RESEARCH PAPER

Evaluation of the immune response to RTS,S/AS01 and RTS,S/AS02 adjuvanted vaccines: randomized, double-blind study in malaria-naïve adults

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Abbreviations: AE, adverse event; ANOVA, analysis of variance; ATP, according-to-protocol; CHMP, Committee for Medicinal Products for Human Use; CI, confidence interval; CMI, cell-mediated immune; CS, circumsporozoite; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; GMT, geometric mean titer; HBs, hepatitis B surface antigen; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MPL, monophosphoryl lipid A; PBMC, peripheral blood mononuclear cell; SD, standard deviation; TNF, tumor necrosis factor

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Abstract

This phase II, randomized, double-blind study evaluated the immune responses elicited by RTS,S vaccines containing adjuvant system AS01 or AS02 as compared to non-adjuvanted RTS,S in healthy, malaria-naïve adults (ClinicalTrials.gov identifier: NCT00443131). Thirty-six subjects were randomized (1:1:1) to receive RTS,S/AS01, RTS,S/AS02, or RTS,S/saline (control) at months 0, 1, and 2. Antibody responses to *Plasmodium falciparum* circumsporozoite (CS) and hepatitis B surface (HBs) antigens were assessed and cell-mediated immune (CMI) responses evaluated by flow cytometry using intracellular cytokine staining on peripheral blood mononuclear cells. Anti-CS antibody avidity was also characterized. Safety and reactogenicity after each vaccine dose were monitored. One month after the third vaccine dose, RTS,S/AS01 and RTS,S/AS02 recipients had significantly higher anti-CS antibody geometric mean titers (GMTs) than recipients of non-adjuvanted RTS,S ($p<0.0001$ and $p=0.0011$, respectively). The anti-CS antibody GMT was significantly higher with RTS,S/AS01 than with RTS,S/AS02 ($p=0.0135$). Anti-CS antibody avidity was in the same range in all groups. CMI responses (CS- and HBs-specific $CD4^+$ T cell responses) were greater for both RTS,S/AS groups than for the non-adjuvanted RTS,S control group. Reactogenicity was in general higher in the RTS,S/AS groups than in the control group. Most grade 3 solicited adverse events (AEs) were of short duration and grade 3 solicited general AEs were infrequent in the three groups. No serious adverse events were reported. In conclusion, in comparison with non-adjuvanted RTS,S, both RTS,S/AS vaccines exhibited better CS-specific immune responses. The anti-CS antibody response was significantly higher with RTS,S/AS01 than with RTS,S/AS02. The adjuvanted vaccines had acceptable safety profiles.

Keywords: adjuvant, AS01, AS02, vaccine, malaria, cell-mediated immunity, humoral immunity

Introduction

The RTS,S/AS candidate malaria vaccine is under clinical development for possible use in the Expanded Program on Immunization for infants and children in sub-Saharan Africa as an addition to existing preventive and treatment measures, such as insecticide-treated bed nets, indoor residual spraying, and intermittent preventive treatment with sulfadoxine-pyrimethamine.[1,2] The antigen component of the candidate malaria vaccine, RTS,S, consists of repeat sequences of the *Plasmodium falciparum* circumsporozoite (CS) protein fused to the hepatitis B surface antigen (HBs).[3] Two adjuvant systems have been evaluated with the RTS,S antigen: AS02, which consists of an oil-in-water emulsion with monophosphoryl lipid A (MPL) and *Quillaja saponaria* Molina, fraction 21 (QS21, Antigenics Inc., a wholly owned subsidiary of Agenus Inc., Lexington, MA, USA), as immunostimulants, and AS01, a related liposome-based adjuvant system that also contains MPL and QS21.[3,4]

Anti-CS antibody titers and, to a lesser extent, CS-specific $CD4^+$ T cells elicited by RTS,S have been identified as immunological markers associated with protection.[5-7] CS-specific $CD4^+$ T cells induced by RTS,S produce a mixture of cytokines, such as interleukin (IL)-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ.[7-11] In phase 2 clinical trials of adults and children, the RTS,S/AS01 formulation had an improved immunogenicity profile, in terms of humoral and cell-mediated immune (CMI) responses, and an equally favorable safety profile as compared with RTS,S/AS02.[11-14] The RTS,S/AS01 formulation was consequently selected for phase 3 development. First results from the ongoing phase 3 trial in Africa show the vaccine candidate provides significant protection against clinical and severe malaria in young children and infants.[15,16]

The Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) recommended to establish in small studies the effect of vaccine adjuvants on immune responses to the antigens with which they are combined.[17] The present study was therefore designed to evaluate the humoral and CMI responses elicited by RTS,S/AS01 and RTS,S/AS02 as compared to non-adjuvanted RTS,S antigen. The study also evaluated antibody avidity against the CS repeat antigen.

This trial was conducted in healthy, malaria-naïve adults in order to control for factors associated with immune responses following malaria exposure. As subjects with pre-existing anti-HBs immunity may have improved immune responses against both HBs and CS when compared to HBs-naïve subjects,[14] for uniformity, only adults seroprotected for HBs at baseline were enrolled in the trial.

Results

Study population

A total of 56 malaria-naïve volunteers were screened of which 36 were randomized (1:1:1) to the vaccination groups (Fig. 1); all participants completed the study. Two were excluded from the according-to-protocol (ATP) cohort for immunogenicity because of incomplete vaccination. The demographic profile of participants was balanced across groups (Table 1). All participants were white (Caucasian/European heritage).

Immunogenicity

Humoral responses

Antibodies to CS were determined by evaluating immunoglobulin G (IgG) responses to the CS-repeat region using a standard enzyme-linked immunosorbent assay (ELISA). The antibody response was evaluated in the ATP cohort for immunogenicity. Before vaccination, none of the subjects had detectable anti-CS antibody responses (Table 2).

One month after each dose, all vaccine recipients in each group were seropositive for anti-CS antibodies (≥0.5 EU/mL), apart from one participant in the RTS,S/saline group who was seronegative after the third vaccine dose.

One month after the third vaccine dose, anti-CS antibody geometric mean titers (GMTs) were significantly higher in the RTS,S/AS01 and RTS,S/AS02 groups than in the RTS,S/saline group ($p<0.0001$, RTS,S/AS01 versus RTS,S/saline; $p=0.0011$, RTS,S/AS02 versus RTS,S/saline) (Table 3). Anti-CS GMTs were 13-fold and 6-fold higher for recipients of RTS,S/AS01 and RTS,S/AS02, respectively, than for recipients of RTS,S/saline. In the adjuvanted RTS,S groups, GMTs increased with subsequent doses (Table 2) and significantly higher responses ($p=0.0135$) were observed with RTS,S/AS01 than with RTS,S/AS02 (Table 3).

Anti-CS antibody avidity (as determined by ELISA using the chaotropic agent, ammonium thiocyanate, and expressed as the avidity index) was in the same range for the three groups at each time point (Fig. 2).

All participants had seroprotective anti-HBs antibody titers (≥10 mIU/mL) before vaccination, and anti-HBs antibody GMTs increased after the first dose of RTS,S (range: 356888–536123 mIU/mL), but did not increase further with subsequent doses (Table 2).

Cell-mediated immunity

CMI responses to the CS and HBs antigens were assessed by flow cytometry using intracellular cytokine staining (ICS) analyses. Following vaccination, CS-specific $CD4^+$ T cell responses, defined as $CD4^+$ cells expressing at least two of the immune markers CD40L, IL-2, TNF-α, and/or IFN-γ, were detected in all vaccine groups with a trend for higher responses in the adjuvanted RTS,S groups over the RTS,S/saline group (Fig. 3A).

As expected from the primed status of the participants in terms of anti-HBs antibody titers, $CD4^+$ T cell frequencies are much higher following stimulation with HBs than with CS (Fig. 3B). HBs-specific $CD4^+$ T cell responses were detected in all groups after vaccination, with a trend for higher median values in the adjuvanted RTS,S groups. Although some $CD8^+$ T cell proliferation was observed following CS stimulation of peripheral blood mononuclear cells (PBMCs) harvested at screening, no vaccine-induced CS- or HBs-specific $CD8^+$ T cell responses were detected in any group (data not shown).

Reactogenicity and safety

Incidences of all solicited adverse events (AEs), apart from gastrointestinal symptoms, tended to be higher with the adjuvanted antigen than with unadjuvanted RTS,S (Fig. 4). There was no trend suggesting an increase in solicited AE incidence with subsequent vaccine doses (data not shown). Injection site pain was the most frequently reported solicited local AE in all vaccine groups (Fig. 4). All grade 3 local AEs resolved within the 7-day follow up, except for two separate episodes of grade 3 redness after the first dose of RTS,S/AS01 that resolved on day 8 (participant received no further vaccine doses) and day 9, respectively. Fatigue and headache were the most frequently reported solicited general AEs (Fig. 4). Grade 3 solicited general AEs were infrequent and resolved within the 7-day follow-up, apart from one report of grade 3 gastrointestinal discomfort following the first dose of RTS,S/AS02, which resolved 14 days after vaccination; the participant received no further vaccine doses.

At least one unsolicited AE was reported in 10 (83.3%) subjects in each of the RTS,S/AS groups and 6 (50.0%) subjects in the RTS,S/saline group. The incidence of unsolicited events reported by more than one subject in a single group is shown in Table 4; few were reported by more than two subjects. Unsolicited AEs that were considered to be

causally related to vaccination were reported by four recipients of RTS,S/AS01 (33.3%), five recipients of RTS,S/AS02 (41.7%), and three recipients of RTS,S/saline (25.0%). Each vaccine-related unsolicited AE occurred in one subject only for each group, except for injection site pruritus (reported in two subjects in the RTS,S/AS01 group), arthralgia (reported in two subjects in the RTS,S/AS01 group), and myalgia (reported in three subjects in the RTS,S/AS01 group). One related unsolicited AE had grade 3 intensity: myalgia, which followed the first dose of RTS,S/AS01 and resolved within 2 days.

No serious AEs were reported during the study. No clinically relevant changes in clinical laboratory parameters were reported as AEs or serious AEs.

Discussion

The present study was designed to evaluate the humoral and cellular immune responses elicited by adjuvanted RTS,S as compared to non-adjuvanted RTS,S in healthy, malaria-naïve adults. As priming with hepatitis B vaccine has been shown to influence immune responses against both CS and HBs,[14] the immunological determinants contained in RTS,S, we enrolled subjects with detectable anti-HBs responses (≥10 mIU/ml) in an attempt to ensure baseline comparability. Adjuvantation was shown to strongly enhance immune responses, with RTS,S/AS01 and RTS,S/AS02 eliciting anti-CS antibody GMT responses that were 13- and 6-fold higher, respectively, than the response to non-adjuvanted RTS,S. CS- and HBs-specific $CD4^+$ T cell responses were also stronger with the adjuvanted RTS,S formulations as compared to RTS,S/saline, with a trend towards higher CMI responses in the RTS,S/AS01 group. Paradoxically one subject in the saline group showed a CS-specific immune response after dose 1 which decreased over time and was undetectable at study end. We have no clear reason for this. However, although highly improbable, we can't completely rule out the possibility that the subject erroneously received an adjuvanted vaccine at month 0.

166 The results of this trial confirm those from a study of malaria-naïve adults conducted in
167 the USA, which reported significantly greater CS-specific humoral immune responses
168 and a tendency towards higher $CD4^+$ T cell responses with RTS,S/AS01 than with
169 RTS,S/AS02.[11] In that study, vaccine efficacy against malaria challenge was 50% with
170 RTS,S/AS01 and 32% with RTS,S/AS02, and significant correlations were found
171 between protection against malaria challenge and both CS-specific antibody responses
172 and CMI responses induced by the RTS,S vaccine.[7,11] $CD4^+$ T cells predominantly
173 expressed CD40L, a co-stimulatory ligand required for T cell help that also induces the
174 differentiation of B cells,[18,19] and IL-2, a cytokine associated with memory T cells and T
175 cell proliferation and differentiation.[20] There was also a strong association between the
176 frequency of IL-2 producing $CD4^+$ T cells and titers of CS-specific antibodies in the same
177 individual, suggesting that IL-2 may contribute to protection by promoting both cellular
178 and humoral responses.[7] Methods available at the time of the study, however, did not
179 allow for a phenotypic analysis of the CS $CD4^+$ T cell data.

180 Induction of $CD4^+$ T cells directed against *P. falciparum* CS protein by RTS,S adjuvanted
181 formulations has been shown in clinical field trials in adults and children.[6,8,9,21-23] No
182 systematic vaccine-induced $CD8^+$ T cell response was detected in PBMCs in our study,
183 which was consistent with other studies that showed RTS,S/AS induces little or no
184 detectable $CD8^+$ T cell response.[6,11,21,24,25]

185 The anti-CS humoral immune responses in this study tended to be lower than those
186 observed following administration of three doses of RTS,S/AS01 or RTS,S/AS02 to
187 malaria-experienced children in Africa[13,14] but higher than those in African adults in a
188 high malaria transmission area.[12] Overall, in all studies including the present trial,
189 RTS,S/AS formulations produced robust anti-CS antibody responses, with the AS01
190 adjuvanted vaccine inducing higher responses than the AS02 adjuvanted formulation.[12-

[14] To further assess the quality of the antibody response, the relative avidity of anti-CS antibodies was measured in an ELISA procedure using the chaotropic agent, ammonium thiocyanate. The use of chaotropic agents is based on their ability to dissociate antibody-antigen complexes of low avidity while complexes of high avidity remain intact.[26] In the present study, the avidity of the anti-CS antibodies was in the same range for the three groups. This suggests that, while adjuvantation can have an impact on magnitude of the anti-CS response, it may have much less influence on the avidity of the elicited antibodies.

Previous HBs-induced immune responses have been shown to enhance the CS-specific antibody response to adjuvanted RTS,S in children, most likely related to the covalently bound CS segment and HBs fusion protein in RTS,S.[14] In this population of HBs-primed subjects, anti-HBs antibody titers increased dramatically after the first dose of study vaccine with no further increase upon subsequent doses. Various hypotheses could explain these observations: (i) more T and B cell epitopes are present in HBs than in the CS antigen, making HBs immunodominant over CS and leading to earlier maximum anti-HBs antibody production than for CS; (ii) relatively lower doses of CS antigen are administered compared to HBs as there are fewer CS antigens than HBs antigens in RTS,S; (iii) competition at the T cell level, resulting in more and earlier T cell responses and B cell help for HBs-specific B cells than for CS; (iv) binding of RTS,S by anti-HBs antibodies followed by uptake and presentation of vaccine-derived peptides by HBs-specific B cells, resulting in a rapid increase in HBs-specific antibodies and minimal priming of CS-specific T and B cells; and (v) higher levels of anti-HBs antibodies interfering with HBs boosting by binding and phagocytosis of vaccine particles. Most likely a combination of all or some of these mechanisms leads to the continuing rise of vaccine-induced anti-CS antibodies, while no further increase of anti-HBs responses is

observed after the second dose. It was also noted that adjuvanted RTS,S did not induce higher anti-hepatitis B booster responses than non-adjuvanted RTS,S.

Reactogenicity was in general higher in the adjuvanted vaccine groups than in the non-adjuvanted control group but was within acceptable limits and in line with previous experience of RTS,S/AS vaccines.[4,27] Most grade 3 solicited symptoms were of short duration and grade 3 solicited general AEs were infrequent in all groups. Further interpretation of the safety results and immunogenicity analyses is limited by the small number of participants in each group. Another limitation of this study was the absence of a group of subjects without seroprotective anti-HBs antibody titers at baseline.

In summary, adjuvanted RTS,S vaccines exhibited superior anti-CS humoral and CMI responses over non-adjuvanted RTS,S, with a tendency towards stronger immune responses induced by RTS,S/AS01 compared to RTS,S/AS02, which was in line with previous studies. The adjuvanted vaccines demonstrated an acceptable safety profile, although reactogenicity was generally higher with the adjuvanted vaccines than with non-adjuvanted RTS,S. These results, together with previously published studies, confirm the immunological basis for adjuvantation of RTS,S.

Methods

Study design and participants

This phase II, randomized, double-blind (observer-blind) study was conducted at the Center for Vaccinology, Ghent University and Ghent University Hospital, Ghent, Belgium, between April and July in 2007 (ClinicalTrials.gov identifier: NCT00443131). Subjects were recruited primarily via advertisements posted at the University Hospital. Healthy malaria-naïve men or women of non-childbearing potential, aged 18 to 45 years at the time of first vaccination, who were seronegative for human immunodeficiency virus

(HIV 1 or 2), HBs, and hepatitis C virus antibodies, with seroprotective anti-HBs antibody titers (≥10 mIU/mL) at screening, were eligible for enrolment. All subjects had been immunized with the hepatitis B vaccine. Written informed consent was obtained from all participants before performing any study procedure.

The study was reviewed and approved by the ethics review committee of the University of Ghent. The trial was undertaken according to the International Conference on Harmonization and Good Clinical Practice guidelines, and was monitored by GlaxoSmithKline Vaccines. The primary objective of the study was to demonstrate superiority of anti-CS antibody responses at 1 month post-dose 3 against RTS,S formulated with AS01 or AS02 compared to RTS,S reconstituted with saline.

The participants were randomized (1:1:1), by a centralized randomization system on the internet administered by the investigator, to receive vaccination at months 0, 1, and 2 with lyophilized RTS,S (50 µg) reconstituted with 500 µL of either $AS01_A$, $AS02_B$ (referred to elsewhere in this paper as AS01 and AS02, respectively), or saline. The RTS,S vaccine has been described previously.[3] The vaccines were administered intramuscularly to the deltoid muscle of the non-dominant arm and vaccine recipients were observed for at least 30 minutes following each vaccination.

All laboratory assays were performed at the Center for Vaccinology, Ghent University and Hospital, or at the laboratories of GlaxoSmithKline Vaccines, Rixensart, Belgium, using standardized, validated procedures.

Humoral immune response assessments

Assessment of anti-CS and anti-HBs antibody titers was conducted on serum samples taken before dose 1 (at enrolment), one month after dose 1 (month 1), one month after dose 2 (month 2), and one month after dose 3 (month 3). Antibodies against CS were measured by evaluating IgG responses to the CS-repeat region, using a standard ELISA

with R32LR as the capture antigen.[28] An anti-CS antibody titer of 0.5 EU/mL or greater was considered to be positive. Anti-HBs antibodies were measured using an in-house ELISA; an antibody titer of 10 mIU/mL or greater was considered to be seroprotective.

The avidity of anti-CS antibodies in sera was assessed at months 1, 2, and 3. The relative avidity of IgG antibodies was determined by ELISA with R32LR as coating antigen. The assay was an adaptation of the anti-CS assay[28] and based on previous methodology on the dissociation of low avidity antibody-antigen complexes by the chaotropic agent, ammonium thiocyanate (NH_4SCN).[26] After sample addition, formed antigen-antibody complexes were treated with a 1M ammonium thiocyanate solution and remaining complexes were quantified. The result was compared to the concentration obtained when no treatment was applied and expressed as the avidity index, indicating the percentage of antibodies that remained bound to antigens.

CMI response assessments

Blood samples for CMI response analysis were collected at months 1, 2, and 3. CMI responses to the CS and HBs antigens were assessed using frozen PBMCs, which were isolated by standard Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen within 12 hours of blood collection.

CS-specific and HBs-specific $CD4^+$/$CD8^+$ T cells expressing the cytokines CD40L and/or IL-2 and/or TNF-α and/or IFN-γ were detected using ICS and flow cytometry, based on previously described methodology.[29,30] Briefly, PBMCs were stimulated *in vitro* for 2 h with antigen or pools of peptides, which covered the entire sequence of the antigens, in the presence of anti-CD28 and anti-CD49d antibodies. The cells were then incubated overnight with brefeldin A to prevent cytokine excretion. The cells were stained for surface markers (CD4 and CD8), fixed and permeabilized, and stained with

fluorochrome-conjugated monoclonal antibodies to detect the immune markers by flow cytometry.

Safety and reactogenicity evaluation

Solicited local (injection site pain, redness, and swelling) and general (fatigue, fever, gastrointestinal symptoms [nausea, vomiting, diarrhea, abdominal pain], and headache) AEs were recorded by participants on diary cards during the 7-day follow-up after each vaccination. Information on unsolicited AEs were collected over 30 days after each vaccination. Serious AEs were reported throughout the study. Duration, causality, and outcome of AEs were recorded. All solicited local reactions were considered causally related to vaccination; the relationship of other AEs was classified as possible or not causally related. AE intensity was scored on a scale from 1 to 3. Grade 3 AEs were defined as preventing normal daily activity, apart from grade 3 solicited fever, which was defined as axillary temperature >39.0°C, and grade 3 solicited swelling or redness, defined as diameter >50 mm. Complete blood count, renal (creatinine) and hepatic functional tests (alanine aminotransferase and aspartate aminotransferase) were taken at screening and one month after the third vaccine dose.

Statistical analyses

A sample size of 10 evaluable subjects per group had 98% power to demonstrate superiority of RTS,S/AS01 over RTS,S/saline, assuming a log standard deviation not exceeding 0.7 and anti-CS GMTs of 5 EU/mL and 143 EU/mL for RTS,S/saline and RTS,S/AS01, respectively, and 91% power to demonstrate superiority of RTS,S/AS02 over RTS,S/saline, assuming a log standard deviation not exceeding 0.7 and anti-CS GMTs of 5 EU/mL and 82 EU/mL for RTS,S/saline and RTS,S/AS02, respectively.

Immunogenicity analysis was performed on the ATP cohort for immunogenicity, defined as those meeting all eligibility criteria, complying with the procedures defined in the

protocol, with no elimination criteria during the study, and for whom data concerning immunogenicity endpoint measures were available. Anti-CS and anti-HBs antibody GMTs were calculated with 95% CIs. The percentages of subjects with seropositive levels of anti-CS antibodies (≥0.5 EU/mL) and seroprotective levels of anti-HBs antibodies (≥10 mIU/mL) were determined. Superiority of RTS,S/AS01 or RTS,S/AS02 over RTS,S/saline in terms of anti-CS antibody GMTs one month after the third vaccine dose was evaluated using a 2-sided T-test on the $\log_{10}$ transformed anti-CS titers (analysis of variance [ANOVA] model, pooled variance). The superiority condition was met if the p value was <0.025.

The avidity of anti-CS antibodies was expressed as the avidity index, indicating the percentage of antibodies that remained bound to antigens after ammonium thiocyanate treatment. CMI responses were determined as the frequency of CS- and HBs-specific $CD4^+$ and $CD8^+$ T cells expressing at least two immune markers (CD40L, IL-2, TNF-α, and/or IFN-γ), presented as the percentage of T cells expressing at least two cytokines per million cells.

The safety analysis was conducted on the total vaccinated cohort. Percentages of solicited or unsolicited AEs were calculated with exact 95% CIs. Clinically relevant abnormal laboratory values were determined according to predefined normal ranges.

Disclosure of Potential Conflicts of Interest

The study was supported by GlaxoSmithKline Biologicals SA. O.O-A., M.L.. E.J., P.M., W.R.B., and J.C. are employees of the GlaxoSmithKline group of companies and own GlaxoSmithKline stock and/or stock options. G.L.-R., I.L.-R., and F.C. received funding from GlaxoSmithKline via their institute to cover study costs. G.L.-R. received payments from GlaxoSmithKline for lectures on HPV vaccines and vaccines in general, and for consultancy on influenza vaccines and adjuvants, from Novartis Vaccine and

Diagnostics and Immune Targeting Systems (UK) for consultancy on influenza vaccines, and from Baxter Vaccines for lectures on influenza vaccines. I.L.-R. received fees from GlaxoSmithKline and Sanofi Pasteur for lectures on vaccine-related topics and received registration and travel expenses from GlaxoSmithKline to attend vaccine-related conferences.

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Contributions

G.L.-R., I.L.-R., and F.C. were investigators in this study and were responsible for the recruitment of subjects, collection and assembly of data, and provided critical input in the protocol, interpretation of results and writing of the manuscript. G.L.R., I.L.R., W.R.B., and O.O.-A. were involved in all steps of the study from study design to analysis and interpretation of results. F.C., P.M., E.J. and J.C. were responsible for the testing and interpretation of humoral and cellular immune response assessments. M.L. was responsible for the design, execution and interpretation of statistical analyses. G.L.R., I.L.R. and O.O.-A. supervised the design of the study, analysis and interpretation of

results. All authors have critically reviewed the manuscript drafts and approved the final article.

Financial Disclosure

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Figure Legends

Figure 1. CONSORT diagram of study flow in phase II randomized, double-blind study of humoral and cell-mediated immune responses against three doses of RTS,S malaria vaccine formulated with AS01 (RTS,S/AS01) or AS02 (RTS,S/AS02) compared to three doses of RTS,S reconstituted with saline (RTS,S/saline).

Figure 2. Box plots of anti-CS antibody avidity index (percentage of antibodies that remained bound to antigen after ammonium thiocyanate treatment) in each group one month after each vaccine dose (ATP cohort for immunogenicity). Box indicates median and Q1 (median minus 25%) and Q3 (median plus 25%) values, whiskers indicate minimum and maximum values. M, month.

Figure 3. Box plots for cytokine-positive T cell frequencies, defined as the percentage of $CD4^+$ cells expressing at least two immune markers (CD40L, IL-2, TNF-α, and/or IFN-γ) per 10^6 $CD4^+$ T cells, on stimulation with circumsporozoite (CS) and hepatitis B surface (HBs) antigens (ATP cohort for immunogenicity). Peripheral blood mononuclear cells were harvested, surface-labeled for CD4 and CD8 and then stained for intracellular detection of immune markers (see Methods). Cells were analyzed by flow cytometry. Box indicates median and Q1 (median minus 25%) and Q3 (median plus 25%) values, whiskers indicate minimum and maximum values. Pre, pre-vaccination; M, month.

A. CS-specific $CD4^+$ T cell responses

B. HBs-specific $CD4^+$ T cell responses

Figure 4. Frequency of solicited local and general adverse events (overall per dose) occurring within 7 days of vaccination (total vaccinated cohort).

Grade 3 defined as preventing normal daily activity, apart from grade 3 fever (>39.0°C) and grade 3 swelling or redness (diameter >50 mm)

507 A. Solicited local adverse events

508 B. Solicited general adverse events

509 **Table 1.** Demographic characteristics (ATP cohort for immunogenicity).

	RTS,S/AS01 (N = 11)	**RTS,S/AS02 (N = 11)**	**RTS,S/Saline (N = 12)**
Mean age ± SD (years)	20.9 ± 2.3	20.7 ± 2.9	21.6 ± 2.3
Age range (years)	18–25	18–28	18–26
Gender (%), female/male	63.6/36.4	63.6/36.4	75.0/25.0

510 SD, standard deviation; N, number of subjects

511

Table 2. Anti-CS and anti-HBs antibody GMTs by vaccine group one month after each vaccine dose (ATP cohort for immunogenicity).

			Anti-CS GMT (EU/mL)			Anti-HBs GMT (mIU/mL)		
Group	**Timing**	**N**	**Value (95% CI)**	**Min**	**Max**	**Value (95% CI)**	**Min**	**Max**
RTS,S/AS01	PRE	11	0.3 (0.3–0.3)	<0.5	<0.5	419 (65–2699)	29	52929
	Month 1	11	43.9 (21.3–90.4)	3.4	259.5	356888 (170662–746324)	71649	1480452
	Month 2	11	93.2 (58.3–149.2)	22.4	231.0	285434 (154715–526599)	60419	1384707
	Month 3	11	160.3 (114.1–225.4)	78.6	363.0	204229 (105211–396436)	29148	1037696
RTS,S/AS02	PRE	11	0.3 (0.3–0.3)	<0.5	<0.5	124 (48–322)	14	1303
	Month 1	11	30.2 (13.3–68.9)	4.4	189.0	536123 (224513–1280230)	37591	2802649
	Month 2	11	58.8 (33.3–103.6)	18.4	263.4	255206 (93038–700038)	12536	863367
	Month 3	11	77.4 (47.3–126.7)	22.2	202.2	216220 (101812–459188)	20510	570058
RTS,S/Saline	PRE	12	0.3 (0.3–0.3)	<0.5	<0.5	404 (120–1358)	11	8844
	Month 1	12	21.4 (8.2–55.6)	1.2	198.9	375772 (125743–1122963)	16770	3876614
	Month 2	12	13.9 (5.9–32.8)	0.7	93.2	245373 (91656–656887)	11206	2436186
	Month 3	12	12.2 (4.8–30.7)	<0.5	65.8	187514 (87264–402930)	20092	1124210

GMT, geometric mean antibody titer calculated on all subjects; N, number of subjects with available results; Min, minimum; Max, maximum; PRE, pre-vaccination; Month 1, one month after first vaccine dose; Month 2, one month after second vaccine dose; Month 3, one month after third vaccine dose.

Table 3. Anti-CS antibody geometric mean titer (GMT) ratios (first group over second group) at one month after the third vaccine dose (ATP cohort for immunogenicity).

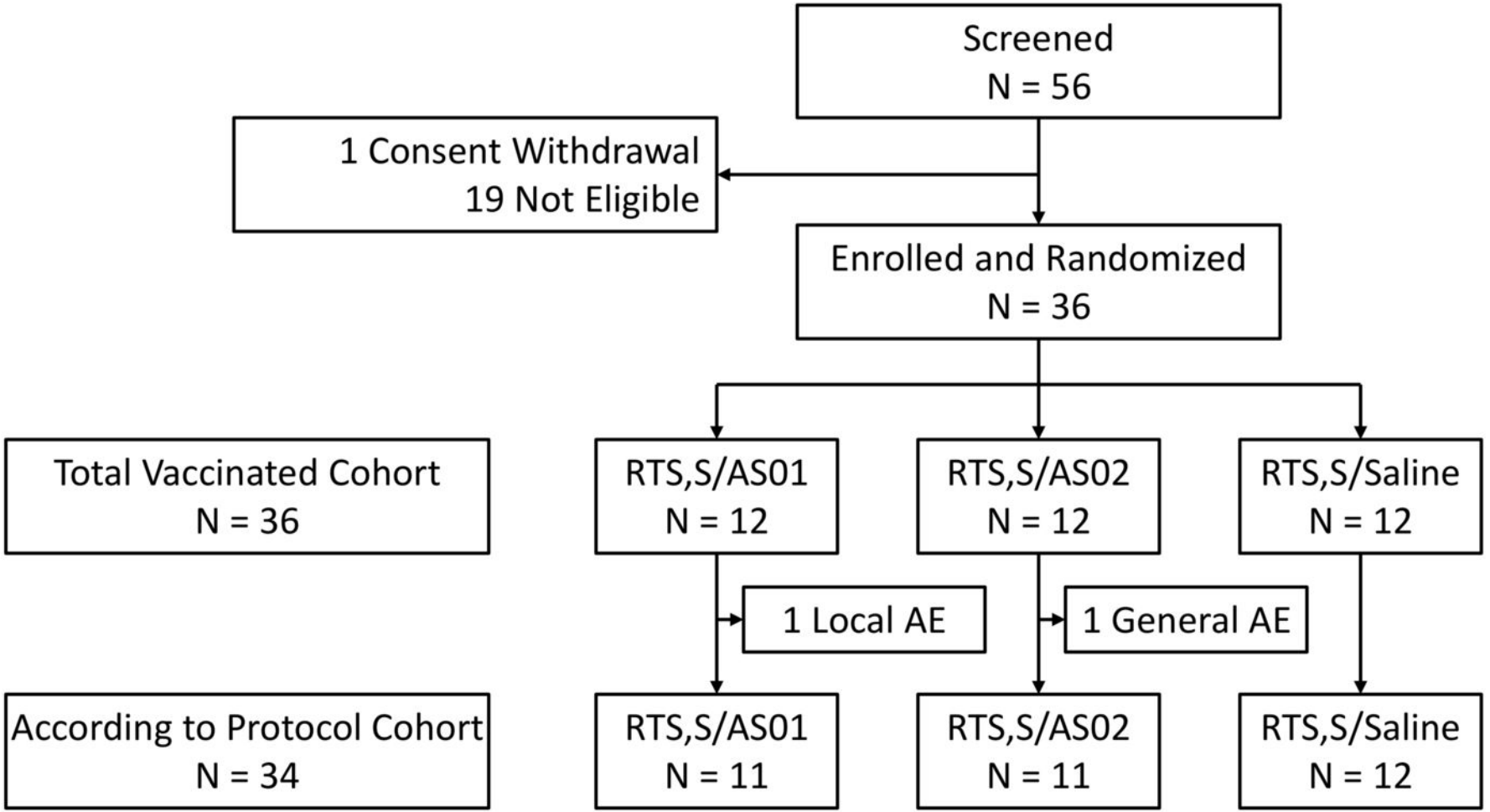
Group comparison	GMT	GMT ratio (95% CI)	p value[a]
RTS,S/AS01 vs. RTS,S/Saline	160.35 vs. 12.19	13.15 (5.02–34.45)	<0.0001
RTS,S/AS02 vs. RTS,S/Saline	77.43 vs. 12.19	6.35 (2.30–17.50)	0.0011
RTS,S/AS01 vs. RTS,S/AS02	160.35 vs. 77.43	2.07 (1.18 – 3.63)	0.0135

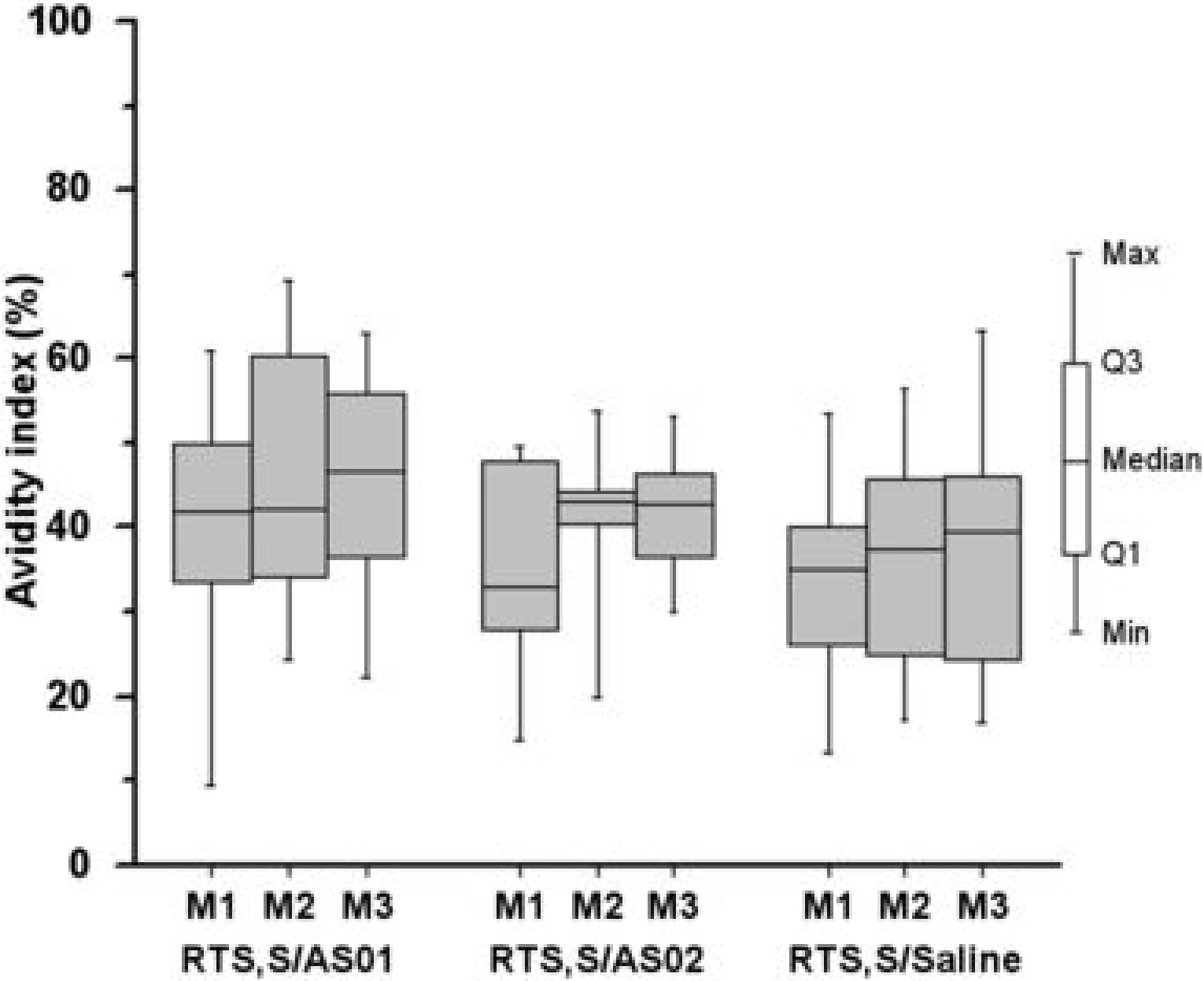
[a] p value for differences in GMT (ANOVA model, pooled variance)
vs., versus

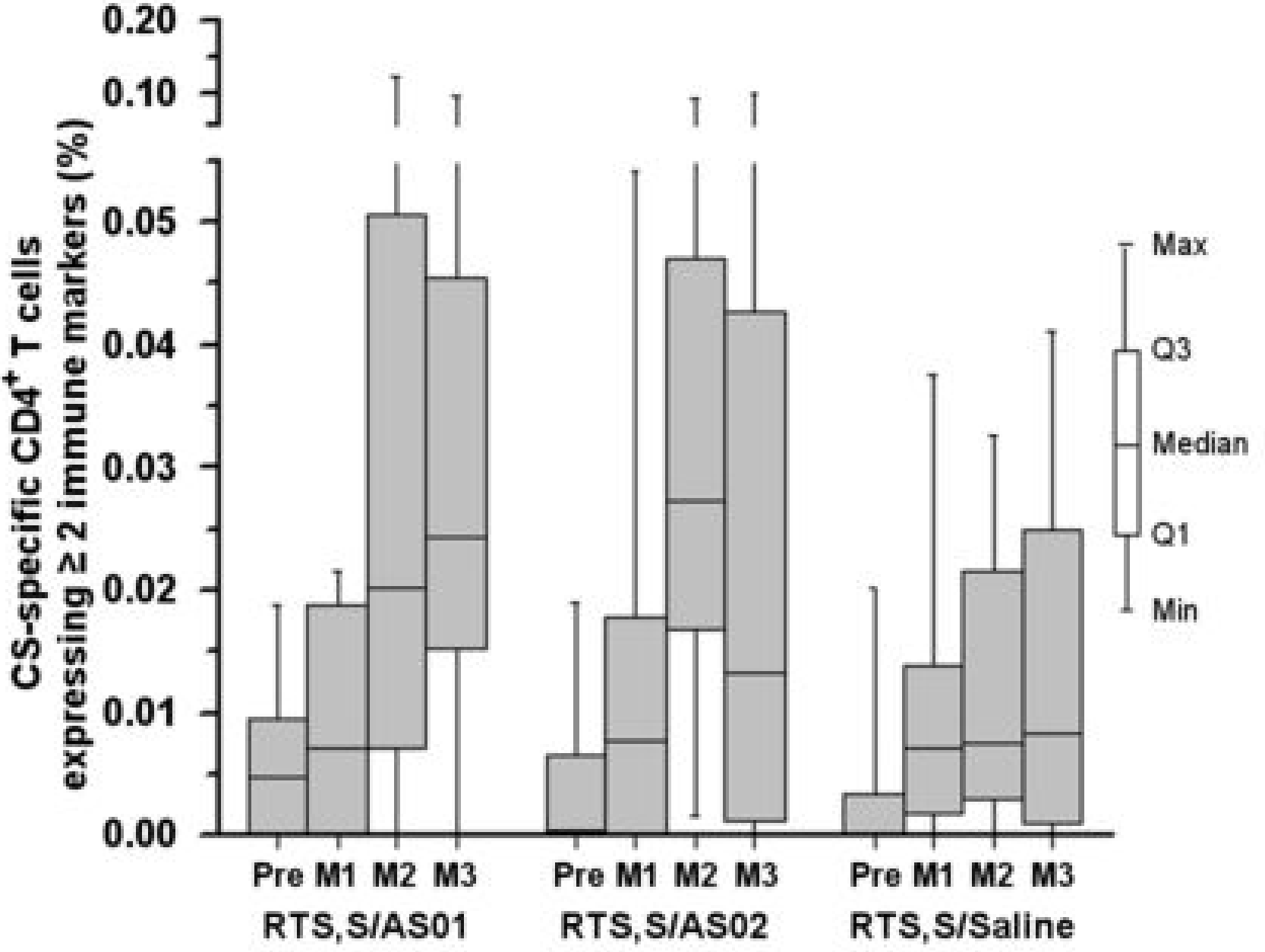
Table 4. Frequency of unsolicited symptoms (reported in more than one subject in a single group) during the 30-day post-vaccination period (total vaccinated cohort).

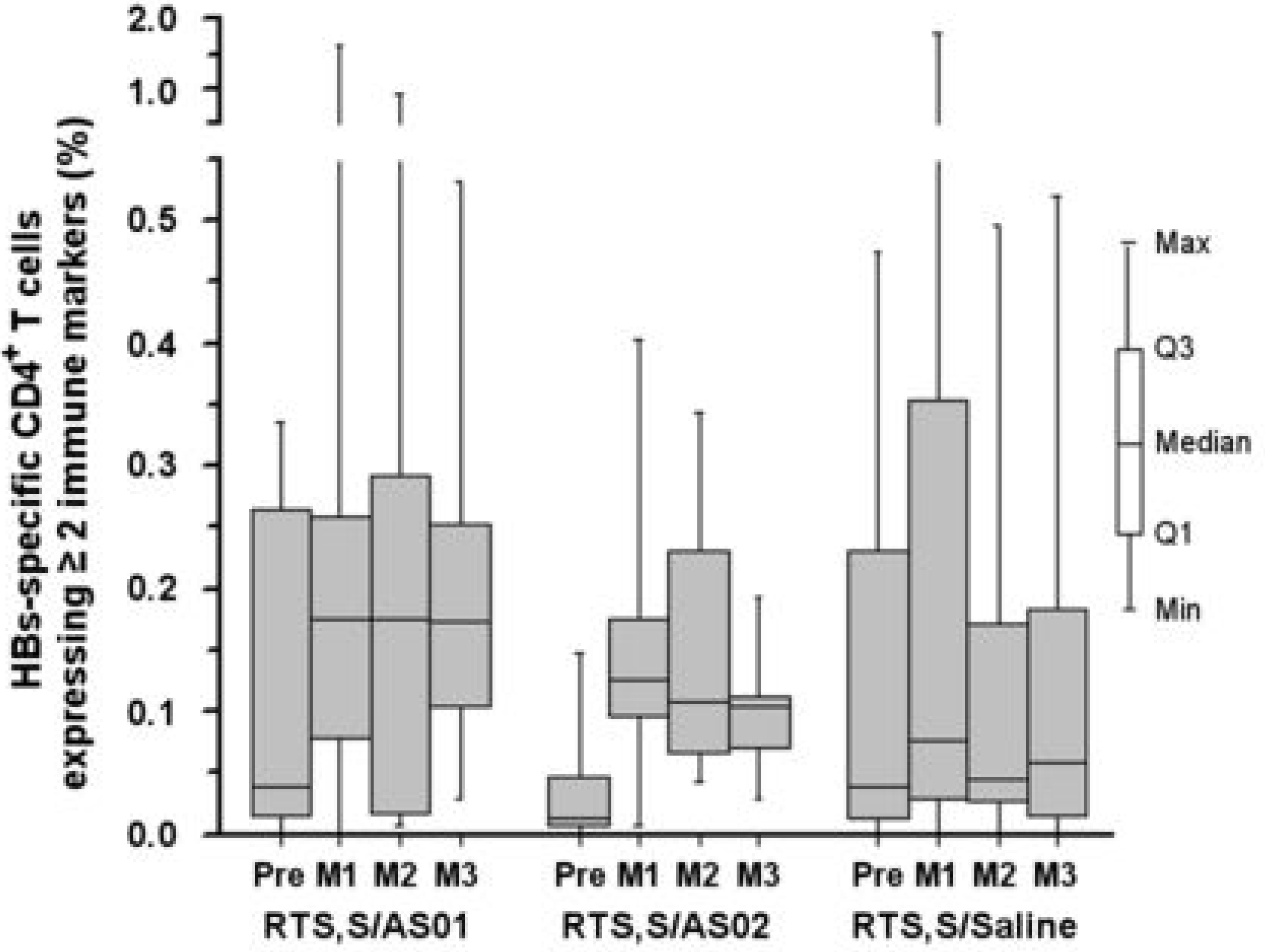
Unsolicited symptom	Percentage of subjects (95% CI)		
	RTS,S/AS01 (N = 12)	**RTS,S/AS02 (N = 12)**	**RTS,S/Saline (N = 12)**
Nausea	16.7 (2.1–48.4)	0.0 (0.0–26.5)	0.0 (0.0–26.5)
Chills	16.7 (2.1–48.4)	0.0 (0.0–26.5)	0.0 (0.0–26.5)
Injection site pruritus	16.7 (2.1–48.4)	8.3 (0.2–38.5)	0.0 (0.0–26.5)
Nasopharyngitis	0.0 (0.0–26.5)	16.7 (2.1–48.4)	16.7 (2.1–48.4)
Upper respiratory tract infection	0.0 (0.0–26.5)	16.7 (2.1–48.4)	0.0 (0.0–26.5)
Arthralgia	16.7 (2.1–48.4)	0.0 (0.0–26.5)	0.0 (0.0–26.5)
Myalgia	25.0 (5.5–57.2)	0.0 (0.0–26.5)	0.0 (0.0–26.5)
Headache	16.7 (2.1–48.4)	33.3 (9.9–65.1)	16.7 (2.1–48.4)
Pharyngolaryngeal pain	8.3 (0.2–38.5)	0.0 (0.0–26.5)	16.7 (2.1–48.4)
Productive cough	16.7 (2.1–48.4)	0.0 (0.0–26.5)	0.0 (0.0–26.5)

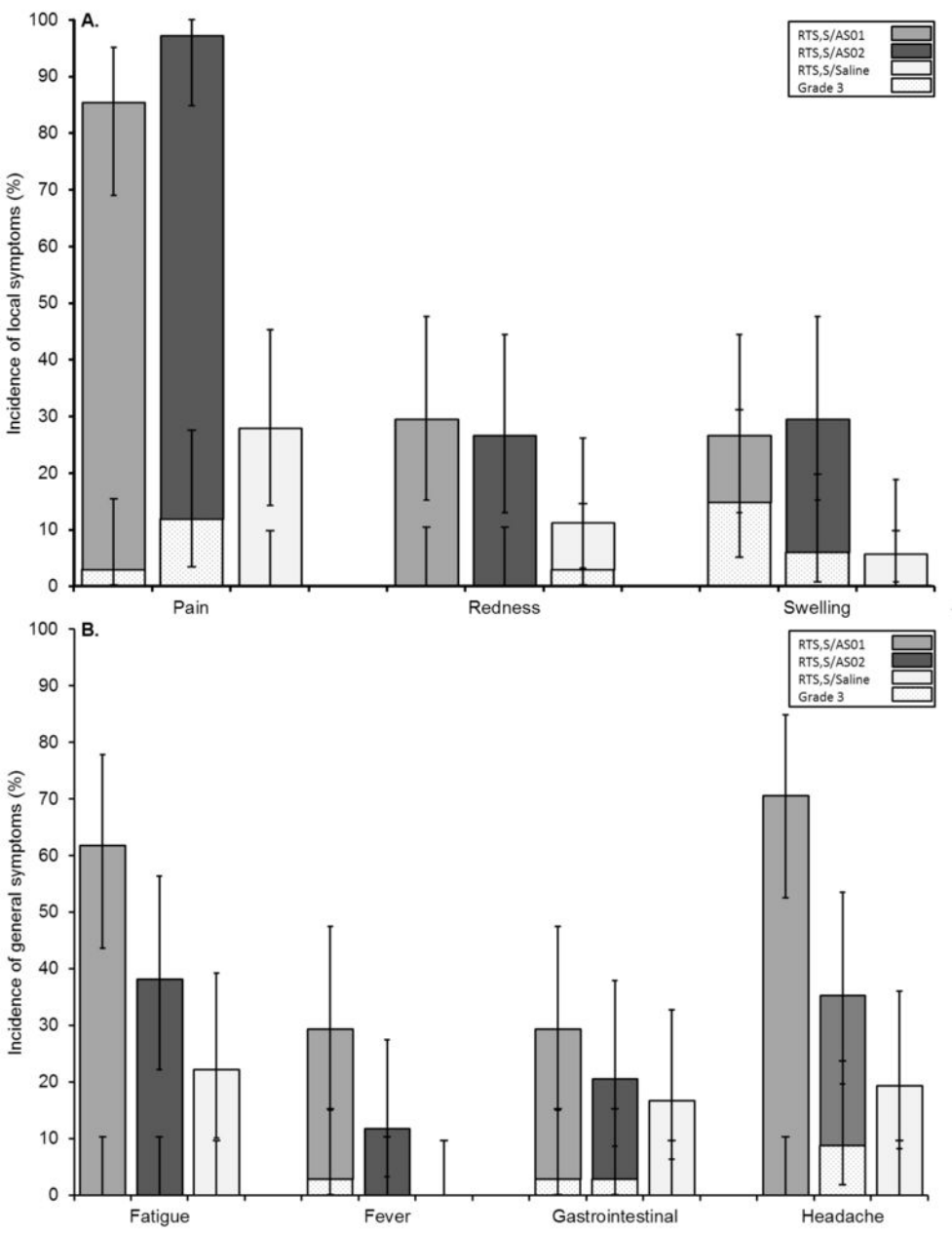
N, number of subjects


Screened
N = 56
1 Consent Withdrawal
19 Not Eligible
Enrolled and Randomized
N = 36
Total Vaccinated Cohort
N = 36
RTS,S/AS01
N = 12
RTS,S/AS02
N = 12
RTS,S/Saline
N = 12
1 Local AE
1 General AE
According to Protocol Cohort
N = 34
RTS,S/AS01
N = 11
RTS,S/AS02
N = 11
RTS,S/Saline
N = 12


100
80
60
40
20
0
Avidity index (%)
Max
Q3
Median
Q1
Min
M1 M2 M3
RTS,S/AS01
M1 M2 M3
RTS,S/AS02
M1 M2 M3
RTS,S/Saline


0.20
0.10
0.05
0.04
0.03
0.02
0.01
0.00
CS-specific CD4+ T cells expressing ≥ 2 immune markers (%)
Pre M1 M2 M3
RTS,S/AS01
Pre M1 M2 M3
RTS,S/AS02
Pre M1 M2 M3
RTS,S/Saline
Max
Q3
Median
Q1
Min


HBs-specific CD4+ T cells
expressing ≥ 2 immune markers (%)
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0.0
Pre M1 M2 M3
RTS,S/AS01
Pre M1 M2 M3
RTS,S/AS02
Pre M1 M2 M3
RTS,S/Saline
Max
Q3
Median
Q1
Min


A.
RTS,S/AS01
RTS,S/AS02
RTS,S/Saline
Grade 3
Incidence of local symptoms (%)
100
90
80
70
60
50
40
30
20
10
0
Pain
Redness
Swelling
B.
RTS,S/AS01
RTS,S/AS02
RTS,S/Saline
Grade 3
Incidence of general symptoms (%)
100
90
80
70
60
50
40
30
20
10
0
Fatigue
Fever
Gastrointestinal
Headache