clearly did not show a significant group difference in Fig. 2 was MSP3, which, interestingly, was also the only antigen expressed as FVO, with the others being 3D7. As the authors are aware, the RTS,S vaccine was most effective against the vaccine strain (3D7) (Neafsey et al NEJM 2015). Thus, a possible explanation for the lack of difference for MSP3 is similarity in incidence of malaria episodes caused by non-vaccine strains. This possibility should be added to the discussion. It would be interesting to compare antibody data for 3D7 antigens vs. antigens from heterologous strains, especially a genetically divergent one such as FVO.

#### Minor Comments:

To harmonize with the main text, it would be more useful for the reader if Fig. 1 showed the best fit curve (fitted to standards) for each antigen with sample values superimposed to provide a sense of how many samples were on the linear versus lower and upper plateaus.

4 or 5 parameter logistic models are more frequently used for ELISA dilutional standard curves as they most often give the best fit. The authors should briefly mention why they opted for a less conventional model.

Also, can the authors explain the rationale for using Poisson regression to estimate malaria risk at each interval in this study when Cox regression was used in their previous study? Did some of the underlying assumptions change when the evaluation was extended from 12 m to 84 m?

Although the timing of the sampling can be somewhat inferred from Fig. 2, Table 1, and the text, it would be helpful for readers if there was a Fig. showing the plasma sampling intervals.

In the Results section, the authors use "age" in the first reference to Fig. 2. However, Fig. 2 plots use months from the 3rd vaccine dose on the X axes. "Age" should be changed to months after vaccine dose as the latter is more accurate.

In the statistical analysis section, please indicate that deciles were determined on a per time point basis if this is the case.

For better readability, semicolons should be used to separate the listed items (months 12, 24, and 36; 24 and 36; and 6, 12, 23, 36, and 84).

For the AMA1 in Fig. 2, the y axis could be re-scaled to better visualize the significant difference between groups at the circled time points. Currently, lines for both groups appear superimposed.

Note that there is a discrepancy in Fig. 2: Text legend states shaded regions are 95% CI whereas Fig. legend denotes these are IQRs. As such, Fig. 2 also needs clarification with regard to which comparisons were means and which were comparisons of medians. If means, then means with 95% CI's should be shown. If medians, then medians with IQR's should be shown. (Typically for comparisons where  $P < 0.05$ , the 95% CI's of means should not overlap.)

For Fig. 2, Table 1, and text, it appears that month 6 and 8.5 all represent that same time point, which is

the March 2008 time point (variable number of days post vaccinations). If this is the case, please use consistent nomenclature to avoid confusion (I suggest March 2008 as this is probably the least ambiguous).

For Fig. 3, the main text and legend mention deciles were used but only 5 categories are shown for AMA1 and 8 categories for the other 3 antigens. If the lowest deciles (5 for AMA1 and 2 for the others) were combined into a single reference group, please indicate in the legend. Also, it might be clearer if X axis was labeled as categorical deciles (0-10%, 11-20%, etc) as the current X axis can be assumed to be continuous.

Typographical notes:

For the last sentence of ELISA methods, extra "s" in "levelss"

For Fig. 3 legend, "The analysis [was] adjusted for..."

In first paragraph of Discussion, "MSP1<sub>24</sub>"

In Data availability, "Ha[r]vard"

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**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** malaria immunology, malaria epidemiology, transcriptomics

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 29 May 2019

<https://doi.org/10.21956/wellcomeopenres.16366.r35551>

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**Robin Stephens**

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In order to test the hypothesis that immunity to clinical malaria is delayed by RTS,S, this manuscript details the findings from a study of serum ELISA antibody levels of blood stage antigens found in blood from RTS,S trial participants or Rabies vaccine controls. As this cohort was studied over 7 years, and active surveillance data is available, it is an invaluable indication of the effects of this vaccine on infection, clinical episodes and immune parameters affected. IgG to MSP1-42, (3D7), MSP3 (FVO), PfEBA175RII (3D7), and AMA1 (3D7) were used as an indication of exposure and/or immune status and found to represent (recent) exposure best, though they do accumulate with time since vaccination/age.

Please describe the extent of exposure of participants using averages or distribution of EIR or episodes. Perhaps the result would depend on this range. All I see is "Exposure Index, as previously described...based on geographical location".

**Fig 1:** I suggest you break the y-axis to show the normal (are they all normal?) part of the curve more clearly. You could label the number of patients represented in the negative fraction on the graph? Is it 500/1735? Is density the correct label for the y-axis, not sure what that means.

**Fig 1 result:** I would say values below the "linear part of the sigmoid curve" of the hyperimmune standard curve, were considered unresponsive. Are these the data points marked as a concentration of 2 in Figure 1?

**Fig 2 text:** The increase with age is apparent for AMA1 for participants with highest levels, the difference may be in the sensitivity of the ELISA.

**Fig 2:** The legend could be more descriptive of the data and less of the method. Define IQR, CTI vs Vac in legend. Also, more could be done to make the figure print well in black and white-change shape of symbols and lines in one group. Could significance be indicated on the graph itself?

**Table 1:** Text - the sentence describing the data is hard to understand. "Antibody levels for A, E, M diverged after vaccination. Levels were higher for Rabies vaccinees than RTS,S vaccinees for AMA1 at 24 months, for EBA at 24-72 months, and for MSP1 8.5, 24, 36 and 84 months. (Unless  $p < .05$  is not the cutoff?) But as is, only two sets of dates are listed with three antigens listed "respectively". The table itself could be improved by adding stars for significance, and by separating antigens with a thicker line for easier readability.

**Figure 3:** Great analysis, please state the meaning of IRR-Incident Rate Ratio in the legend, or on the graph.

Can you tell from your data, if the larger error bars for MSP1 slide positive group, are due to the faster decline of these antibodies compared to other specificities? It will be great when robust neutralizing assays have been developed that could distinguish effective blood stage antibody from ELISA positive/exposure induced.

In the last sentence, maybe you mean among the children with asymptomatic parasitemia? or without symptomatic parasitemia?

**Discussion:** Please check the run-on sentence "In the presence of asymptomatic infection, anti-EBA175 antibodies at all the deciles were higher than the lowest (i.e. non-reactive) decile (I'm already lost), and some of the higher deciles for AMA1...".

I would add a sentence about the positive side-reduced exposure leading to reduced clinical episodes. How does that balance out with the potential for increased episodes from delayed immunity, are they predicted to be worse episodes, as in later severe disease?

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

No source data required

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.



**Reviewer Expertise:** Immunology of malaria

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 18 March 2019

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**Wilson L. Mandala**

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<sup>2</sup> Malawi University of Science and Technology, Thyolo, Malawi

This is an extension of a previously published work (Bejon *et al.*, 2011<sup>1</sup>) on the effect of the RTS,S vaccine on the development of the natural immunity against blood stage malaria. The authors have previously reported some reduction in antibody titers against specific *P. falciparum* antigens. What did not come up clearly in this previous report was the longevity of this RTS,S-related effect. This recent report therefore was aimed at addressing that point.

Some major points:

1. There are some discrepancies in the presentation of the time points mentioned in the Methods section [0, 4, 6.5, 8, 14, 24, 36, 48, 60, 72 and 84 months] whereas what is being presented in the Results section (Table 1) seems to have slightly different time points [0, 4, 8.5, 12, 24, 36, 48, 60, 72, 84]. This needs to be checked and the correct time points used in both sections and throughout the manuscript.
2. The authors state that the geometric means of antibodies against the four *P. falciparum* antigens were lower in the RTS,S group compared to the group that had the Rabies vaccine administered. Going through Table 1 the trend seems to be different from this. The trend shown in Figure 2 does agree with the authors' report but not with the trend observed in Table 1. The authors therefore need to clarify how the results presented in Table 1 differ from those presented in Figure 2.
3. The statement "antibodies to four specific merozoite antigens were not associated with clinical protection, rather anti-merozoite antibodies were positively associated with risk of clinical malaria for the group as a whole." is a strong and potentially damaging report which does not augur well with the overall aims of out-rolling this vaccine candidate. It might be considered to water down the whole point of introducing this vaccine and may need to be revisited and presented in a more promising manner. More importantly, the authors seem to contradict this strong statement with the follow up statement: "it is possible that higher antibodies titers might have been protective (i.e. those above a protective threshold)." To marry these two rather disjointed/conflicting statements the authors may wish to start off by introducing the results and proposed concept emanating from this study reported by Kinyanjui *et al.* (2004<sup>2</sup>) and say more about this proposed threshold of

antibody titers above which they confer protection against malaria infection but below which they do not. They can then proceed by stating if the antibody titers detected in this study are either above or below that threshold level and then proceed to speculate if there is merit on the statement that the administration of the RTS,S vaccine directly (or indirectly) impairs the development of the P.f. specific antibodies. It's worth noting that it is the same team who reported a 53% efficacy for the RTS,S vaccine with 38 episodes of clinical malaria in the RTS,S arm compared to 89 episodes observed in the control group vaccinated with a Rabies vaccine that was associated with higher anticircumsporozoite antibody titers (Bejon *et al.*, 2008<sup>3</sup>). This detail needs to come in their discussion.

Minor comments:

1. The authors need to justify the choice of Rabies vaccine in the control group either by referring to their previous works or other related works.
2. There is need for a paragraph on the limitations of the study in the Discussion section.
3. Their previous paper on the same Kenyan cohort combined data from Kenyan and Tanzanian participants. It's worth mentioning why this time they have decided to report only on the Kenyan cohort.
4. The observation that antibody levels do not correlate positively with immunity against malaria has been discussed in detail in a number of good papers (Fowkes *et al.*, 2010<sup>4</sup>, Osier *et al.*, 2008<sup>5</sup>, Osier *et al.*, 2014<sup>6</sup>, Stanisic *et al.*, 2015<sup>7</sup> and Crompton *et al.*, 2010<sup>8</sup>). The authors might wish to refer to these in their introduction and discussion sections.
5. The authors raise the point about malaria transmission control measures and vaccine approaches being effective in controlling the pre-erythrocytic stages of the malaria infection but, not only failing to confer robust antibody mediated immunity, but might even be compromising/impairing the development of the P.f. antibody-mediated immunity against the blood stage in the affected children. Maybe they need to point out how a combination of these measures might have a compounded effect on the development of merozoite-stage immunity.
6. In the discussion section the authors propose that the administration of the RTS,S vaccine should be combined with the provision of other malaria control measures like ITNs. They also suggest that the four-dose vaccine regimen should include long-term follow up in the implementation trials. Adding a paragraph on informed speculations on what could be achieved by combinations of vaccine candidates (especially one specific for the sporozoite stage and one specific for the merozoite stage) might add weight to their argument. This is in light of their observation that RTS,S might actually be predisposing the recipient children to acquiring blood stage malaria infection presumably due to the impaired development of this stage-specific immunity.
7. They only introduce about the four-dose in passing in the discussion section. Could they please expand on that and say more on what is being proposed to be implemented?
8. The fact that seasonality was not entirely eliminated as a potential confounded in this second report needs to be expanded in the discussion section with appropriate reference to how seasonality was observed to affect the outcome of the vaccine in the previous study/report.

9. In the introduction please consider change the sentence to "...based on the circumsporozoite protein (CSP) that targets the pre-erythrocytic STAGE (not cycle) of the *P. falciparum* life cycle in humans."
10. The main objective was to determine the duration of the effect of the RTS,S vaccine. Has this been achieved and reported appropriately? Could they do this better by referring to what they had reported in their JID paper (Bejon *et al.*, 2011<sup>1</sup>) and build on that in this report?
11. Since they are using pool serum samples from healthy controls, could the authors clearly state how blood samples were collected from the study participants, what volume, in what tubes, and what anticoagulant was used?
12. The plasma samples were collected at different stages of the seven year duration implying some were stored for longer than others. What measures were put in place to account for a possible deterioration of the antibodies which could account for the reported decline of antibody titers with time?
13. The legend for Figure 1: "Distribution of anti-merozoite antibody levels. Antibody levels were measured by ELISA" requires more details.
14. The legend for Table 1 needs to be revised and delete "of antibody levels" as this sounds like a repetition.
15. In Figure 3, the Y-axis label (IRR) needs to be written in full either in the legend or on the axis.

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Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

I cannot comment. A qualified statistician is required.

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Malaria Immunology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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